"Potato and beneficial fungal inoculants associations : in vitro and within field studies under different agricultural factors"

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Abstract

Potato is a key food security crop worldwide. Under conventional agriculture, this crop is characterized by high levels of fertilizers and pesticides applications which can have detrimental effects on the environment. An alternative or complementary approach to chemicals is the use of beneficial fungal inoculants such as arbuscular mycorrhizal fungi (AMF) or Trichoderma spp. These microorganisms are known to improve plant growth and health and are thus key players in agro-ecosystems. However, their application in the field is complex and results sometimes inconclusive. Consequently, it is important to understand the impacts of agricultural factors on their fitness and functions in the field to guarantee their successful application. In this context, the first objective of the thesis was to study the impact of some fungicides and herbicides on AMF under in vitro culture conditions. Results suggested that fungicides at high concentrations, in particular fungicides with a systemic actio...

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Potato and beneficial fungal inoculants associations

In vitro and within field studies under different agricultural factors

Thèse de doctorat présentée par **Catherine Buysens** en vue de l'obtention du grade de Docteur en Sciences agronomiques et ingénierie biologique

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List of abbreviations

ACC	Acetyl CoA carboxylase
ALP	Alkaline phosphatase
ALS	Acetohydroxyacid synthase
AMF	Arbuscular mycorrhizal fungi
AMPA	Aminomethyl phosphonic acid
Arg	Arginine
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
BAS	Branched absorbing structure
BCA	Biocontrol agent
BCCM	Belgian Coordinated Collections of Microorganisms
BM	Beneficial microorganism
CC	Cover crop
CESAMM	Center of Study on Arbuscular Mycorrhizal Monoxenics
CFU	Colony-forming unit
CMN	Common Mycorrhizal Network
CRA-W	Walloon Agricultural Research Centre
CSSP	Common Symbiosis Signaling Pathway
Cq	Quantification cycles
DAOM	Canadian National Mycological Herbarium
DCM	De Ceuster Meststoffen NV
DGA	Direction générale opérationnelle de l'Agriculture, des ressources naturelles et de l'Environnement
DHP	Dihydropteroate
DNOC	Dinitro-ortho-cresol (herbicide)
dNTP	Nucleoside triphosphate
(ds)DNA	(dubble strain) Deoxyribonucleic acid
DW	Dry Weight
EAPR	European Association for Potato Research.
ELIB	Earth and Life Institute Biodiversity
ELIM	Earth and Life Institute Applied Microbiology

EPSP	Enolpyruvylshikimate-3-phosphate
ER	Endoplasmic Reticulum
ERM	Extraradical mycelium
FAO	Food and Agriculture Organization of the United Nations
FNRS	Fonds de la Recherche Scientifique
GINCO	Glomeromycota *in vitro* collection
HAM-P	Half-closed arbuscular mycorrhizal–Plant (*in vitro* culture system)
HC	Hyphal compartment
HPPD	Hydroxyphenyl-pyruvate-dioxygenase
ICC	Inoculation at cover crop
ICOM	International Conference on Mycorrhiza
IC_{50}	Half maximal inhibitory concentration
ID_{90}	Inoculation density necessary to infect 90% of plants
IPP	Inoculation at potato plantation
IRM	Intraradical mycelium
ISR	Induced Systemic Resistance
ITS	Internal Transcribed Spacers
(L)CO	**(**Lipo)chitooligosaccharide
LSU	Large SubUnit (gene)
MAPK	Mitogen-activated protein kinases (gene)
MBC	Methyl Benzimidazole Carbamate (fungicide)
MDP	Mycelium donor plant (*in vitro* culture system)
MHB	Mycorrhiza Helper Bacteria
MIP	Mycorrhizal Infective Potential
MIR	Mycorrhiza Induced Resistance
MP	Mycorrhizal Plant
MPN	Most Probable Number
MS	Murashige and Skoog (medium)
MSR	Modified Strullu-Romand (medium)
mt	Mitochondrial
(M)UCL	**(**Mycothèque de l')Université catholique de Louvain
NADH	Nicotinamide adenine dinucleotide (reduced)

$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NGS	Next Generation Sequencing
nr	Nuclear
PAL	Phenylalanine ammonia lyase (gene)
PAM	Peri-arbuscular Membrane
PDA	Potato dextrose agar (medium)
PCNB	Pentachloronitrobenzene (fungicides)
PDS	Phytoene desaturase step
PGPM	Plant growth promoting microorganism
PM	Perifungal Membrane
PolyP	Polyphosphate
PPA	Pre-penetration Apparatus
PPF	Photosynthetic Photon Flux
PPO	Protoporphyrinogen oxidase
PR	Pathogenesis related (gene)
(q)PCR	(quantitative) polymerase chain reaction
RC	Root compartment
RFLP	Restriction Fragment Length Polymorphism
RH	Runner hyphae
ROC	Root organ culture
(r)RNA	(ribosomal) Ribonucleic acid
SA	Salicylic Acid
SARDI	South Australian Research and Development Institute
SAS	Statistical Analysis System (software)
SBI	Sterol Biosynthesis Inhibitors
SDH	Succinate dehydrogenase
SDWP	Sterile distilled water-peptone
SE	Standard error
SLS	Sodium dodecyl sulfate
SSU	Small SubUnit (gene)
TAG	Triacylglycerides
THSM	*Trichoderma harzianum* selective medium
VLCFA	very long chain fatty acid

Summary

Potato is a valuable food security crop worldwide. However, under conventional agriculture it is characterized by heavy applications of fertilizers and pesticides which can have detrimental effects on the environment. An alternative or complementary approach to these chemicals is the use of beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF) in combination with adapted agricultural itineraries. AMF are obligate root symbionts, forming associations with most terrestrial plants. The plants provide the fungi with carbohydrates in exchange for inorganic nutrients (e.g. N, P). AMF improve plant growth, health and productivity and are thus key organisms in agro-ecosystems. However, their application in the field is complex and sometimes inconclusive. It is thus important to understand the impacts of agricultural factors on AMF to guarantee their successful application under field conditions. In this context, the first objective of the thesis was to study the impact of some fungicides and herbicides/defoliation treatments on AMF under *in vitro* conditions. Results suggested that fungicides at adequate dosage to control *Rhizoctonia solani* or decrease its impact on potato may be combined with the AMF *Rhizophagus irregularis* MUCL 41833. In contrast, at higher concentrations, they inhibit growth of AMF intra- and extra-radical structures. This effect was particularly marked with the systemic fungicide flutolanil who accumulate in the root. Similarly, systemic herbicide glyphosate, in contrast to the contact herbicide diquat or to mechanical defoliation was shown to have a faster and stronger impact on *R. intraradices* MUCL 49410 and *R. irregularis* MUCL 41833 sporulation or colonization abilities. A possible explanation is linked to the systemic nature of glyphosate that is translocated from shoots to roots and released in the medium via root

decay, thus in direct contact with the intra- and extraradical mycelium, respectively. The second objective of the thesis was to identify agricultural itineraries optimal for the application of AMF both within conventional and organic cultivation systems. Interestingly, cover crop inoculation with *R. irregularis* MUCL 41833 in combination with another beneficial microorganism *Trichoderma harzianum* MUCL 29707 increased potato yield of cultivars Sarpo Mira and Bionta. Finally, in a third objective, the importance of tracing the inoculated AMF within field was investigated via Real-Time quantitative PCR of the mitochondrial Large SubUnit (mtLSU). This approach helped to ascertain the potential of AMF in increasing health and yield of potato, whilst decreasing mineral fertilizers and pesticides applications.

Outline of the thesis

Arbuscular mycorrhizal fungi (AMF) are obligate root symbionts that promote the growth and health of an estimate of 80% of land plants among which most agricultural crops. Their use in commercial product is thus highly recommended. However, most results so far have been obtained from growth chamber and greenhouse experiments while field observations are less numerous and frequently inconclusive. This is because crops naturally become colonized by native AMF strains making it difficult to separate the effects of applied inoculum, from local AMF communities, in particular if they harbor similar species to the inoculated ones. In addition, the introduced AMF may be impacted by agricultural factors, thus becoming inefficient in promoting plant growth. Here, we investigated, *in vitro* and within field, the effects of some agricultural factors (e.g. the application of fungicides and herbicides, the co-inoculation with *T. harzianum*, the use of cover crops prior to plantation…) on the AMF and their subsequent benefits to potato crop. We further identified some technical itineraries that increase the AMF (native and introduced) population, favor their association with the potato plant and improve their functioning, under conventional as well as organic potato cropping systems. Sustainability of these itineraries were discussed. Finally, we proposed some molecular tools allowing to trace the introduced AMF within field (Figure 1).

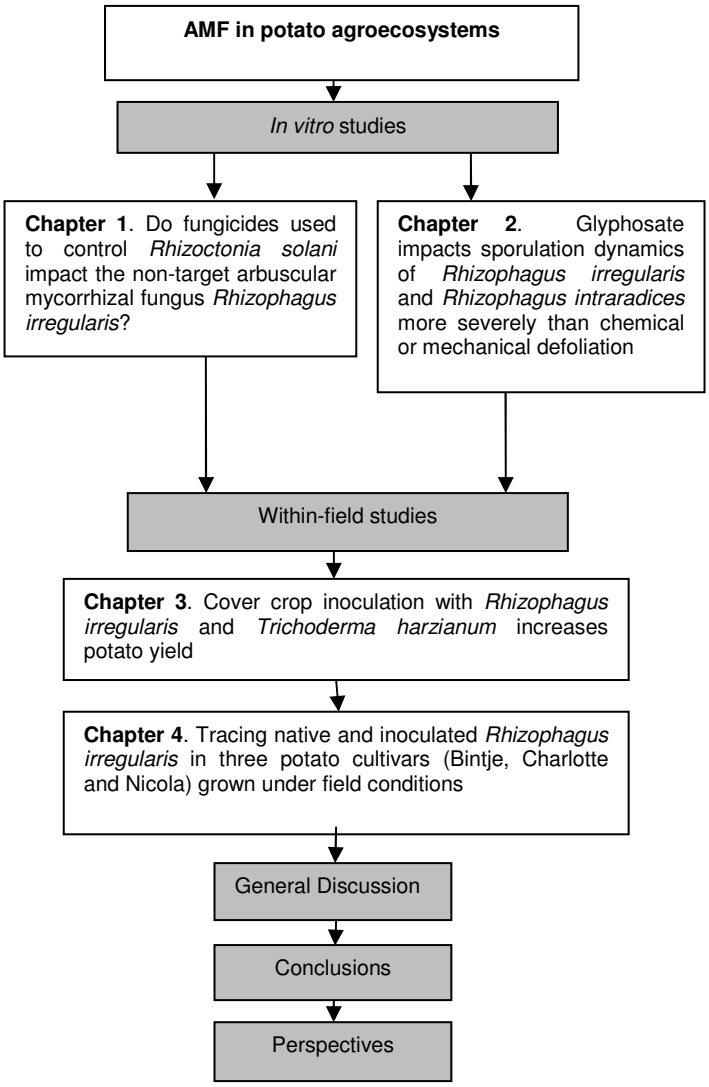

Figure 1. Outline of the thesis

In the **introduction** section, the context and the general objective of the study were presented. In the **State of the Art** section, the most recent and pertinent literature on AMF and their functions in agro-ecosystems were reviewed. This section also detailed the importance of AMF in potato crops and highlighted the impact of

some agricultural factors on them. Finally the possible field application of AMF and their efficacy in the field was reviewed.

The **Materials and Methods** section detailed the biological materials considered and briefly described the most important techniques used throughout the study.

Because of the complexity of soil environments, it was decided to conduct the first experiments using *in vitro* culture systems (**Part I. *In vitro* studies).** These systems are based on whole plants grown on synthetic medium in controlled growth chambers. They are particularly adequate for the non-destructive microscopic observations of the three-dimensional arrangements of AMF and to investigate the responses of the fungus to anthropogenic stresses such as the application of fungicides or herbicides.

The objective of **chapter I** was to evaluate the impact of fungicides with different modes of actions on growth and development of *Rhizophagus irregularis* MUCL 41833 in an integrated pest management strategy. To do so, the half maximal inhibitory concentration (IC_{50}) of three fungicides widely used to control *Rhizoctonia solani* in potato crops was determined on Potato Dextrose Agar medium supplemented with different concentrations of fungicides. Based on the IC_{50} necessary to control *R. solani* MUCL 49235, the impact of the fungicides on spore germination of the AMF was evaluated. Mycelium Donor Plant (MDP) *in vitro* culture systems (Voets *et al.*, 2009) were then used to evaluate the impact of the fungicides on root colonization of potato, spore production and fungal growth.

The results of chapter I were published in Mycorrhiza (Buysens et al. 2014).

The objective of **Chapter II** was to evaluate and compare, under *in vitro* culture conditions, the impact of two defoliation treatments (mechanical defoliation or chemical defoliation with a contact defoliant (diquat-formulation)) or a systemic herbicide (glyphosate-formulation) applied on potato or barrel medic, on the dynamics of spore production of *R. irregularis* MUCL 41833 or *R. intraradices* MUCL 49410. The half-closed mycorrhizal (H-AMP) *in vitro* culture system (Voets *et al.*, 2005) was used for the mycorrhization of potato and barrel medic plantlets and the weekly spore production rate was evaluated at different times.

The results of chapter II were submitted to Fungal biology.

In the second part of our study, the experiments were conducted within the field (**Part II. Within-field studies**).

The objective of **chapter III** was to investigate the impact of co-inoculation of the beneficial micro-organisms *R. irregularis* MUCL 41833 and *Trichoderma harzianum* MUCL 29707, applied at cover crop or at potato plantation, on potato yield. This study was conducted under organic or conventional agricultural systems over two growing seasons.

The results of chapter III were submitted to Applied Soil Ecology.

In **chapter IV** we developed haplotype- and species-specific molecular markers to trace and/or quantify AMF in potato crops. Direct inoculation of *R. irregularis* MUCL 41833 on three potato cultivars grown under field conditions and in competition with indigenous AMF populations was evaluated.

The chapter IV is a research paper in preparation for Mycorrhiza

In the **General Discussion** section, the major findings of the thesis were summarized and discussed.

Finally, in the **Conclusions and Perspectives** section, the major outcomes of the thesis were given and commented. Further studies to improve our understanding of the impacts of agricultural factors on AMF and conversely, the agricultural factors that favor the AMF, their adequate application and sustainability in potato crops were proposed.

Author's contribution

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

The Introduction and state of the art as well as the General Discussion, Conclusions and Perspectives were written by myself. These parts were not published.

Chapter I is a research paper published in Mycorrhiza (2014). My contribution to this chapter was approximately 80 %. The data collection on *R. solani* and *R. irregularis* (i.e. estimation of mycelial growth, spore germination, root colonization, spore production and fungal regrowth) and the practical work (*in vitro* plant system maintenance and follow-up) of this experiment were performed by myself. The data analysis with JMP and SAS Enterprise guide was performed with the help of Catherine Rasse ("Support en Méthodologie et Calcul Statistique", UCL). Writing of the paper was conducted by myself.

Chapter II is a research paper submitted to Fungal biology. My contribution to this chapter was estimated to 60 %. The practical work (i.e. estimation of spore production) was performed by myself with the help of Tankoano Yentema Priscile (Master student at the University Blaise Pascal – Clermont-Ferrand – France) which is acknowledged. Statistical analysis was performed with the help of Prof. Nicolas Schtickzelle (ELIB – UCL). Writing of the paper was conducted by myself.

Chapter III is a research paper submitted to Applied Soil Ecology. My contribution to this chapter was estimated to 60 %.The

practical work, data analysis and writing of the paper were conducted by myself. Field trial was performed by Nathalie De Jaeger (ELIM – Laboratory of mycology – (first year)) and myself (second year) with the help of Vincent César (Engineer at the Walloon Agricultural Research Centre - CRA-W) and Hugues Seutin (Technician at the Walloon Agricultural Research Centre – CRA-W). Inoculant preparation, root colonization analyses and MPN was performed with the help of Vanessa Lonnoy and François Ferrais (Technicians at ELIM – Laboratory of mycology - UCL).

Chapter IV is a research paper in preparation for Mycorrhiza. My contribution to this paper was estimated to 70 %. The practical work, data analysis and writing of the paper were conducted by myself. Field trial was performed with the help of Vincent César (Engineer at the Walloon Agricultural Research Centre - CRA-W) and Hugues Seutin (Technician at the Walloon Agricultural Research Centre – CRA-W). Inoculant preparation, root colonization analyses and Real-Time qPCR was performed by myself. Preliminary greenhouse experiment was performed by Pierre-Louis Alaux (PhD student at ELIM – laboratory of mycology - UCL) and myself.

Special thanks are addressed to the co-authors for their help and good advices in the revision of the four papers.

I. INTRODUCTION

Potato (*Solanum tuberosum*) is the most important tuber crop worldwide (FAO, 2013), which is also highly vulnerable to shoot (e.g. late blight caused by *Phytophthora infestans*) and root (e.g. root and tuber rot caused by *Rhizoctonia solani*) diseases. Potato is likewise among the highest demanding crop in fertilizers (Maynard and Hochmuth, 2007) because of its poorly developed and shallow root system that make its nutrient exploration and exploitation zone limited. Potato cropping need thus adequate disease control strategies and fertilization to maximize yield.

Nowadays, the application of synthetic fungicides and herbicides remain the primary agricultural factors to control diseases and weeds in the field, and the application of synthetic fertilizers the most common to sustain crop production. All these chemicals could have detrimental effects on the environment. There is thus an increasing need of integrated potato cultivation systems combining different management strategies with reduced use of mineral fertilizers and pesticides.

An alternative approach to fertilizers and pesticides is the valorization of soil beneficial microorganisms. These below-ground inhabitants can exert multiple functions (e.g. plant growth promoters and plant resistance enhancers) favorable to crop yield and health. Among the below-ground microorganisms, arbuscular mycorrhizal fungi (AMF) and *Trichoderma* sp. have been repeatedly reported to benefit plant growth and health (Arriola *et al.*, 2000, Camprubí *et al.*, 1995, Chandanie *et al.*, 2009). They are thus crucial players for the sustainable management of agricultural systems.

So far, most studies on the effects of AMF as single inoculant or as mixed inoculant with other beneficial microorganisms (e.g.

Trichoderma sp.) on potato were conducted under greenhouse or *in vitro* conditions. Most documented an increase in potato production as well as an improvement in resistance to above- and below-ground pathogens (Duffy and Cassells, 2000, Gallou *et al.*, 2011, McArthur and Knowles, 1993). To the contrary, field experiments that indubitably demonstrate the effect of AMF on crop performance are rare. This is because crops naturally become colonized by native AMF making it difficult to distinguish the effects of applied inoculum from local AMF communities, in particular if they harbor similar species to the inoculated ones. Moreover, the agricultural factors and environmental conditions may impact the development and performance of inoculated microorganisms.

For instance, fungicides could have similar target sites for beneficial fungi as for phytopathogenic fungi (Calonne *et al.*, 2012). Their application could thus represent a threat to AMF and in particular fungicides applied on the soil, seeds or roots. In contrast, herbicides may impact AMF indirectly via their effects on weeds or crops, which are hosts of these obligate root symbionts. Direct effects of non-fungicidal biocides are not excluded but are still not well understood (Gosling *et al.*, 2006).

Conversely, some agricultural factors may benefit AMF. For instance, the use of mycorrhizal cover crops before cash crop or as weed control strategy during cash crop, could increase the populations of AMF (native or inoculated) in soil and subsequent root colonization of cash crop (Boswell *et al.* (1998), Kabir and Koide (2000), Ramos-Zapata *et al.* (2012) and Lehman *et al.* (2012)). Presence of other beneficial microorganisms could even stimulate AMF populations.

The environmental compatibility, localisation and competition with other AMF and microbes for the establishment of communities also influence the success of AMF in the field (Dickie *et al.*, 2012).

In this complex soil environment it is thus necessary to trace the inoculated AMF within the potato roots and rhizosphere throughout the cultivation season and in comparison to the local AMF communities to comprehend the impact of agricultural factors and/or local microbes on the population dynamics of the inoculants introduced. Beneficial fungal inoculants should be environmental but also economical sustainable.

In the present thesis we investigated, *in vitro* and within field, the effects of some agricultural factors on the AMF and their subsequent benefits to potato crop. The AMF strains were provided by the Glomeromycota *in vitro* collection (GINCO) to save the time needed for the isolation of native strains from Belgian potato fields. *Rhizophagus* spp. were selected because of their ubiquitous distribution and occurrence in disturbed environments such as agricultural soils (Chagnon *et al.*, 2013). *Rhizophagus irregularis* MUCL 41833 and *Rhizophagus intraradices* MUCL 49410 are furthermore widely studied under *in vitro* conditions. They grow under temperate (70% relative humidity and 22/18°C (day/night) (e.g. IJdo *et al.* (2010)) as well as tropical conditions (70% relative humidity and 27/25°C (day/night)) (e.g. Koffi *et al.* (2013)).

In a first part (conducted *in vitro*) of the thesis, we studied the impact of fungicides and herbicides/defoliation treatments on *R. irregularis* MUCL 41833 or *R. intraradices* MUCL 49410. Numerous fungicides are used in potato crops. The choice was made for fungicides azoxystrobin, flutolanil and pencycuron controlling an

important below-ground pathogen, i.e. *Rhizoctonia solani*. These fungicides are directly applied on soil or on tubers and are thus in close contact with AMF. It was hypothesized that these fungicides impact more AMF than fungicides applied on shoots. The impact of these fungicides, with distinct mode of action and translocation into the plant, was investigated on the AMF *Rhizophagus irregularis* MUCL 41833. Then we analyzed how a contact[1] or systemic[2] herbicide affected the dynamics of spore production of *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410 in comparison with mechanical defoliation. Herbicides or defoliation treatments influence AMF development principally by inhibiting photosynthesis and thus C allocation from the plant to the fungus. But herbicides could also come in close contact with AMF depending on the absortion and translocation properties of the herbicide. The most worldwide used systemic herbicide glyphosate was tested because it impacts the whole plant (shoots and roots). It was compared with a contact herbicide diquat impacting only the foliar part of the plant. Both are authorized on young or old plants/weeds.

In a second part (conducted within-field), we evaluated the impact of different technical itineraries (e.g. cover crop co-inoculation) on AMF population densities, root colonization and growth promotion in potato crops under conventional as well as organic cropping systems. *Medicago sativa* was used as cover crop. This plant is an overwintering legume strongly mycorrhizal-dependent. It is also commonly used as host plant for the determination of AMF density of soils or commercial products (via Most Probable Number (MPN)

[1] A contact herbicide is not taken up into the plant tissue and act only on the plant part where the spary is deposited.

[2] A systemic herbicide is capable of being translocated to sites other than where it was adsorbed in suffiecient quantities to be biologically effective.

technique). *R. irregularis* MUCL 41833 was inoculated in combination with another beneficial microorganism *T. harzianum* MUCL 29707 because preliminary experiments (unpublished data) have shown that both microorganisms inoculated separately via a cover crop did not increase significantly potato yield in contrast to co-inoculation. Indeed, synergistic effects on plant growth were noticed between both micro-organisms. Interestingly, *T. harzianum* MUCL 29707 co-entrapped with *R. irregularis* MUCL 41833 stimulated AMF spore production (De Jaeger *et al.*, 2011). Finally, a molecular tool to trace and quantify AMF and in particular *R. irregularis* MUCL 41833 strain in the field and to distinguish from native *R. irregularis* was proposed. The mitochondrial Large SubUnit (mtLSU) gene of AMF was selected because it was shown to be homogeneous within the same isolate (Raab *et al.*, 2005) in contrast to the nuclear Large SubUnit (nrLSU) gene. Moreover, higher-resolution power of the mtLSU compared to that of nrLSU was shown (Börstler *et al.*, 2010).

II. STATE OF THE ART

1. Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts that, with a few exceptions (e.g. *Brassicaceae*, *Chenopodiaceae*), colonize the root system of all land plants (Smith and Read, 2008). Their presence have been detected in plant fossils from the Devonian period (400 million years) suggesting their probable co-existence with the first plants that have colonized the earth (Remy *et al*., 1994). These fungi seem to have remained unchanged over hundreds of millions of years in comparison to plants that have highly diverged with evolution and adapted their life cycle to contrasting ecological niches (Croll and Sanders, 2009).

AMF develops within the roots and extends their hyphae in the soil scavenging for nutrients (particularly phosphorus and nitrogen) and water that are subsequently transported to the plant in exchange for carbohydrates produced by photosynthesis (Smith and Read, 2008). They thus develop in two interconnected niches; the root and the soil as intraradical and extraradical mycelium (IRM and ERM, respectively). The transport of minerals and carbohydrates from AMF to plant and vice versa is facilitated by the coenocytic nature (i.e. the absence of septa in living hyphae) and protoplasmic continuity of the mycelium (Smith and Read, 2008). The ERM extends far beyond the root exploration zone, takes up nutrients and water and translocate them to the IRM within the roots (Jakobsen *et al*., 1992, Johansen *et al*., 1993). The minerals are then transferred from the fungal cells to the plant cells and vice versa for the carbohydrates, via the arbuscules, the main site of nutrient exchange between the two partners of the symbiosis (Smith and Read, 2008).

The consequence of nutrients accumulation in plants is their improved growth and yield. The association of the AMF with plants also increases their resistance/tolerance to biotic and abiotic stresses (Gianinazzi *et al.*, 2010). In addition, these root symbionts stabilize soil aggregates via the production of glomalin, a glycoprotein (Rillig and Mummey, 2006, Schloter *et al.*, 2003).

AMF have been sampled on all continents except Antarctica (Kivlin *et al.*, 2011, Öpik *et al.*, 2010). The occurence of Glomeromycota in soil is influenced by environmental factors such as climate (Öpik *et al.*, 2010), soil (Lekberg *et al.*, 2007) and plant community (Öpik *et al.*, 2010). AMF diversity vary across many environmental gradients. The tolerance of AMF to freezing and drying is known to shift between AMF taxa (Klironomos *et al.*, 2001). AMF become less frequent and diverse at higher soil nutrient and moisture concentrations (Augé, 2001). Along altitude gradients, AMF composition often becomes less diverse on mountain tops than at lower altitudes, but this effect is driven by the composition of plant species (Gai *et al.*, 2012).

1.1 AMF life cycle

AMF cannot complete their life cycle without being associated to a suitable host. Their dissemination within soil is done via spores produced on the IRM or ERM and structures produced strictly within root fragments (i.e. vesicles or intraradical spores), all termed propagules.

The fungal life cycle starts with a reciprocal exchange of diffusible signals between roots and AMF propagules (Gutjahr and Parniske, 2013). AMF detect the host plant via root exudates containing specific signal molecules (i.e. strigolactones - (Akiyama *et*

al., 2005, Besserer *et al.*, 2006)) that induce germination and hyphal branching (Buee *et al.*, 2000, Giovannetti *et al.*, 1993, Nagahashi and Douds D.D, 2000). Other plant diffusible molecules, i.e. the cutin monomers, seem to play a role in the differentiation of AMF tip-growing hyphae into hyphopodia (also known as appressoria) at the surface of epidermic cells before penetration into the root cortex (Murray *et al.*, 2013, Wang *et al.*, 2012). These first steps are controlled by a plant signaling pathway, referred to as the common symbiosis signaling pathway (CSSP), so named because in legumes, this pathway is also required for the formation of symbiosis with rhizobia (Harrison, 2012). In return, the AMF release signal molecules that induce the expression of plant genes, calcium spiking in rhizodermal cells, starch accumulation in roots, and lateral root formation prior to colonization (Chabaud *et al.*, 2011, Gutjahr *et al.*, 2009, Kosuta *et al.*, 2008, Kuhn *et al.*, 2010, Maillet *et al.*, 2011, Oláh *et al.*, 2005). These molecules include lipochitooligosaccharides (LCOs) (Maillet *et al.*, 2011) and chitooligosaccharides (COs) (Genre *et al.*, 2013) which are highly similar to Nod factors released by rhizobia (Maillet *et al.*, 2011) (Figure 2).

Following contact with the epidermal cells, the plant nucleus migrates toward the hyphopodium. Then the nucleus moves away from the site of hyphopodium contact and crosses the plant cell vacuole. Fully differentiated cortical cells develop a transvacuolar structure, the pre-penetration apparatus (PPA) (Genre *et al.*, 2005) that open the passage for hyphae to enter deeper in the cell layers (Figure 2).

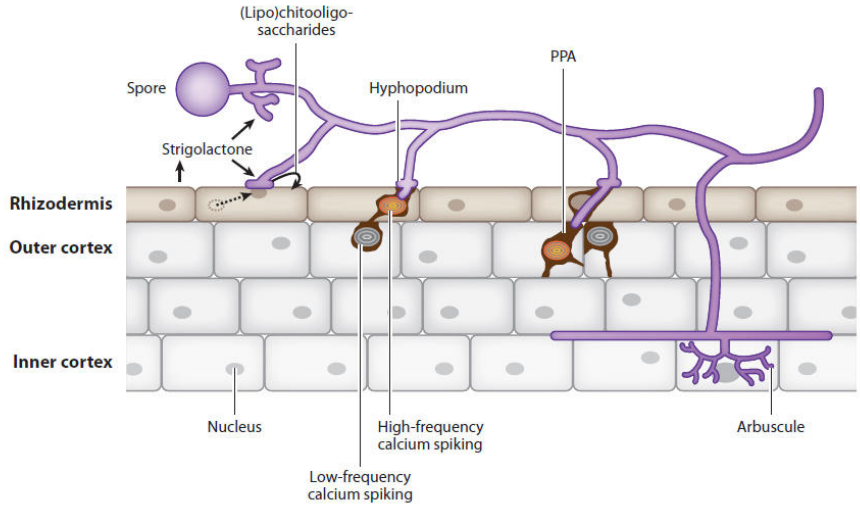

Figure 2. Colonization of AMF into root cortical cells (from Gutjahr and Parniske (2013) in Annual Review of Cell and Developmental Biology).

Within the root cortex, AMF develop following the *Arum*- or *Paris*-type morphology (Smith and Read, 2008). 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 (Figure 3A). 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). Most plants support intermediate forms to these patterns, which are called the "*Arum*-*Paris*-type continuum" (Dickson, 2004).

The first stages of arbuscules formation resemble that of the PPA development (Genre *et al.*, 2008). In the case of the *Paris*-type, PPA formation reaches several cortex cells ahead of the colonization front. For the *Arum*-type, separated PPA-like endoplasmic reticulum

(ER) aggregates develop in the inner cortical cells upon contact with intracellular hyphae (Genre *et al.*, 2008). Exocytosis of Golgi vesicles at the tips of the growing hyphae is involved in the extension of the perifungal membrane (PM) surrounding intracellular hyphae and the plant-derived periarbuscular membrane (PAM) (Genre *et al.*, 2012, Ivanov *et al.*, 2012). The PAM is in continuum with the plasma membrane and surrounds each branch of the arbuscule, preventing direct contact with the plant cytoplasm. The plant-membrane surface increases during arbuscule formation, which increases the interface available for nutrient and metabolite exchange between the symbiotic partners (Gutjahr and Parniske, 2013).

In the final step of the root colonization, the members of all genera, with the exception of the *Gigasporaceae*, develop vesicles (Figure 3A) in the cortex. The vesicles are reproductive and storage structures (Smith and Read, 2008).

Following root colonization, AMF produce an ERM network in the soil by the proliferation of straight-growing thick-walled hyphae called runner hyphae (RH). The ERM develop branched absorbing structures (BAS) (Figure 3B) which are supposedly involved in the uptake of minerals (Bago *et al.*, 1998). During the symbiosis, spores (Figure 3C, D) and auxiliary cells (Figure 3E) (in the case of *Gigasporaceae*) are formed on the ERM. Spores are formed apically or intercalary along RH of higher order and represent the final stage in the fungal life cycle (Declerck *et al.*, 2004).

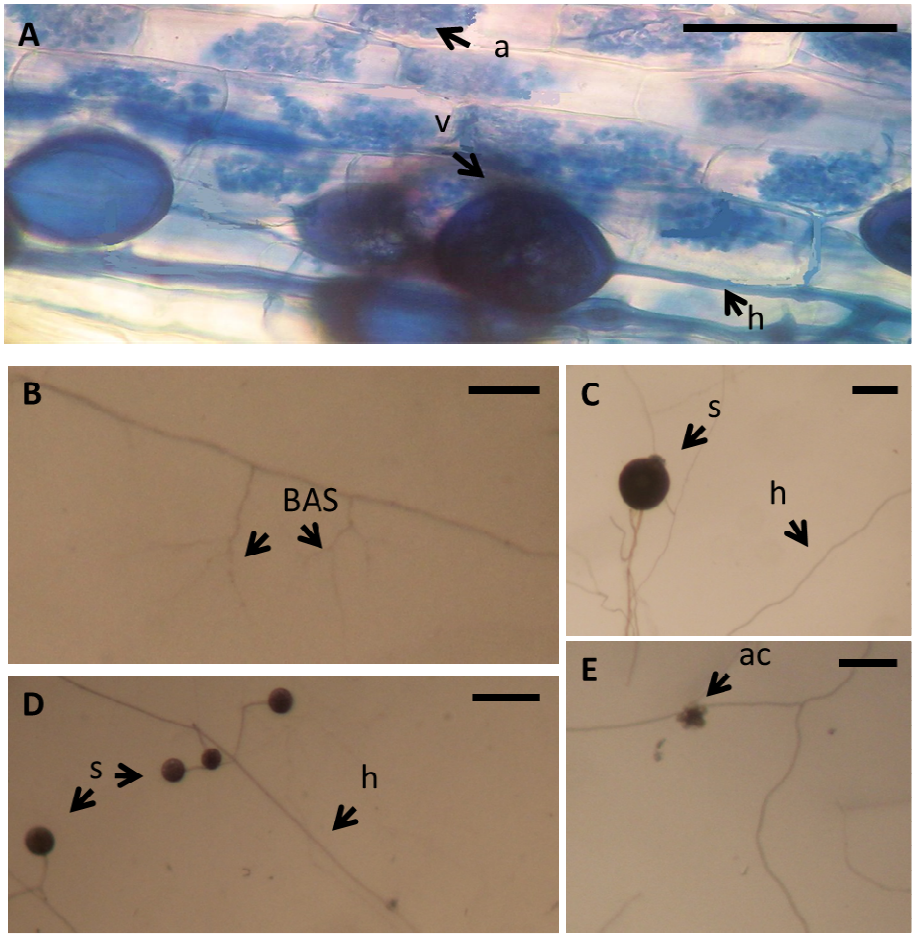

Figure 3. (A) Intraradical AMF structures with arbuscules (a), vesicles (v) and hyphae (h); Extraradical structures with **(B)** Branched absorbing structures (BAS) **(C)** *Gigaspora* sp. spores (s) and hyphae (h); **(D)** *Rhizophagus irregularis* spores (s) and hyphae (h) and **(E)** *Gigaspora* sp. auxiliary cell (ac). Bars 200 µm.

1.2 Plant-AMF interface

The mutualism between AMF and a host plant is characterized by a transfer of minerals (e.g. N and P) from the fungus to the plant in exchange for C from the plant to the fungus (Smith and Read, 2008) and schematized in Figure 4. Host C is transferred across the plant-fungal interface, taken up by the fungus and translocated to the ERM.

Fungal C is subsequently allocated to the IRM and ERM. In the symbiotic interface, C is taken up from the host in the form of hexose and converted to carbohydrates and storage lipids as triacylglycerides (TAGs) in the IRM (Bago *et al.*, 2002). Lipids and glycogen are translocated from IRM to ERM. Storage (glycogen and trehalose) and structural (chitin) carbohydrates are synthesized in the ERM from hexose that is derived from exported carbohydrates as well as from lipids. In exchange, P is taken up from the soil as inorganic P (Pi) and converted into polyphosphates (PolyP). PolyP plays a key role in transferring nutrients to the IRM. Nitrogen, as NH_4^+ and NO_3^-, is likewise taken up from the soil by AMF, and converted mainly in arginine (Arg). PolyP are negatively charged polyanions that can also bind the basic amino acid Arg. In the IRM, PolyPs are remobilized and release inorganic phosphate (Pi) and Arg. Arg is further broken down to inorganic N (specifically NH_4^+), and then transferred across the plant-fungal interface.

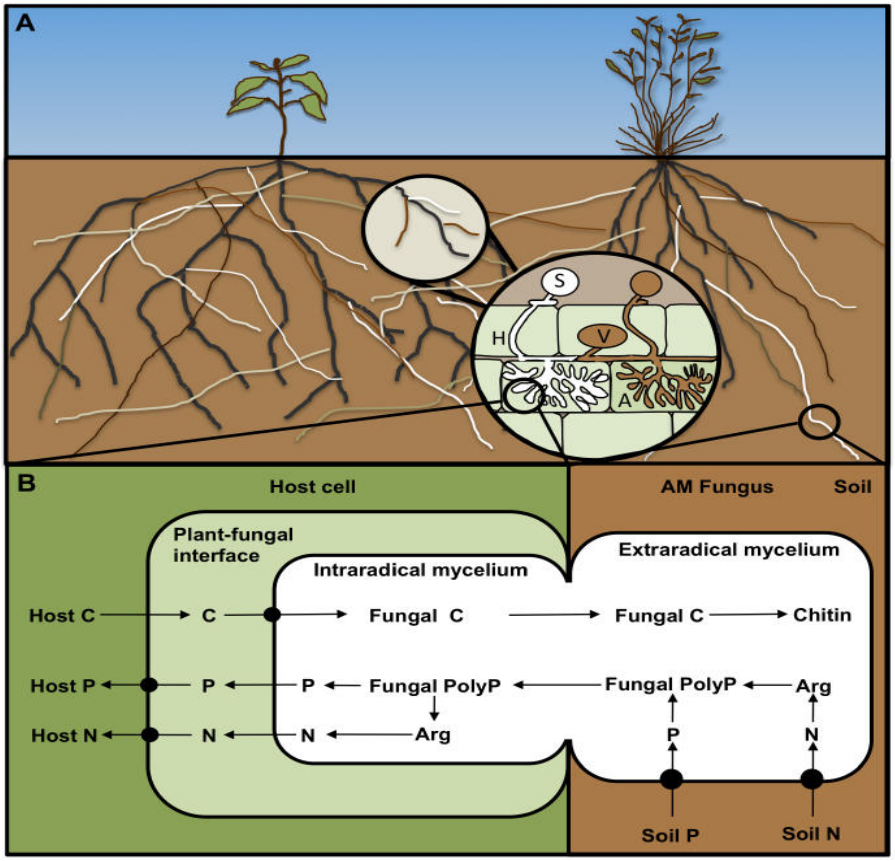

Figure 4. Schematic drawing of the arbuscular mycorrhizal mutualism and resource exchange mechanisms. **(A)** Plants interact with diverse AMF communities and AMF interact with multiple hosts. The mutualism is characterized by a transfer of minerals (e.g. N and P) from the fungus for to the plant in exchange for C from the plant to the fungus. The transfer of nutrients occurs across the arbuscules (A). Fungal C is subsequently allocated to the hyphae (H), vesicles (V) and/or spores (S). **(B)** Nutrient exchange between plant and fungal partner. (From Kiers *et al.* (2011) Science).

1.3 Taxonomy and phylogeny of AMF

Before the emergence of molecular techniques, the only way to identify AMF was by microscopic examination of spores (Figure 5). However, these structures are relatively simple with only a few

discriminating characters. In addition, AMF are pleomorphic and spores may differ in morphology, size or color in response to environmental factors (Krüger *et al.*, 2012, Krüger *et al.*, 2009). Finally, spores sampled from soil are often not matching fresh reference material produced in pots. Molecular techniques enabling the identification of AMF taxa, which colonize the plant (Clapp *et al.*, 1995, Hempel *et al.*, 2007), in addition to morphological observations of soil-born spores (Johnson *et al.*, 1991), and the access to a large collection of well-characterized cultures are thus required for an accurate identification (Krüger *et al.*, 2009).

Different molecular markers have been used to identify and classify AMF species. Among these, the nuclear (nr) ribosomal (r)RNA genes are the most frequently considered because they can be amplified from small quantities of DNA and several primers targeting the rDNA regions were claimed to be AMF-specific. In addition, the presence of different rDNA regions allows both robust phylogenetic analyses and species resolution (Gamper *et al.*, 2009, Stockinger *et al.*, 2009). Four rDNA regions, individually or in combination, are used as molecular markers: the partial small subunit rRNA gene (SSU), the partial large subunit rRNA gene (LSU), 5.8S rRNA gene (5.8S) and the Internal Transcribed Spacers (ITS).

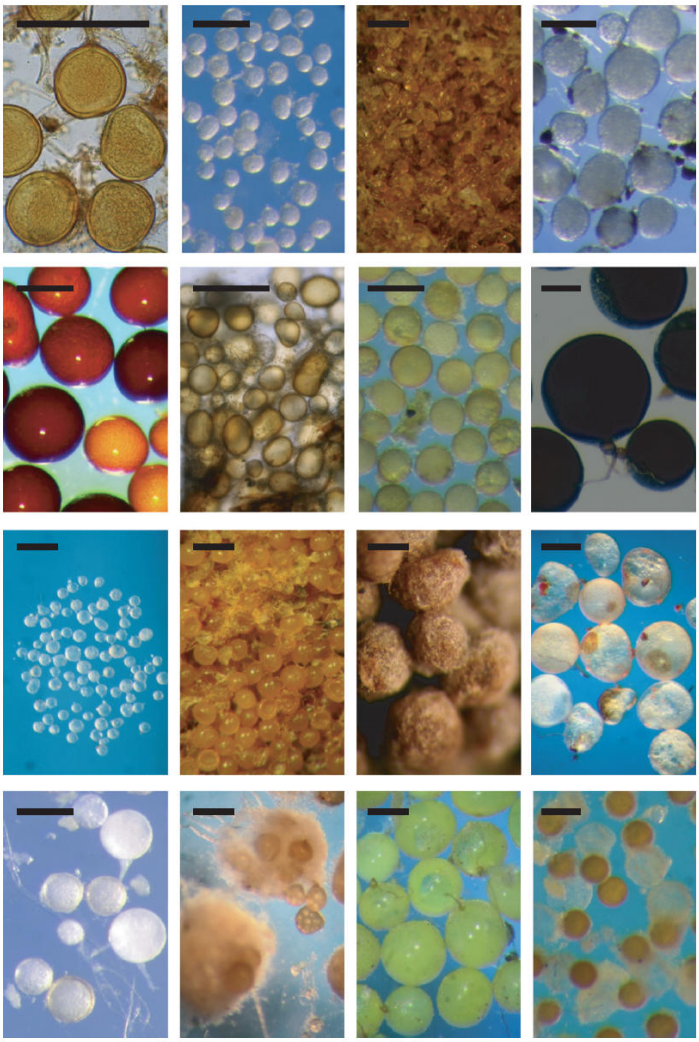

Figure 5. Spores of arbuscular mycorrhizal fungi representing the different genera of the Glomeromycota. Row 1, *Glomus, Archaeospora, Redeckera, Pacispora*; row 2, *Acaulospora, Rhizophagus, Claroideoglomus, Racocetra*; row 3, *Paraglomus, Diversispora, Sclerocystis, Scutellospora*; row 4, *Ambispora, Funneliformis, Gigaspora, Entrophosphora*. Bars, 200 µm. (From Young (2012) in New Phytologist).

Kohout *et al.* (2014) compared four routinely-used AMF-specific primers covering i) the partial small subunit (SSU), ii) the partial large subunit (LSU), iii) the partial SSU, ITS and 5.8S (Redecker, 2000, Redecker *et al.*, 2003) and iv) the partial SSU-ITS-5.8S-partial LSU region (Krüger *et al.*, 2009) (Figure 6). The primers used by Krüger *et al.* (2009) seemed to yield higher AMF diversity than the SSU primers. AMF mitochondrial (mt)DNA have also been used for phylogenetic analysis (Börstler *et al.*, 2008). The problems of apparent polymorphism of nr rDNA and protein-coding genes within single spores could be circumvented by using this independent genetic system within the fungal organism. AMF mtDNA is homogeneous within single isolates (Raab *et al.*, 2005), making it a good target for marker development. The exon phylogeny of a region of the mtLSU showed superior resolution among subclades of *R. irregularis* compared to nuclear-encoded rDNA ITS (Börstler *et al.*, 2008). Particularly, the mtLSU introns were shown to be highly sensitive molecular markers to genotype different isolates of *R. irregularis* (sensu lato) and it was used to differentiate mtLSU haplotypes directly from colonized roots (Börstler *et al.*, 2008) which is a promising approach to better understand the diversity and dynamics of field communities and populations of AMF.

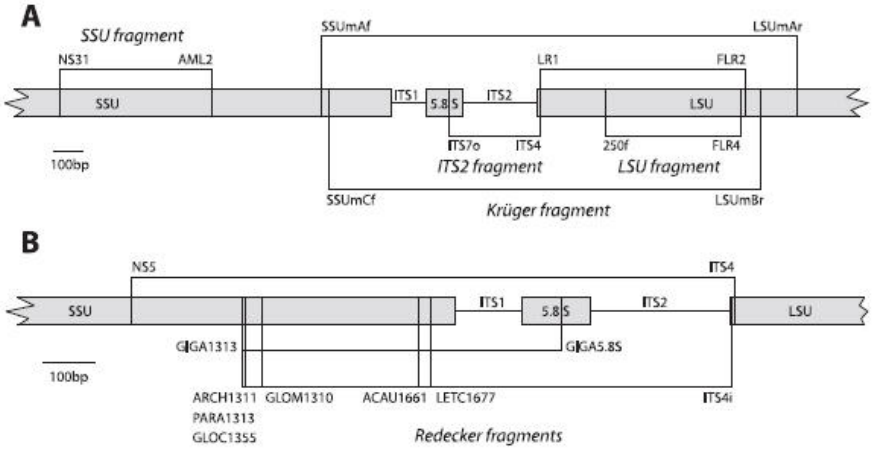

Figure 6. Target nuclear ribosomal DNA regions and primers locations. Lines above the rDNA represent products of the first PCRs. Lines below the rDNA represent products of the second PCRs. The regions studied are shown on the upper **(A)** figure except for those amplified by the primers developed by Redecker (2000) and Redecker *et al.* (2003) which are shown on the below figure **(B)**. (From Kohout *et al.* (2014) in Soil Biology and Biochemistry).

In the last decades, frequent taxonomic changes have been proposed for the classification of AMF. Initially, these fungi were classified in the Zygomycota due to an observational error: a sporocarp was found with spores belonging to both Endogone (belonging to the Endogonaceae) (Zygomycota) and *Glomus*. Because of their superficial similarity, it was presumed that one was an anamorph of the other (Thaxter, 1922). A review of the group was carried out by Gerdemann and Trappe (1974), during which two new genera (*Acaulospora* and *Gigaspora*) were identified within the Endogonaceae. Later the monophyletic classification of the AMF was brought into doubt. From analysis of the near full-length SSU rRNA gene sequences of 51 described and some undescribed species, Schüßler *et al.* (2001) demonstrated that AMF belong to a distinct

monophyletic group quite separate from other fungi. These authors reclassified them in a new Phylum, the Glomeromycota. Schüßler *et al.* (2001) subdivided the Glomeromycota into 4 orders (Paraglomerales, Archaeosporales, Diversisporales and Glomerales) and 8 families (Paraglomeraceae, Archaeosporaceae, Geosporaceae, Acaulosporaceae, Diversisporaceae, Gigasporaceae, Glomeraceae Glomus-group A, Glomeraceae Glomus-group B). Later, a major revision of the *Glomerales* was published by Schüßler and Walker (2010). One example is *Rhizophagus irregularis* (syn. *Glomus irregulare*, formerly named *Glomus intraradices*; see Stockinger *et al.* (2009)). The list is constantly updated (http://schuessler.userweb.mwn.de/amphylo/) as illustrated in Table 1. Recently, Sieverding *et al.* (2014) proposed *Rhizoglomus* gen. nov. (Glomeraceae, Glomeromycetes), typified by *Glomus intraradices* [= *Rhizoglomus intraradices*] in the place of *Rhizophagus intraradices*. Phylogenetically, the genus forms a separate clade in the Glomeraceae comprising *R. intraradices, R. aggregatum, R. antarcticum, R. arabicum, R. clarum, R. custos, R. fasciculatum, R. invermaium, R. irregulare, R. manihotis, R. microaggregatum, R. natalense,* and *R. proliferum*. These authors argued that *Rhizophagus* genus was previously assigned to *Rhizophagus* (type: *R. populinus*), a pathogenic genus that does not belong to the Glomeromycota. They defined the *Rhizoglomus* genus as species of AMF that frequently form abundant spores in soil and roots and that are morphologically characterized by spores with cylindrical subtending hyphae (usually with an open pore at the base) and at least two or three (rarely up to five) distinct wall layers (Sieverding *et al.*, 2014).

Table 1. Classification of 'AMF', based on Redecker *et al.* (2013) and Schüßler and Walker (2010).

Phylum *Glomeromycota*		
Class *Glomeromycetes*		
Orders (4)	**families (11)**	**genera (25)**
Glomerales	***Glomeraceae***	***Glomus***
		Funneliformis (former *Glomus* Group Aa, '*Glomus mosseae* clade')
		Rhizophagus (former *Glomus* Group Ab, '*Glomus intraradices* clade')
		Sclerocystis (basal in former *Glomus* Group Ab)
		Septoglomus
	Claroideoglomeraceae	***Claroideoglomus*** (former *Glomus* Group B, '*Glomus claroideum* clade')
Diversisporales	***Gigasporaceae***	***Cetraspora***
		Dentiscutata
		Gigaspora
		Intraornatospora (insufficient evidence, but no formal action was taken)
		Paradentiscutata (insufficient evidence, but no formal action was taken)
		Racocetra
		Scutellospora
	Acaulosporaceae	***Acaulospora*** (including the former *Kuklospora*)
	Pacisporaceae	***Pacispora***
	Diversisporaceae	***Corymbiglomus*** (insufficient evidence, but no formal action was taken)
		Diversispora (former *Glomus* Group C)
		Otospora (insufficient evidence, but no formal action was taken)
		Redeckera
		Tricispora (insufficient evidence, but no formal action was taken)
	Sacculosporaceae	***Sacculospora*** (insufficient evidence, but no formal action was taken)
Paraglomerales	***Paraglomeraceae***	***Paraglomus***
Archaeosporales	***Geosiphonaceae***	***Geosiphon***
	Ambisporaceae	***Ambispora***
	Archaeosporaceae	***Archaeospora*** (including the former *Intraspora*)

2. Functions of AMF in agroecosystems

Arbuscular mycorrhizal fungi are present in nearly all (agro)-ecosystems and exert multiple functions. These microorganisms

a) Improve soil stability and water retention

b) Improve plant mineral nutrition

c) Improve plant resistance/tolerance to abiotic stresses

d) Improve plant resistance/tolerance to biotic stresses

e) Modify plant metabolism and physiology

2.1 Soil stability and water retention

AMF develop a complex and ramified network of hyphae into the surrounding soil which can reach up to 30 m of fungal hyphae per g of soil (Wilson *et al.*, 2009) and represent from 20 to 30% of the soil microbial biomass (Leake *et al.*, 2004). This mycelium can have a binding action on the soil and improve soil structure. In addition, AMF participate in the production of soil micro-aggregates via the release of a hydrophobic, “sticky” proteinaceous substance called glomalin. This glycoprotein is excreted by the ERM, bind soil micro-particles (Rillig *et al.*, 2002) and contributes to soil stability and water retention (Bedini *et al.*, 2009).

2.2. Plant nutrition

The ERM takes up minerals (principally phosphorus) at distances far beyond the roots exploration zone and transports them to the plants. Phosphorus is an essential mineral nutrient for plant growth, but inorganic phosphates (Pi) have limited diffusion capacities

in soil causing a Pi depletion zone around the roots (Roose and Fowler, 2004). The mycelium growing from the roots extends far beyond this depletion zone increasing the volume of soil explored and thus the 'indirect' access to P sources for plants (Smith and Read, 2008). At low phosphate availability for the plant, the phosphate-uptake route of AMF-colonized plants switches entirely from direct rhizodermal-uptake pathway to symbiotic-uptake pathway, which involves a switch between two different sets of phosphate-uptake transporters (Gai *et al.*, 2012, Smith *et al.*, 2003). The capability of AMF to store P in the form of polyphosphates (PolyPs), allows the fungus to keep the internal Pi concentration relatively low, and allows an efficient transfer of P from the ERM to the IRM (Hijikata *et al.*, 2010). Moreover, the production and secretion of acid phosphatases and organic acids by AMF facilitate the release of P from organic complexes (Ezawa *et al.*, 2005) and phosphate solubilizing bacteria appear also to be promoted in the presence of AMF (Toro *et al.*, 1997). The ERM also has the ability to assimilate nitrogen (N) in the forms of NH_4^+ and possibly NO_3^- (Govindarajulu *et al.*, 2005, Tian *et al.*, 2010). Nitrogen is then translocated to the IRM as arginine and, seemingly, transferred to the plant in the form of NH_4^+ (Govindarajulu *et al.*, 2005, Tian *et al.*, 2010). The more thorough colonization of soil environment of AMF colonized plants and efficient high-affinity NH_4^+ and NO_3^- transporters makes the assimilation of N by the plant via the AMF more efficient (Johansen *et al.*, 1993). AMF contributes also to N-mineralization directly through the secretion of N-mineralization enzymes or indirectly through the modification of the microbial community that is able to degrade organic matter (e.g. Griffiths and Swanson (2001); Liebich *et al.* (2007)). Potassium (K) and micronutrients such as copper (Cu), iron (Fe), magnesium (Mg), calcium (Ca) and zinc (Zn) are also transported by the AMF to the

host plant (Cavagnaro, 2008, Clark and Zeto, 2000, Smith and Read, 2008).

2.3 Plant resistance/tolerance to abiotic stresses

Abiotic stresses such as drought, salinity or mineral depletion are responsible for major losses in agricultural systems. The potential of AMF to increase plant resistance/tolerance to abiotic stress conditions is largely documented and involve several processes. For example, under salinity and drought stress conditions, the increase of osmolytes concentration (i.e. carbohydrates and electrolytes) in plant roots (Daei *et al.*, 2009, Ruiz-Lozano *et al.*, 1996), the increase in root hydraulic conductivity, the improved stomatal regulation and improved contact with soil particles through the binding effect of hyphae, enabling water to be extracted from smaller pores (Augé, 2001) have been reported.

2.4 Plant resistance/tolerance to biotic stresses

Several studies have demonstrated that AMF increase plant resistance/tolerance to biotic stresses caused by soil-borne and above-ground pathogens such as fungi or Oomycetes (e.g. *Fusarium*, *Rhizoctonia*, *Verticillium*, *Phytophthora* and Pythium – see review by Azcon-Aguilar and Barea (1996); Harrier and Watson (2004); Whipps (2004)). Reduction in the severity of disease has been frequently reported rather than a total inhibition. This often resulted in a significant increase in yield, despite the fact that pathogen infection generally reduced AMF colonization (Hassan Dar *et al.*, 1997, Karagiannidis *et al.*, 2002, Torres-Barragán *et al.*, 1996). Multiple mechanisms of resistance are operating simultaneously (Whipps, 2004). One is the exclusion or competition for space (Azcon-Aguilar and Barea, 1996). Pre-colonized plants with AMF are generally more

resistant to pathogen attack (Matsubara *et al.*, 2001, Slezack *et al.*, 1999, Sylvia and Chellemi, 2001). Other mechanisms are changes in root exudates (Filion *et al.*, 1999, Norman and Hooker, 2000) that cause changes in rhizosphere microbial community (Hassan Dar *et al.*, 1997) or changes to the host root architecture (Yano *et al.*, 1996). But the protective effects conferred by AMF were principally attributed to the changes in root biochemistry and subsequent induction of several defense pathways in the mycorrhizal plants, via the Mycorrhiza Induced Resistance (MIR) mechanism (Cameron *et al.*, 2013, Jung *et al.*, 2012, Pozo and Azcón-Aguilar, 2007, Pozo *et al.*, 2010). Other pests and diseases caused by nematodes (e.g. Talavera *et al.* (2001), above-ground fungal pathogens (e.g. Feldmann and Boyle (1998)) and herbivores (e.g. Gange *et al.* (2002)) may also be suppressed by AMF. In most cases the degree of control varies between AMF species (Gange *et al.*, 2003, Matsubara *et al.*, 2000).

2.5 Modification in plant metabolism and physiology

AMF have been reported to increase mineral content and the production of plant secondary metabolites (e.g. organosulfides, polyphenols (phenolic acids, anthocyanins, flavonoids), phytosterols, stilbenes, vitamins and terpenoids including carotenoids) in crops (Hooper and Cassidy, 2006, Kirby and Keasling, 2009, Stan *et al.*, 2008). For example, an increase of 67% of total phenolics concentration in shoots of *Echinacea purpurea* was reported in presence of *G. intraradices* DAOM 181602 (Araim *et al.*, 2009).

3. Importance of AMF in potato crop

3.1 Potato crop

Potato (*Solanum tuberosum*) is the most important tuber crop worldwide (FAO, 2013). It is cultivated in more than 125 countries and consumed almost daily by more than a billion of people. Potato tubers are rich in several micronutrients and vitamin C. In Europe it is highly mechanized and cultivated as monoculture. Nowadays, the vast majority of cultures are grown under conventional agricultural systems and a minority (below 4 %) under organic agricultural systems (Canali *et al*., 2012).

Potato production is one of the most demanding crop in fertilizers, due to its poor developed and shallow root system (Maynard and Hochmuth, 2007). For instance, P deficiency can be a limiting factor to yield in commercial potato production (Mackay and Barber, 1984). Microorganisms such as AMF could reduce the potato dependency for nutrients because of their capacity to take up and translocate nutrients from places far beyond the roots exploration zone.

Potato crops are also facing numerous pathogens, causing a constant menace to its production worldwide. Diseases are spread generally by seed but also by wind, running water, soil, bags and equipment. The soilborne fungus *Rhizoctonia solani* and the late blight causal agent *Phytophthora infestans*, an Oomycete, are among the most damaging microbes. This last decade, the occurrence of *R. solani* has significantly increased in Europe. Yield losses are estimated to 20 % (Grosch *et al*., 2006). Potato late blight, caused by *P. infestans*, was responsible for the European potato famine in 1845. Nowadays, the annual losses in Europe (i.e. costs of damage and

control) caused by this pathogen are estimated above 1 billion € (Haverkort *et al.*, 2008). The control of these two diseases by cultural factors is limited, while the use of fungicides are the most recommended strategies for their control. However, in the recent decade, microorganisms such as *Pseudomonas* sp., AMF and fungal antagonists (e.g. *Trichoderma* spp.) (Brewer and Larkin, 2005, Whipps, 2004) have been considered as promising alternatives to control plant pathogens and to minimize the impact of chemicals on the environment. However, costs of beneficial fungal inoculants should not exceed costs of chemical inputs.

3.2 Diversity and abundance of AMF in potato cropping systems

To our knowledge, four studies reported on the diversity of AMF within potato fields. Three were conducted in the Andean highlands, the centre of origin and diversity of potato (Davies *et al.*, 2005c, Senés-Guerrero and Schüßler, 2015, Senés-Guerrero *et al.*, 2013) and one in Italy in a potato farming area (Cesaro *et al.*, 2008). (Davies *et al.*, 2005c) reported the predominance of *Gigaspora*, *Glomus* and *Scutellospora* species within potato soils. This study was conducted via spore morphotyping. More recently, Senés-Guerrero *et al.* (2013) used molecular tools to evaluate the AMF diversity in Andean potato roots and soil. They identified sequences of species belonging to 8 of the 11 families in the Glomeromycota, *Paraglomeraceae*, *Archaeosporaceae*, *Ambisporaceae*, *Glomeraceae*, *Claroideoglomeraceae*, *Gigasporaceae*, *Acaulosporaceae* and *Diversisporaceae*. The three more abundant species were *Funneliformis mosseae* (syn. *Glomus mosseae*), an unknown *Claroideoglomus* and *Rhizophagus irregularis.* The same samples were later analysed with 454-pyrosequencing (Senés-

Guerrero and Schüßler (2015). 67% of the potato plants, analyzed from 12 Andean sites, were colonized by species belonging to *Acaulospora, Cetraspora, Claroideoglomus* and *Rhizophagus* simultaneously. These genera appeared to host most of the species associated to potato in the Andean region. They also demonstrated that potato roots could be colonized by more than 25 AMF species simultaneously. In the study conducted in the Italian potato crop area (Cesaro *et al*., 2008), no sequences corresponding to Gigasporaceae or Acaulosporaceae were detected. All sequences corresponded to the genus Glomus (group A and B) and more precisely to the species *Rhizophagus, Glomus, Funneliformis* or *Claroideoglomus.* After phylogenetic analyses eight monophyletic ribotypes could be distinguished. Three groups, *G. mosseae* (syn. *Funneliformis*), *G. intraradices* (syn. *Rhizophagus*), and *G. claroideum* (syn. *Claroideoglomus*) sequences, clustered with previously identified AMF sequences. Five other groups did not cluster with any known Glomus sequence. *Gigaspora* species were not detected in the studies of Senés-Guerrero *et al.* (2013) and Cesaro *et al.* (2008), in contrast to the study of Davies *et al.* (2005c) where spores of *Gigaspora* were found in the soil.

G. intraradices (syn. *Rhizophagus*) was reported to be a preferential colonizer of potato in agricultural soils in Italy and more dominant in potato roots (>90%) than in soil (<10%) (Cesaro *et al*., 2008). Senés-Guerrero *et al.* (2013) established by Sanger sequencing that *R. irregularis* was the third most abundant potato root colonizer in Andean fields. This last specie is usually described to be ubiquitous (Sýkorová *et al*., 2007), occurring frequently in, or dominating, agricultural systems. But Senés-Guerrero *et al.* (2013) observed in Andean potato crop that *F. mosseae* was much more

widespread and frequent than *R. irregularis* in potato roots, whereas in the study of Cesaro *et al.* (2008) *F. mosseae* was detected only in soil and not in roots. Indeed, differences can exist among AMF communities in the potato rhizosphere soil and roots (Cesaro *et al*., 2008). The very frequent appearance of *Acaulospora* spp. in the Andes (Senés-Guerrero and Schüßler, 2015) might directly or indirectly be related to altitude, since members of this genus were also frequently reported at altitudes around 3000 m in the Alps and in the Chilean Andes (Oehl *et al*., 2011, Oehl *et al*., 2006), as well as in the South American Puna grassland at 3520 m altitude (Lugo *et al*., 2008) and at the Tibetan Plateau (Gai *et al*., 2012, Li *et al*., 2014).

Senés-Guerrero *et al.* (2013) observed that the number of species and diversity index was relatively constant in soil, while in roots it seemed to differ with the developmental stage of the potato plant. AMF were most diverse in roots at the emergence stage with *F. mosseae* being the most abundant. At the flowering stage, diversity in the roots was the lowest with an unknown *Claroideoglomus sp*. as dominant fungus. In contrast, analysis with 454-pyrosequencing (Senés-Guerrero and Schüßler, 2015) showed that plant stage did not significantly change community composition. Hannula *et al.* (2012) nevertheless, reported that the plant growth stage significantly affected AMF communities in rhizosphere soil of potato plants, regardless of the plant cultivar studied. At the senescence stage, soil hosted the most diverse fungal community (Hannula *et al*., 2012).

3.3 Benefits of AMF to potato

A number of studies have reported a beneficial effect of AMF on potato. Improved plant growth and development was noticed under greenhouse and *in vitro* conditions with AMF applied as a

single microorganism or as a mixture of several isolates (Davies *et al*., 2005a, Duffy and Cassells, 2000, McArthur and Knowles, 1993). Increased plant growth was related to an increase in P concentration in potato plants associated with three different AMF species (*G. intraradices*, *G. dimorphicum* and *G. mosseae*) (McArthur and Knowles, 1993). These fungi also increased the concentrations of N, K, Mg, Fe, and Zn in potato plants. *G. intraradices* was the most efficient in promoting plant growth, whereas *G. dimorphicum* was less efficient and *G. mosseae* produced intermediate growth response with the two other fungi.

Reduced severity of the root rot disease caused by *R. solani* was reported by Yao *et al.* (2002) in a greenhouse experiment with micro-propagated potato plants pre-inoculated with either *G. etunicatum* or *R. irregularis* and challenged three weeks later with *R. solani*. Increased resistance was also observed under strict *in vitro* culture conditions at the pre (before root colonization), early (first hyphae colonizing roots), and late (heavy root colonization with arbuscules and spores/vesicles formed) stage of potato root colonization by *R. irregularis* MUCL 41833 (Gallou *et al*., 2011). Gallou *et al.* (2011) investigated also *in vitro* the impact of an AMF on *P. infestans* in potato plants. The leaf infection index was decreased in mycorrhizal potato plants and Real-Time quantitative PCR revealed the induction of two pathogenesis related genes (*PR1* and *PR2*) in the leaves of mycorrhizal plants shortly after infection with *P. infestans*. These results suggested a systemic resistance in mycorrhizal plants, related to the priming of the two *PR* genes in potato.

3.4 Application of AMF in potato crop

Inoculation of AMF within the field and reports on potato yield and health are rare. In a two years experiment, Douds *et al.* (2007) observed in the first cropping season an increase in yield of 33% under conventional fertilization following application of AMF and a yield increase of 45% when AMF was inoculated in addition to compost. During the second cropping season, yield increase was less marked but still higher by 10 to 20% as compared to the control treatments.

4. Impact of agricultural factors on AMF

Agricultural factors may adversely impact AMF (Hildebrandt *et al.*, 2007, Miransari, 2010, Rillig, 2004). For instance, the application of fertilizers and pesticides, tillage, monocultures or crop rotations with non-host plants have been reported to reduce the abundance and diversity of AMF in agricultural soils (Table 2) (reviewed by Gosling *et al.* (2006)).

Within agricultural soils, AMF communities are usually dominated by species belonging to the Glomeraceae, more specifically to the former genus *Glomus* (group A and B) (e. g. Helgason *et al.* (1999); Jansa *et al.* (2002); Maherali and Klironomos (2012)). One possible reason explaining this predominance above other AMF genera (in particular *Gigaspora* and *Scutellospora*) is related to the reproductive structures. Indeed, it is estimated that the Gigasporaceae are only capable of propagation via spore dispersal or infection from an intact mycelium. In contrast, the Glomaceae are also able of colonizing roots via fragments of mycelium (Bierman and Linderman, 1983). In addition, Glomeraceae and Gigasporaceae have developed different strategies for colony to survive under

adverse conditions (De La Providencia *et al.*, 2007). Giovannetti *et al.* (1999) demonstrated that *Glomus* spp. formed anastomoses (i.e. hyphal fusions) between different colonies of the same strain and might therefore have the ability to re-establish an interconnected network after mechanical disruption. This is not the case for the Gigasporaceae. In addition, in Gigasporaceae, the hyphal healing mechanism (i.e. mechanism of hyphal fusion of damaged hyphae by which different branches of the same or different hyphae fuse (De La Providencia *et al.*, 2007)) was oriented towards the complete recovery of hyphal integrity, while in Glomeraceae this mechanism was oriented towards hyphal recovery, regrowth into the environment or root colonization (De La Providencia *et al.*, 2007). Many studies reported higher levels of AMF colonization, higher propagule numbers or higher diversity in organic farming (reviewed by Gosling *et al.* (2006)). Encouraging agricultural factors that favor AMF diversity may increase the chances of obtaining beneficial effects for crops (Van Der Heijden *et al.*, 1998).

Table 2. Agricultural factors known to impact AMF. Beneficial and detrimental agricultural factors (Adapted from Gosling et al. 2006).

Agricultural factors	Impact on AMF	Beneficial factors	Detrimental factors
Heavy fertilizers use	●Plants reduce resource allocation to their fungal partners ●Reduction of AMF diversity	●Use of slow release mineral fertilisers ●Reasonable use of organic sources	●Use of readily soluble fertilisers
Pesticides	●Potentially toxic to AMF: reduction of root colonization, spore viability… ●Reduction of abundance and diversity	●Reduction of pesticide applications ●Determination of compatibility of pesticides with AMF	●High pesticide applications without evaluation of disease warning
Tillage	●Repeated destruction of hyphal network reduces AMF nutrient transport capability ●Reduction of AMF abundance and diversity	●Low till returning organic redsidues to soil ●No-till	● Ploughing and tillage for weed control
Poor crop rotations/ Monocultures	●Reduction of AMF abundance and diversity	●Diverse rotations ●Polycultures ●Ley periods ●Use of cover crops before cash crop (i.e. high mycotrophic legumes) ●Intercropping host crops with non-host crops	●Non-mycorrhizal crops ●Bare fallow periods ●Use of non-mycorrhizal legumes

4.1 Fertilizers and soil amendments

An excess of total or easily available P in soil following fertilization, decreases the dependence of crops on the AMF association (Kogelmann *et al.*, 2004). A reduced number of spores in soil as well as a decreased root colonization was observed under increasing P fertilization (Jensen and Jakobsen, 1980). However, the reverse was also reported (Al-Karaki and Clark, 1999, Miller and Jackson, 1998). Other readily soluble fertilizers, such as N, have also been reported to impact AMF colonization and diversity (Burrows and Pfleger, 2002, Liu *et al.*, 2000, Miller and Jackson, 1998) although less systematically than for P (Jumpponen *et al.*, 2005). Reynolds *et al.* (2005) observed a change in AMF community composition and the predominance of *R. irregularis* in N-enriched soils. Reasonable use of organic sources of nutrients or slow release mineral fertilizers does not seem to suppress AMF and may even stimulate them. Gosling *et al.* (2006) suggested that the use of compost and slow release mineral fertilizers should be used instead of readily soluble fertilizers.

4.2 Pesticides application

4.2.1 Fungicides

Several authors have evaluated the impact of fungicides on AMF in the field, in the greenhouse (e.g., Burrows and Ahmed (2007); Diedhiou *et al.* (2004); Ipsilantis *et al.* (2012); (Jin *et al.*, 2013)) and under *in vitro* culture conditions (e.g., Wan *et al.* (1998); Zocco *et al.* (2008); Calonne *et al.* (2012); Campagnac *et al.* (2008); Gong *et al.* (2014)). Several studies reported a reduced root colonization at recommended application rates (Plenchette and Perrin, 1992, Schreiner and Bethlenfalvay, 1997, Schweiger *et al.*, 2001), while

others reported no effect or increased AMF colonization or sporulation, especially at reduced application rates (Burrows and Ahmed, 2007, Plenchette and Perrin, 1992).

Fungicides impact vital functions of pathogenic fungi such as lipid synthesis, respiration or cell division (Leroux, 2003), but can also impact non-target organisms such as AMF (Table 3).

Table 3. Mode of action and possible non-target effects of fungicides on AMF

Mode of action	Target site	Fungicide chemical group	Common name (*)	Mode of application	Host plant	Non-target effects	Reference
Sterol biosynthesis	Δ14 demethylation (Class I)	Triazoles	Propiconazole (systemic)	Medium application (*in vitro*)	Transformed carrot roots	-Modification of sterol metabolism (AMF + host roots) -Reduction of root growth and AMF colonization -Reduction of sporulation and hyphal development -Inhibition of spore germination	(Calonne *et al*., 2012)
				Medium application (*in vitro*)		-Inhibition of germination of *G. monosporum*, *G. mosseae* but not *G. geosporum*	(Dodd and Jeffries, 1989)
				Foliar application	Wheat	-Reduction of root colonization by *G. monosporum*, *G. mosseae* and *G. geosporum*	(Dodd and Jeffries, 1989)
				Foliar application	Wheat and Leek	-No effect on *G. intraradices* root colonization	(Plenchette and Perrin, 1992)
		Imidazole	**Imazalil** (systemic)			Unknown	
	Δ14-reduction and/or Δ8-> Δ7 isomerisation (Class II)	Morpholine	Fenpropimorph (systemic)	Medium application (*in vitro*)	Transformed carrot roots	-Modification of sterol metabolism (AMF + host roots) -Reduction of root growth and AMF colonization	(Campagnac *et al*., 2008, Zocco *et al*., 2008)

						-Reduction of hyphal growth and spore production -Fungistatic effect on spore germination and germ tube elongation	
				Medium application (*in vitro*)	Barrel medic	-Decrease of AMF metabolic activity (ALP and SDH) -Decrease of P transport by AMF	(Zocco *et al*., 2011)
	C4 demethylation (class III)		Fenhexamid (contact)	Medium application (*in vitro*)	Transformed carrot roots	-Limited impact on AMF colonization -Reduction of hyphal growth and spore production -Fungistatic effect on spore germination and germ tube elongation	(Campagnac *et al*., 2008, Zocco *et al*., 2008)
				Medium application (*in vitro*)	Barrel medic	-Decrease of AMF metabolic activity (ALP and SDH)	(Zocco *et al*., 2011)
Respiration	Succinate dehydrogenase (Complex II)	Pyridine carboxamide	**Boscalid** (systemic)			Unknown	
		Gxathiin carboxamide	Carboxin (systemic)			Unknown	
		Benzamide	**Flutolanil** (systemic)			Unknown	

	ATP synthesis disruption (Complex III)	Strobilurines	**Azoxystrobin** (systemic)	Soil drench	Maize	-Reduction of *G. coronatum* colonization and SDH activity	(Diedhiou *et al.*, 2004)
				Leaf application		-No reduction of AMF colonization	
	Uncoupler of oxidative phosphorylation halting synthesis of ATP		**Fluazinam** (contact)			Unknown	
Cell wall biosynthesis		Carboxylicacid amide (CAA)	**Mandipropamid** (contact)			Unknown	
Mitosis and cell division	Inhibitor of spindle microtubules assembly	Methyl benzimidazole carbamate	Benomyl (systemic)		Maize	-Reduction of AMF colonization by *G. mosseae, G. etunicatum* and *G. intraradices*	(Samarbakhsh *et al.*, 2009)
					Tomato	-Reduction of AMF colonization	(Salem *et al.*, 2003)
				Medium application (*in vitro*)	Transformed carrot roots	-IC_{50} on *G. intraradices* extraradical mycelium growth = 0.003 ± 0.0008 ppm and on extraradical mycelium sporulation = 0.003 ± 0.0005 ppm	(Wan *et al.*, 1998)
						-Reduction of spore germination, hyphal growth and root	(Schreiner and Bethlenfalvay, 1997)

					colonization	
			Foliar application	Wheat and Leek	-Reduction of *G. intraradices* colonization	(Plenchette and Perrin, 1992)
		Carbendazim (systemic)	Foliar application	Groundnut	-Reduction of AMF colonization	(Sugavanam *et al*., 1994)
			Foliar application	Leek	-Reduction of *G. intraradices* colonization	(Plenchette and Perrin, 1992)
				Wheat	-Reduction of colonization by *G. monosporum*, *G. mosseae* and *G. geosporum*	(Dodd and Jeffries, 1989)
			Medium application (in vitro)		-Inhibition of germination of *G. monosporum, G. mosseae* but not *G. geosporum*	(Dodd and Jeffries, 1989)
	Phenylurea	**Pencycuron** (contact)			Unknown	
Signal transduction	Phenylpyrroles	**Fludioxonil** (contact)	Seed application	Soybean	-Favoring AMF colonization	(Murillo-Williams and Pedersen, 2008)
			Seed application	Pea	-Reduction of AMF colonization (most sensitive specie *G. hoi*), reduced plant biomass and P uptake	(Jin *et al*., 2013)

				Seed application	Chickpea	-Reduction of AMF colonization (most sensitive species *Acaulospora*), reduced plant biomass and P uptake	
Nucleic acids synthesis	RNA polymerase I inhibitors	Acylalanines	**Metalaxyl** (systemic)	Soil drench	Orange and lemon tree	-Reduction of spore number and diversity	(Carrenho *et al.*, 1998)
				Seedling treatment	Leek	-Reduction of *G. intraradices* root colonization	(Jabaji-Hare and Kendrick, 1987)
				Seed application	Pea	-Reduction of *G. viscosum*, *G. hoi* and *G. proliferum* colonization	(Jin *et al.*, 2013)
					Chickpea	-Reduction of *Acaulospora* colonization	
				Seed application	Maize	-Promotion of AMF inoculum production	(Seymour *et al.*, 1994)
					Wheat	-low concentration stimulated AMF and high concentration reduced *G. fasciculatum* colonization	(Shetty and Magu, 1997)
Multisite activity		Copper	**Copper sulfate** (contact)	Medium application (*in vitro*)	Transformed carrot roots	-IC_{50} on *G. intraradices* extraradical mycelium growth = 0.7 ± 0.2 ppm and on extraradical mycelium sporulation = 0.4 ± 0.2 ppm	(Wan *et al.*, 1998)

Phthalimide	Captan (contact)	Seed application	Pea and Chickpea	-No effect or slight stimulatory effect on *G. viscosum* and *G. sp.* colonization and P uptake	(Jin *et al.*, 2013)
			Tomato	-Reduction of *G. etunicatum* hyphal length and vesicle formation	(Salem *et al.*, 2003)
			Pea	-Reduction of AMF colonization in *G. mosseae* and *G. Rosea* > *G. etunicatum*	(Schreiner and Bethlenfalvay, 1997)
			Maize	-Reduction of AMF colonization in *G. mosseae* < *G. etunicatum* and *G. intraradices*	(Samarbakhsh *et al.*, 2009)
			Muskmelon	-No effect or slight stimulatory effect on AMF colonization and P uptake	
			Maize and Zucchini	-Reduction of AMF colonization	(Burrows and Ahmed, 2007)
Dithiocarbamate	Thiram (contact)	Seed application	Pea and Chickpea	-No effect or slight stimulatory effect on AMF colonization and P uptake	(Jin *et al.*, 2013)
		Foliar application	Leek	-No reduction of *G. intraradices* colonization	(Plenchette and Perrin, 1992)
			Wheat	-Reduction of *G. intraradices* colonization	

	Mancozeb (contact)	Foliar application	Wheat	-Reduction of *G. intraradices* colonization	(Plenchette and Perrin, 1992)
			Leek	-No reduction of *G. intraradices* colonization	
	Manèbe (contact)			Unknown	
Phosphonate	Fosetyl-aluminium (systemic)	Foliar application	Wheat and Leek	-No or slightly stimulatory effect on AMF colonization	(Plenchette and Perrin, 1992)
Phthalonitrile	**Chlorothalonil** (contact)	Medium application (*in vitro*)	Transformed carrot roots	-IC_{50} on *G. intraradices* extraradical mycelium growth = 0.03 ± 0.002 ppm and on extraradical mycelium sporulation = 0.01 ± 0.007 ppm	(Wan *et al.*, 1998)
		Soil drench	Leucaena leucocephala	-Reduction of *G. aggregatum* colonization at concentrations > 50 µg/g soil	

In bold are fungicides generally used in potato crop
(*) Mode of absorption

Sterol Biosynthesis Inhibitors (SBI) are the most frequently used fungicides in agriculture. They impact the (I) Δ14 demethylation (e.g. prochloraz, imazalil, propiconazole, epoxyconazole), (II) Δ14-reduction and/or Δ8-> Δ7 isomerisation (e.g. fenpropidin, fenpropimorph), (III) C4 demethylation (e.g. fenhexamid), (IV) squalene epoxidation (e.g. terbinafine, naftifine) (Leroux, 2003) (Figure 7).

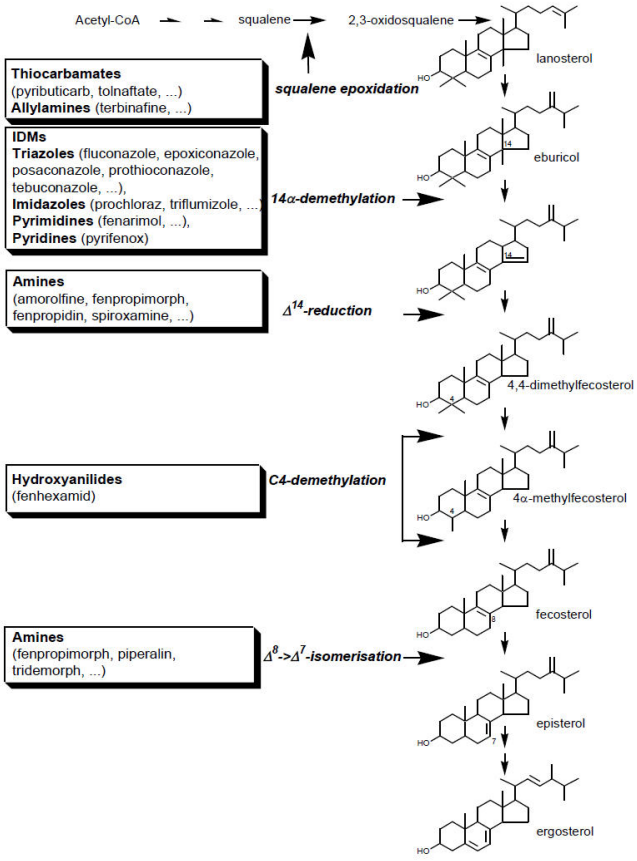

Figure 7. Pathway of fungal ergosterol synthesis and target sites of SBI fungicides (from Leroux *et al.* (2008) in Modern Fungicides and Antifungal Compounds V).

Calonne *et al.* (2012) investigated the impact of class I SBI fungicides on AMF and Campagnac *et al.* (2008), Campagnac *et al.* (2009), Campagnac *et al.* (2010), Zocco *et al.* (2008) and Zocco *et al.* (2011) class II and class III. Calonne *et al.* (2011) and Calonne *et al.* (2012) showed a direct impact of propiconazole (class I fungicide) on the extraradical mycelium (i.e. production of spores and fungal growth) with a decrease in sterols contents (24-methylcholesterol and in 24-ethylcholesterol) and an accumulation of 24-methylenedihydrolanosterol indicating the inhibition of the sterol 14α-demethylase like in phytopathogenic fungi. A reduction of sterol content in propiconazole-treated roots indicated also a decrease of sterol metabolism in plant. Dodd and Jeffries (1989) observed that propiconazole inhibited spore germination of *G. monosporum* and *G. mosseae* but not of *G. geosporum*. Wheat colonization by *G. monosporum*, *G. mosseae* and *G. geosporum* was reduced after foliar application of propiconazole (Dodd and Jeffries, 1989). However, Plenchette and Perrin (1992) did not observe colonization reduction in wheat and leek by *G. intraradices*. The application of fenpropimorph (class II fungicide) also induced a strong modification in sterol content of the host roots accompanied by a reduction of root growth and root colonization (Campagnac *et al.*, 2008, Zocco *et al.*, 2008). Moreover, increased concentrations of fenpropimorph had an impact on spore germination, germ tube elongation, extraradical fungal development (hyphal growth, spore production) (Zocco *et al.*, 2008) and on AMF metabolic activity or P transport (Zocco *et al.*, 2011). The effect on the fungal development was probably due to a decrease of sterol content and the increase of a precursor, the squalene suggesting inhibition of an unusual target enzyme in AMF, the squalene epoxidase (Campagnac *et al.*, 2009). By contrast, fenhexamid (class III fungicide) did not modify the plant sterol profiles

and had a very limited impact on mycorrhizal colonization (Campagnac *et al.*, 2008), while a significant oxidative stress was highlighted in roots (Campagnac *et al.*, 2010). However, Zocco *et al.* (2008) observed that spore germination after fenhexamid application at high concentrations was inhibited and that extraradical fungal development (Zocco *et al.*, 2008) and AMF metabolic activity was impacted (Zocco *et al.*, 2011). Respiration inhibitors are also widely used in agriculture and act on NADH oxidoreductase (complex I), on succinate-dehydrogenase (complex II), cytochrome bc1 (complex III) and oxidative phosphorylation (Figure 8). Three widely used complex II inhibitors, boscalid, carboxin and flutolanil cause disfunction of succinate dehydrogenase (SDH). They have been shown to inhibit denitrifying bacterial activity (Milenkovski *et al.*, 2010) but no studies where performed on AMF. Others have been reported to block mitochondrial respiration complex III via ATP synthesis disruption (Bartlett *et al.*, 2002) such as azoxystrobin or kresoximmethyl. Diedhiou *et al.* (2004), demonstrated that direct application on the soil of these strobilurins reduced root colonization and SDH activity of AMF but that foliar application did not impact colonization by *G. coronatum*. No studies reported on the impact of fungicides inhibiting oxidative phosphorylation such as fluazinam on AMF. Mitosis or cell division inhibitors such as methyl benzimidazole carbamate (MBC) fungicides including benomyl and carbendazim negatively affect AMF (Bashan, 1998, Plenchette and Perrin, 1992, Salem *et al.*, 2003, Samarbakhsh *et al.*, 2009, Sugavanam *et al.*, 1994, Wan *et al.*, 1998). In the same family, phenylurea such as pencycuron affect soil bacteria (Pal *et al.*, 2005) but no studies were performed on AMF. Similarly, no studies were found with fungicides inhibiting cell wall biosynthesis such as mandipropamid. Nevertheless, signal transduction inhibitor fludioxonil applied on seeds reduced AMF

colonization in pea and chickpea (Jin *et al.*, 2013) but favored colonization in Soybean (Murillo-Williams and Pedersen, 2008). This last result was attributed to a reduced competition between AMF and the pathogen. Some AMF were particularly sensitive to metalaxyl, a nucleic acid inhibitor (Carrenho *et al.*, 1998, Jabaji-Hare and Kendrick, 1987, Jin *et al.*, 2013), even if some authors observed a stimulatory effect at low concentrations (Seymour *et al.*, 1994, Shetty and Magu, 1997). Finally fungicides with multisite activity reduced colonization of some AMF in some plants (Jin *et al.*, 2013, Plenchette and Perrin, 1992, Salem *et al.*, 2003, Samarbakhsh *et al.*, 2009, Schreiner and Bethlenfalvay, 1997, Wan *et al.*, 1998) or had no or stimulatory effect at low concentrations on AMF (Burrows and Ahmed, 2007, Jin *et al.*, 2013, Plenchette and Perrin, 1992).

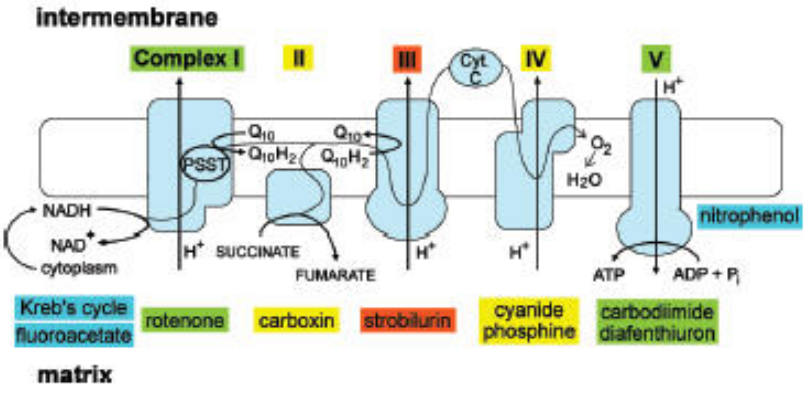

Figure 8. Mitochondrial respiratory electron transport chain with fungicide inhibition sites in each of the five membrane-bound complexes (from Casida (2009) in Chem. Res. Toxicol.).

The period of application (early or late stage of plant development), the mode of application (leaf application, seed

treatment or soil drench) and mode of absorption (systemic or contact) could modify the impact of fungicides on AMF. For example, no AMF inhibition was observed with azoxystrobin at recommended field dosage (1000 mg L^{-1} a.i.) when applied as leaf application, while there was a strong impact on fungal activity when applied as soil drench (Diedhiou *et al.*, 2004). Burrows and Ahmed (2007) showed that seed treatments had minor effects on AMF, while soil drench showed the reverse. Jin *et al.* (2013) observed that systemic seed applied fungicides (acylalanine, phenylpyrrole, carboximide, benzimidazole and strobilurine) affected negatively AMF in contrast to contact seed applied fungicides (phthalimide and dithiocarbamate). They attributed this to the concentrations of systemic fungicides in the root zone penetrating into host and AMF cells in opposite to contact fungicides not reaching the root zone. Plenchette and Perrin (1992) observed that the effect of fungicides on AMF could vary from one plant to another.

Diversity of AMF community in host roots was altered in response to seed applied fungicides (Jin *et al.*, 2013) indicating that suppressive effect depend on specific fungicide-AMF interactions. Indeed, Schreiner and Bethlenfalvay (1997) found that benomyl, PCNB and captan reduced AMF colonization of pea roots by *F. mosseae* and *Gi. rosea* more than *G. etunicatum*. Samarbakhsh *et al.* (2009) found that the colonization of *F. mosseae* in maize roots in presence of benomyl and captan was less affected than *G. etunicatum* and *R. irregularis.*

4.2.2 Herbicides

Herbicides have different modes of action (Table 4) or sites of absorption and translocation (Figure 9). In agriculture, they are

generally used to control weeds, eliminate cover crops or defoliate crops to facilitate harvest. The soil ecosystem can be impacted by herbicides through direct and indirect effects on various components of the microflora, including saprophytes, plant pathogens, pathogen antagonists or mycorrhizal fungi (Ghorbani *et al.*, 2008, Levesque and Rahe, 1992, Sanyal and Shrestha, 2008).

In the recent years, an increasing attention has been devoted to the impact of herbicides on AMF (Druille *et al.*, 2013, Karpouzas *et al.*, 2014, Li *et al.*, 2013) Herbicides could impact AMF directly via contact with fungal structures or indirectly via changes occurring in the host plant. Known impacts of herbicides on AMF are synthetized in Table 4.

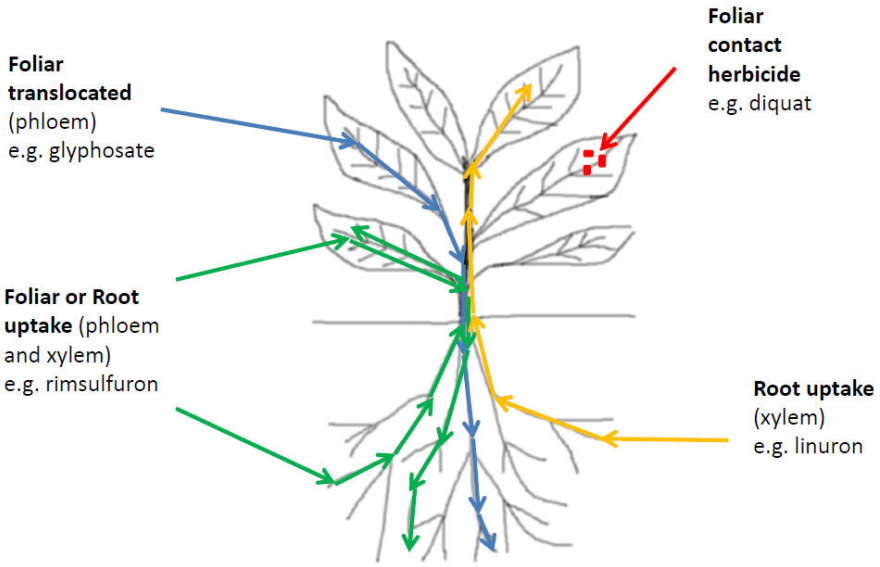

Figure 9. Schematic drawing of absorption and translocation sites of herbicides

Table 4. Mode of action and possible non-target effects of herbicides on AMF

Mode of action	Herbicide chemical group	Common name	Mode of application	Host plant	Non-target effects	reference
Inhibition of acetyl CoA carboxylase (ACC-ase)	Aryloxyphenoxy-propionate "fops"	**Quizalofop-P-ethyl**			Unknown	
		Propaquizafop			Unknown	
		Fluazifop-p-butyl	Soil and weed application	Bean	-Reduction of root colonization	(Santos *et al*., 2006)
	Cyclohexanedione "dims"	**Clethodim** **Tepraloxydim** **Cycloxydim**			Unknown Unknown Unknown	
Inhibition of acetolactate synthase ALS (acetohydroxyacid synthase AHAS)	Sulfonylurea	Nicosulfuron	Soil and weed application	Maize	- Reduction of colonization capacity and AMF diversity at doses 100x higher than recommended	(Karpouzas *et al*., 2014)
		Rimsulfuron	Soil and weed application	Maize	-Reduction in root growth and AMF colonization	(Bilalis *et al*., 2012)
	Imidazolinone	Imazethapyr	Soil and weed application		Unknown	
Microtubule assembly	Dinitroaniline	**Pendimethalin**	Soil and weed application	Cowpea and Soybean	-Reduction of AMF colonization -Reduction of diversity of AMF at conc. ≥ 8 kg a.i. ha^{-1}	(Chikoye *et al*., 2014)
photosystem II	Triazine	Atrazine	Soil and weed application	Maize	-Reduction in root colonization	(Demir *et al*., 2011)
		Prometryn		Transformed carrot roots	- Reduction of spore germination at doses ≥ 10	(Li *et al*., 2013)

					mg/ l -Reduction of hyphal growth of *G. etunicatum* -Reduction of spore production and root colonization -Decrease SDH and ALP activity	
		Metribuzine			Unknown	
	Urea	**Linuron**			Unknown	
Action like indole acetic acid (synthetic auxins)	Benzoic acid	Dicamba			Unknown	
Inhibition of glutamine synthetase	Phosphinic acid	**Glufosinate-ammonium**			Unknown	
Bleaching : Inhibition of carotenoid biosynthesis at the phytoene desaturase step (PDS)	Isoxazolidinone	**Clomazone**	Soil and weed application	Rice	-Unknown but reduction in strigolactone production	(Jamil *et al*., 2010)
	Diphenylether	**Aclonifen**			Unknown	
Bleaching : Inhibition of carotenoid biosynthesis (unknown target)	Triazole	Amitrole	Soil and weed application	Rice	-Unknown but reduction in strigolactone production	(Jamil *et al*., 2010)
Inhibition of protoporphyrinogen oxidase (PPO)	Diphenylether	Fomesafen	Soil and weed application	Bean	-Reduction of root colonization	(Santos *et al*., 2006)
	Triazolineone	**Carfentrazone-ethyl**			Unknown	
	Phenylpyrazole	**Pyraflufen-ethyl**			Unknown	

Inhibition of lipid synthesis – not ACCase inhibition	Thicarbamate	**Prosulfocarb**			Unknown	
Inhibition of auxin transport	Semicarbazone	Diflufenzopyr-Na			Unknown	
Bleaching: Inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD)	Triketone	Mesotrione			Unknown	
Inhibition of EPSP synthase	Glycine	**Glyphosate**	soil Soil or foliar application	*Paspalum dilatatum* *Lotus tenuis*	-Reduction of spore viability -Reduction of root colonization	(Druille *et al*., 2013)
Photosystem-I-electron diversion	Bipyridylium	**Diquat**			Unknown	
Inhibition of VLCFAs (Inhibition of cell division)	Chloroacetamide	**Metazachlor**			Unknown	
		Acetochlor		Transformed carrot roots	-0,1, 1 and 10 mg/ l did not impact spore germination -Reduction of hyphal growth of *G. etunicatum* -Reduction of spore production and root colonization -Decrease in SDH and ALP activity	(Li *et al*., 2013)
	Oxyacetamide	**Flufenacet**			Unknown	
Inhibition of DHP (dihydropteroate) synthase	Carbamate	Asulam			Unknown	
Inhibition of cell wall (cellulose) synthesis	Nitrile	Dichlobenil			Unknown	
Inhibition of mitosis/ microtubule organization	Carbamate	Chlorpropham			Unknown	
Uncoupling (Membrane disruption)	Dinitrophenol	DNOC			Unknown	

In bold are herbicides generally used in potato crop

Most impacts of herbicides on AMF are unknown but in general herbicides disrupt community structure and ecosystem functions of target as well as non-target organisms (Zaltauskaite and Brazaityte, 2011). Glyphosate for example, a systemic non-selective herbicide widely used in the world, was shown to reduce spore viability by 5.8- to 7.7-fold, in treated soils in comparison to non-treated soils (Druille *et al.*, 2013). Total root and arbuscules colonization was also significantly lower after glyphosate application (Druille *et al.*, 2013, Zaller *et al.*, 2014). The direct effect on spores may depend on the AMF species. Herbicide inhibition on germination of spores and growth of germ tubes showed a decreasing tendency from *G. etunicatum* to *Scutellospora heterogama* and *Gigaspora margarita* (Malty *et al.*, 2006), and was even absent in *F. mosseae* (Giovannetti *et al.*, 2006). The impact on arbuscules was higher when glyphosate was applied on plant foliage (indirect pathway) than when it was applied on soil (direct pathway) (Druille *et al.*, 2013). This suggests a higher sensitivity of this structure to changes in the supply of carbohydrates by the plant. Another herbicide, nicosulfuron, applied to maize significantly reduced colonization capacity and diversity of the AMF community when repeatedly applied at rates 100x higher than the recommended dosage (Karpouzas *et al.*, 2014). It is noteworthy to note that application of nicosulfuron at lower dosages (10x higher than the recommended or lower) did not induce alterations of AMF colonization and on the structure of the AMF community. In the case of this study the effects observed were supposed to be plant-driven. Moreover clone libraries showed that the majority of AMF in this study belonged to the *Glomus* group and were sensitive to the high levels of nicosulfuron accumulated in soil at the latter culture cycles. In contrast, a *Paraglomeraceae* and a *Glomus etunicatum* ribotype were present in maize roots in all cycles and

dose rates suggesting a tolerance to nicosulfuron-induced stress (Karpouzas *et al.*, 2014). In contrast, recent *in vitro* tests with the herbicides prometryn and acetochlor showed a direct negative effect on the physiology and metabolic activity of a *G. etunicatum* isolate (Li *et al.*, 2013). Effects depend thus on the herbicide used, dosage, mode of application and AMF species.

4.3 Tillage

Soil tillage is known to increase N mineralization and soil temperature, to reduce weed density and improve soil physical properties. However, soil tillage can also disrupt the common mycorrhizal network (CMN), resulting in a reduction of soil volume that is explored and exploited by AMF with a reduction in root colonization and subsequent nutrient uptake by plants (Evans and Miller, 1990). Tillage may also cause a shift in the AMF community structure (Jansa *et al.*, 2002) and may favor species that have the most efficient hyphal healing mechanism (De La Providencia *et al.*, 2005, Oehl *et al.*, 2009), many asexual spores, high hyphal turnover and fast growth rates, rather than other species with low hyphal healing or asexual spore growth (reviewed by Chagnon *et al.* (2013)). Unfortunately, these species may not be the most beneficial species for the host, because they allocate a large proportion of their resources to spore production (Johnson *et al.*, 1992). Moreover, it was shown that AMF spore density and species richness increased in the top soils (0-20 cm) under reduced tillage as compared to ploughed plots (Säle *et al.*, 2015). Tillage reduced AMF activity (Sieverding, 1991), community structure (Jansa *et al.*, 2002) and glomalin production (Wright *et al.*, 2007). In contrast, low-till agriculture returning organic residues to soil had a positive effect on soil characteristics (Chesworth, 2008). Tillage should thus be limited

to a certain level to limit damages caused to AMF growth and diversity while maintaining adequate weed control.

4.4 Crop rotations

Adding non-mycorrhizal host crops in the rotation impact AMF colonization and nutrient uptake of subsequent AMF reliant crops. For example AMF colonization of maize decreased following non-mycorrhizal mustard (*Sinapis alba*) compared with the mycotrophic sunflower (*Helianthus annuus*). Bare fallow periods have the same effect as non-mycorrhizal crops (e.g. Hamilton *et al.* (1993)). Growing a cover crop such as winter wheat or a green manure can avoid bare fallow periods and increase AMF abundance for subsequent crops (e.g. Boswell *et al.* (1998)). A well-designed, diverse rotation can aid the management of crop nutrient requirements, pests and diseases. Highly mycotrophic legumes such as clovers (*Trifolium spp.)* medics (*Medicago spp.)* and vetches (*Vicia spp.)* result in increased AMF inoculum potential in soil (Menéndez *et al.*, 2001, Oehl *et al.*, 2003) that will benefit the subsequent crop. It was also demonstrated that the utilization of mycotrophic cover crops (e.g. Mucuna deeringiana (Bort.) Merr. (legume used in tropical regions)) used before cash crop or as weed control strategy during cash crop, increased the natural population of AMF in soil and subsequent root colonization of cash crop (Boswell *et al.*, 1998, Kabir and Koide, 2000, Lehman *et al.*, 2012, Ramos-Zapata *et al.*, 2012). Non-mycorrhizal legume crops such as mustard, oilseed rape, stubble turnips and buckwheat should be avoided.

4.5 Intercropping or polyculture

Intercropping AMF host crops with non-host main crops might be effective for the propagation and survival of AMF (Karasawa and

Takebe, 2011). These authors showed that intercropped vetch, red clover and white clover sustained the AMF population in soil during non-host cropping but the duration of cropping and vigorous growth of the host plant were important factors determining AMF colonization in succeeding crops. The low diversity of AMF in agricultural soils is probably due to the low diversity of hosts and monocultures (An *et al.*, 1993, Burrows and Pfleger, 2002, Oehl *et al.*, 2003). Increasing plant diversity such as with a polyculture of Mexican maize was found to increase AMF colonization and nutrient uptake (Negrete-Yankelevich *et al.*, 2013).

5. *Trichoderma* spp.

Trichoderma spp. is a genus of asexually reproducing fungi belonging to the Hypocreaceae family. *Hypocrea* are teleomorphs (sexual form of a fungus) of *Trichoderma* (Kubicek *et al.*, 2001). *Trichoderma* spp. have a high level of genetic diversity which makes them easily adaptable to diverse environments (Harman *et al.*, 2004). Currently *Trichoderma* genus includes 89 accepted species. They are free-living fungi common in soil (Harman *et al.*, 2004) and characterized as saprotrophic organisms, although some of them are (or have the ability to be) mycoparasites (De Jaeger *et al.*, 2010). Many species in this genus have been described as opportunistic avirulent plant symbionts and act as antagonists of many phytopathogenic fungi protecting plants from diseases (Harman *et al.*, 2004). *Trichoderma* spp. are among the most studied biocontrol agents (BCA). In addition to the effectiveness of *Trichoderma* spp. in controlling plant pathogens (i.e. *Rhizoctonia solani*...), many studies have shown that these BCAs have properties that directly stimulate plant growth (Harman, 2000, Vinale *et al.*, 2009, Yedidia *et al.*, 2001). For instance, *Trichoderma* spp. synthesise auxins which promote

lateral plant root development (Benítez *et al*., 2004, Contreras-Cornejo *et al*., 2009) or modify host synthesis of nitric oxide under conditions of pathogen attack (Gupta *et al*., 2014). Depending upon the strain, the activity of *Trichoderma* spp. may include (i) mycoparasitism (Benhamou and Chet, 1996, Rousseau *et al*., 1996), (ii) competition for nutrients (i.e. C, N,…) and space (i.e. specific infection sites) (Gullino *et al*., 1999), (iii) production of antibiosis and secondary metabolites (Ghisalberti and Sivasithamparam, 1991) (iv) improved root and plant growth (Chacón *et al*., 2007, Harman, 2000), and (v) induction of resistance in plants (Gallou *et al*., 2009). Different crops, including Solanaceae, Gramineae and Cucurbitaceae were more resistant to disease development when the plants were treated with *Trichoderma* spp. (review in Harman *et al.* (2004)).

6. Field application of beneficial fungal inoculants

Direct inoculation of either the soil or the host plant with AMF have resulted in increased P uptake, and in some cases yield, and in reduced diseases in AMF-dependent crops (e.g. Davies *et al.* (2005a), Vosátka and Gryndler (2000), Yao *et al.* (2002)). Beneficial effects of *Trichoderma* spp. inoculation on plant health and growth have also been reported. Most experiments were conducted in the greenhouse and their relevance under field conditions is thus questionable. However, in a few field experiments, increased nutrient uptake and/or yield, or reduced disease severity was noticed (Al-Karaki and Clark, 1999, Douds *et al*., 2007, Vosátka, 1995). The problem of field inoculation is the competition with native species and to find the most appropriate species/ host/ inoculation method. Moreover, it is difficult to know if the benefit can be attributed to the inoculated strain. It is thus pertinent to understand the mechanisms

that govern community composition and performance. The effects of inoculum addition in the presence of a pre-established AMF community was evaluated in a greenhouse experiment (Janoušková *et al.*, 2013). These authors found a transient positive response in AMF abundance to the intraspecific inoculation only in the competitively weakest isolate. Other isolates responded negatively to intra- and interspecific inoculations suggesting that increasing the AMF density may lead to increased competition among fungi and a trade-off with their ability to promote plant productivity.

6.1 Inoculants

The most classical commercial inoculants containing AMF species are produced by growing host plants in controlled conditions with AMF propagules (spores or hyphae) or mycorrhizal root pieces (IJdo *et al.*, 2011). For these inocula, substrates of sand/soil and/or other materials as well as substrate-free culture techniques (aeroponics and hydroponics) and *in vitro* cultivation methods can be used to mass-produce AMF inoculum (IJdo *et al.*, 2011). A variety of media have also been found for production of *Trichoderma* spp.

Mixed inoculants combining AMF with *Trichoderma* spp. have been widely studied and are applied for their beneficial effects on plant growth and disease suppression (Srivastava *et al.*, 2010). *Trichoderma* spp. is a mycoparasite of several phytopathogenic fungi suggesting a potential risk to the non-target AMF (Martinez *et al.*, 2004, McAllister *et al.*, 1994). *Trichoderma* sp. was shown to hinder colonization of plant roots by AMF. Similarly, AMF was reported to decrease the population of *T. harzianum* in the rhizosphere indicating that this root symbiont may also exert some antagonistic effects (Fracchia *et al.*, 1998, Green *et al.*, 1999). Recently, co-entrapment of

R. irregularis and *T. harzianum* did not obstruct germination and regrowth of *R. irregularis* or *T. harzianum*. The saprotroph did not affect the AMF symbiosis establishment but rather stimulate the extra- and intra-radical AMF development and fitness (De Jaeger *et al.*, 2011). According to the diversity of results reported, there are clear evidences that the interaction between AMF and *Trichoderma* spp. may produce a different effect (i.e. synergistic to antagonistic) on plant growth and fungal development depending among other things on the genus considered as well as on the environmental condition.

For commercial use, inoculants need a carrier to deliver a suitable amount of beneficial fungi in good physiological condition. Malusá *et al.* (2012) have proposed two different types of carriers: natural carriers (coal, clays and inorganic soils) (Smith, 1995) or polymer-based carriers (alginate (Smidsrød and Skjåk-Bræk, 1990), carragenan and hydroxyethylcellulose (Sylvia and Jarstfer, 1992)).

Alginate, a natural polymer, is the most commonly used compound for microbial cell encapsulation (Smidsrød and Skjåk-Bræk, 1990). Sodium alginate is a hydrophilic biopolymer obtained from marine brown algae. It has the property to form water insoluble calcium alginate gel through ionotropic gelation with Ca^{2+} ions. This method offers the possibility to encapsulate bio-active agents easily and under eco-friendly conditions. The immobilization of cells within beads is frequently reported (Bashan, 1998, Lamberti and Sefton, 1983), but also enzymes (Burns *et al.*, 1985), proteins (Polk *et al.*, 1994) and vaccines (Bowersock *et al.*, 1999).

Entrapment in beads offers an excellent protection of micro-organisms against many environmental stresses (Bashan, 1998) and increases microbial survival and thus shelf-life following inoculation

into field (Vassilev *et al.*, 2001). Entrapment of bacteria (Bashan and Gonzalez, 1999, Jankowski *et al.*, 1997), or AMF and co-entrapment of AMF and *Trichoderma* spp. together has also been reported (De Jaeger *et al.*, 2011, Vassilev *et al.*, 2001).

Freshly prepared alginate beads tend to agglomerate due to their high water content. For storage and field application it is therefore important to dry the beads. Dried alginate beads can develop several undesirable qualities such as distorted shape, uneven size and collapse of the matrix because of the low percentage of alginate content. Fillers can be used to avoid these three problems. In some studies the quality of the alginate beads was improved by incorporating a solid filler, which may have a significant influence on cell viability during drying and storage (Chan *et al.*, 2010, O'Riordan *et al.*, 2001). Encapsulation and air-drying of bacteria into a mixture made of alginate (3%), standard starch (44.6%), and modified starch (2.4%) has already been reported (Ivanova *et al.*, 2005). Other fillers for encapsulation of micro-organisms as kaolin and chitin (Zohar-Perez *et al.*, 2003) can also be used. The addition of a filler in alginate beads containing AMF and *Trichoderma* spp was not yet reported.

6.2 Inoculation methods

Inoculation of AMF and *Trichoderma* spp. in the field is a potential solution to increase benefits to crops in agro-ecosystems that are limited in AMF or *Trichoderma* spp. abundance, diversity or efficacy. However, several factors may impact inoculation success as reviewed by Verbruggen *et al.* (2012). These authors identified three principal factors that determine inoculation success and persistence in soils: species compatibility; field carrying capacity and priority

effects. Species must be compatible with the environment, including soil type, crop host, tillage and nutrient levels, as well as resident community. The carrying capacity (i.e. habitat niche) for microorganisms must be compatible and introduced species must reach unoccupied patches sooner than competing species to benefit from priority effect. There is no information, to our knowledge on the adequate timing in a rotation to apply AMF inoculum. Direct inoculation of plants grown in the greenhouse, which are then transplanted into the field, can establish strong mycorrhizal colonization (Douds and Reider, 2003, Vosátka, 1995). However pre-inoculation is not practical for most crops and other strategies must be developed.

6.3 Traceability

6.3.1 AMF

Neither different AMF species nor different isolates of the same species can be distinguished microscopically based on fungal structures formed in roots. In order to assess the success of inoculation in terms of fungal symbiont establishment in roots of inoculated plants, molecular tools specific for fungal strains are necessary. Different authors tested molecular markers for *R. irregularis* (Croll *et al.*, 2008, Koch *et al.*, 2004, Raab *et al.*, 2005). Only the mitochondrial large subunit (mtLSU) could be used to characterize and distinguish different haplotypes of AMF in the field (Börstler *et al.*, 2008, Börstler *et al.*, 2010). Raab *et al.* (2005) showed that the mtLSU is homogeneous within the same isolate and can be used to distinguish different isolates. Using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach and sequencing, Börstler *et al.* (2008) and Börstler *et al.*

(2010) revealed that the diversity of mtLSU haplotypes of *R. irregularis* is very high in cultured isolates collected world-wide as well as in field populations. Moreover Krak *et al.* (2012) was able to quantify by Real-Time PCR of mtLSU, coexistence and dynamics of two *G. intraradices* strains in roots. Formey *et al.* (2012) sequenced the complete mitochondrial genome of some *R. irregularis* strains and found high polymorphic intergenic and intronic sequences of importance in order to design powerful mt markers for the typing and monitoring of *R. irregularis* in genetic and population studies. The advantage of mt genome for the quantification of AMF consists thus mainly in its higher discriminative power to lower taxonomic levels.

6.3.2 *Trichoderma* spp.

Persistence and distribution of inoculated *Trichoderma* spp. into the environment need to be accurately monitored. Real-time qPCR methods for genus/species/strain identification are currently being developed to overcome the difficulties of classical microbiological and enzymatic methods for monitoring these populations. Savazzini *et al.* (2008) for example developed and validated a specific Real-Time qPCR-based method for detecting a *Trichoderma atroviride* strain in soil. In the same way, a set of primers and a TaqMan probe for the ITS region of *T. harzianum* fungal genome were designed and tested in order to trace *T. harzianum* in soil (Beaulieu *et al.*, 2011).

III. RESEARCH OBJECTIVES

Potato (*Solanum tuberosum*) is the most important root and tuber crop worldwide. Its cultivation is characterized by heavy mechanization and the application of high levels of fungicides to control shoot (e.g. late blight caused by *Phytophthora infestans*) and root (e.g. root and tuber rot caused by *Rhizoctonia solani*) diseases. Potato is likewise among the highest demanding crop in fertilizers (Maynard and Hochmuth, 2007) because of its poorly developed and shallow root system that makes its nutrient exploration and exploitation zone limited.

An alternative approach to these chemicals is the use of beneficial microorganisms such as AMF or *Trichoderma* spp. They have been amply reported to improve plant growth and health.

In the last decade, a particular attention has thus been addresses to the commercial use of these plant beneficial microorganisms. However, their application in the field is more complex than under controlled conditions, because inoculants have to develop in a multi-interactions environment (soil, microbes, climate…) and may be impacted by agricultural factors. Therefore, the traceability of inoculated strains is necessary in order to link the observed benefits to the inoculated strain. Finally, in a context of field application, the economic and ecological impact need to be evaluated.

The first objective of the thesis was to evaluate the effects of some agricultural factors (i.e. the application of widely used fungicides and herbicides/defoliants) on the growth and development of *R. irregularis* MUCL 41833 or *R. intraradices* MUCL 49410 under *in vitro* culture conditions. Numerous fungicides are used in potato crop. Here, we focused on three fungicides (azoxystrobin, flutolanil and

pencycuron) widely used to control a major below-ground fungal pathogen (i.e. *Rhizoctonia solani*). The impact of these fungicides used to control *R. solani* was thus investigated on the AMF *R. irregularis* MUCL 41833. In addition, we investigated the impact of an important contact or systemic herbicide (diquat and glyphosate, respectively) on the dynamics of spore production of *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410, two species highly succeptible to C limitations, in comparison with mechanical defoliation.

The second objective was to assess itineraries for optimal use of beneficial fungal inoculants (*R. irregularis* combined with *Trichoderma harzianum*) in potato crop and to evaluate their subsequent effect on yield. AMF need a host plant to complete their life cycle. In this context, the use of a cover crop before potato crop is evaluated to increase native or inoculated microorganisms. *Medicago sativa* was used as cover crop because this legume is highly mycotrophic and improves soil fertility and stability.

The third objective was to find a tool to trace, quantify and distinguish inoculated from native AMF in the field in order to link benefits of crop production to inoculated AMF and to evaluate their persistence in the field.

IV. RESEARCH QUESTIONS

Four research questions were addressed:

1. Do fungicides used to control *Rhizoctonia solani* impact the non-target AMF *R. irregularis* MUCL 41833?

2. Is a systemic herbicide (i.e. glyphosate) more detrimental to the obligate root symbionts *R. irregularis* or *R. intraradices* than chemical (i.e. diquat) or mechanical defoliation?

3. Is the application of *R. irregularis* combined with *T. harzianum* beneficial to potato crops and what are the more pertinent agricultural itineraries to increase potato yield in presence of the microbial inoculants?

4. How can we trace and quantify an AMF (i.e. *R. irregularis* MUCL 41833) applied in potato fields and distinguish from native AMF?

V. MATERIALS AND METHODS

1. Biological material

The beneficial micro-organisms used are:

***Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler MUCL 41833**: This obligate biotroph was supplied by the Glomeromycota *in vitro* collection (GINCO – http://www.mycorrhiza.be/ginco-bel). It was isolated from a soil of Canary Islands (Spain). This AMF was formerly named *Glomus sp*. MUCL 41833 and was renamed *Rhizophagus sp.* MUCL 41833 according to the new classification of Schüßler and Walker (2010) (http://schuessler.userweb.mwn.de/amphylo/). In addition, this fungus formerly identified as *G. intraradices* (now *R. intraradices*) Schenck and Smith was reclassified in a clade that contains the described species *G. irregulare* (*R. irregularis*) Błaszk., Wubet, Renker and Buscot (Stockinger *et al*., 2009). *R. irregularis* MUCL 41833 is the most studied AMF under *in vitro* culture conditions. Its life cycle and development have been amply described under these conditions (Voets *et al*., 2009).

***Rhizophagus intraradices* (N.C. Schenck & G. S. Sm) C. Walker & A. Schüßler MUCL 49410**: This obligate biotroph was supplied by the Glomeromycota *in vitro* collection (GINCO – http://www.mycorrhiza.be/ginco-bel). It was isolated from roots of a citrus plantation in Florida, Orlando, Clermont-Minneola (USA). This AMF was formerly named *Glomus intraradices* Schenck & Smith but was renamed *Rhizophagus intraradices* (Schenck & Smith) Walker & Schüßler MUCL 49410 according to the new classification of Schüßler and Walker (2010) (http://schuessler.userweb.mwn.de/amphylo/).

***Trichoderma harzianum* Rifai MUCL 29707**: This opportunistic plant symbiotic fungus was supplied by the Mycothèque de l'Université catholique de Louvain (MUCL - http://bccm.belspo.be/about/mucl.php). This fungal strain was isolated from a soil of Heverlee (Belgium). Its life cycle was described under *in vitro* plant culture conditions by (De Jaeger *et al.*, 2010).

The fungal plant pathogen used in this thesis was:

***Rhizoctonia solani* Kuhn MUCL 49235:** This fungus was supplied by the Mycothèque de l'Université catholique de Louvain (MUCL - http://bccm.belspo.be/about/mucl.php). It belongs to the anastomosis group 3. It was originally isolated from a potato field culture (Belgium).

Five potato cultivars and two barrel medic species were used in the thesis.

Cultivars Sarpo Mira and Bionta were selected for their moderate to high resistance to late blight (*Phytophthora infestans*) (Rietman *et al.*, 2012) and used under organic agricultural itineraries. The three following potato cultivars (i.e. Bintje, Nicola and Charlotte) were used because of their high yield characteristics and importance in Europe. Because of their higher susceptibility to late blight, they were cultivated under conventional agricultural itineraries.

***Solanum tuberosum L. cv.* Sarpo Mira:** This cultivar was supplied by Danespo (Danemark) and is a very late ripening potato variety bred in Hungary by Sarpo KFT, Zirc in 2002 from (76 PO 12 14 268 x D187).

***Solanum tuberosum L. cv.* Bionta:** This cultivar was supplied by NÖS (Austria) and is a very late ripening potato variety bred by Asche-Saatzucht in 1992 from (Franzi x Granola).

Solanum tuberosum L. cv. **Bintje:** This cultivar is a middle-early ripening potato variety bred in the Netherlands by K.L. de Vries in 1904 from (Munstersen x Fransen) and marketed for the first time in 1910. It is used for boiling, baking, and for French fries, mashed potato and potato chips. Bintje is immune to Potato virus A. It has moderate resistance to Potato leafroll virus. It is susceptible to netted scab and common scab (*Streptomyces* spp.), Fusarium dry rot (*Fusarium oxysporum*), potato wart (*Synchytrium endobioticum*), Potato virus X, Potato virus Y, stem canker and black scurf (*R. solani*) and late blight (*P. infestans*).

Solanum tuberosum L. cv. **Nicola:** This cultivar is a middle-early ripening potato variety bred in Germany by Saatzucht Soltau-Bergen eG in 1973 from (Clivia x 6430 1011). This high yielding variety is widely grown in Europe. It is used for boiling, baking and mashed potato. Nicola has high resistance to stem canker (*R. solani*), common scab (*Streptomyces* spp.), blackleg (*Erwinia* spp.) and Potato virus X. Resistance to late blight (*P. infestans*) on tubers is moderate and on foliage low. Resistance to Potato leafroll virus and Potato virus A is low.

Solanum tuberosum L. cv. **Charlotte:** This cultivar is a middle-early ripening potato variety bred in France by Unicopa et Société L. Clause from (Hansa × Danae) and on the market in 1981. Very productive and excellent for cooking. Suitable for steam, the blow but not roasted. Charlotte has medium resistance to common scab (*Streptomyces* spp.), late blight (*P. infestans),* Potato virus A and Potato virus Y. Resistance to Potato leafroll virus is low to medium.

Two species of barrel medic were used.

For the *in vitro* study following barrel medic species was used because it is a model organism for legume biology forming symbiosis with AMF.

***Medicago truncatula Gaertn.* Jemalong A17 clone:** This plant is a small annual legume and was supplied by SARDI Genetic Resource Centre (Australia). It is a low-growing, clover-like plant 10–60 cm tall with trifoliate leaves. Each leaflet is rounded, 1–2 cm long, often with a dark spot in the center. The flowers are yellow, produced singly or in a small inflorescence of 2-5 together; the fruit is a small spiny pod.

In the field studies we used a more accessible legume in the same genus.

Medicago sativa L., It is a perennial flowering plant cultivated as an important forage legume in many countries around the world. It was supplied by Philip-Seeds (Belgium). It superficially resembles clover, with clusters of small purple flowers followed by fruits spiralled in 2 to 3 turns containing 10-20 seeds.

2. *In vitro* AMF-plant culture systems

Two *in vitro* AMF-plant culture systems were used in this thesis. These systems are based on whole plants growing in growth chambers and were proved particularly efficient for (1) studying the molecular and physiological bases of the pre-symbiotic and symbiotic steps involved in the AMF root colonization process (Dupré De Boulois *et al.*, 2005, Gallou *et al.*, 2010, Voets *et al.*, 2005), (2) performing non-destructive microscope observations of the three-dimensional arrangements, configuration and responses of AMF cells to environmental conditions (Zocco *et al.*, 2011), (3) studying the metabolism and transfer of nutrients from fungus to plant and vice

versa (Zocco *et al.*, 2011) and (4) examining microbial interactions with beneficial as well as pathogenic fungi (De Jaeger *et al.*, 2010, Gallou *et al.*, 2011) under highly-controlled conditions.

2.1 Half-closed Arbuscular Mycorrhizal Plant (H-AMP) system

The Half-closed Arbuscular Mycorrhizal plant (H-AMP) *in vitro* culture system was developed by Voets *et al.* (2005). AMF was associated to plant roots on a gelled medium under strict *in vitro* culture conditions, while the shoot developed under open air conditions (Figure 10). In this autotrophic culture system AMF was able to develop typical colonization structures and to produce an abundant extra-radical mycelium. Numerous spores were produced, morphologically similar to those observed in the monoxenic culture systems on excised root organs and in pot cultures (Koffi *et al.*, 2009, Sosa-Rodriguez *et al.*, 2013, Voets *et al.*, 2005).

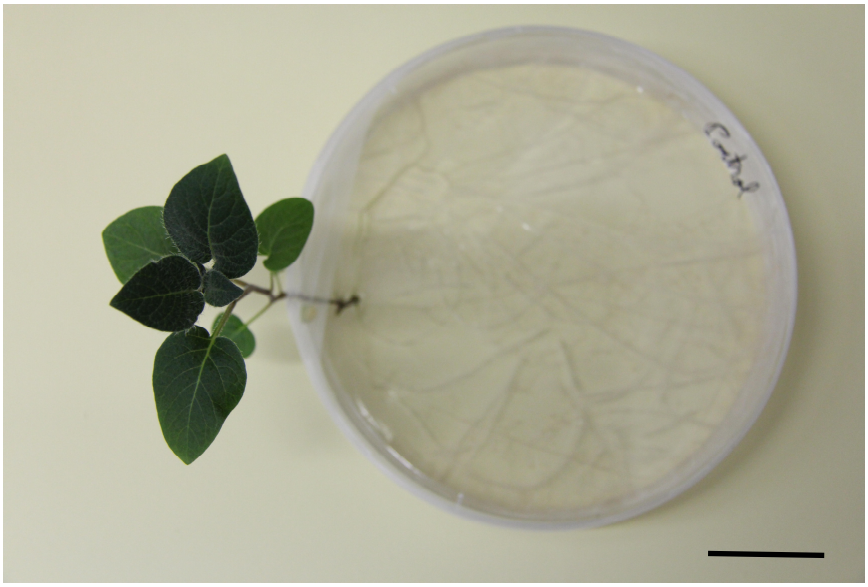

Figure 10. Half-closed Arbuscular Mycorrhizal plant (HAMP) *in vitro* culture system used for the mycorrhization of potato plantlets. Bar 18 mm.

Briefly, the system consisted of a Petri plate containing 50 ml of Modified Strullu Romand (MSR) (Declerck *et al.*, 1998) medium lacking sucrose and vitamins, and solidified with 3 g l^{-1} Phytagel. A hole, in the lid and base of the Petri plate is used to insert a potato plantlet with the roots placed on the surface of the medium and the shoot extending out of the Petri plate via the hole. The Petri plate is then closed and sealed with Parafilm and the hole cautiously plastered with sterilized silicon grease to avoid contaminations. The Petri plate is finally covered with an opaque plastic bag and incubated horizontally in a growth chamber under controlled conditions (i.e. 22/18°C (day/night), 70% relative humidity, a photoperiod of 16 h day^{-1} and an average photosynthetic photon flux density of 225 µmol m^{-2} s^{-1}).

2.2 Mycelium Donor Plant (MDP) *in vitro* culture system

The mycelium donor Plant (MDP) *in vitro* culture system was developed by Voets *et al.* (2009). This system was developed for the fast and homogenous mycorrhization of seedlings. As compared to the H-AMP *in vitro* culture system, plant colonization is faster (within 9 to 12 days instead of an approximate of 4 weeks) because the mycelium network is used as inoculum rather than isolated spores (Voets *et al.*, 2009) (Figure 11).

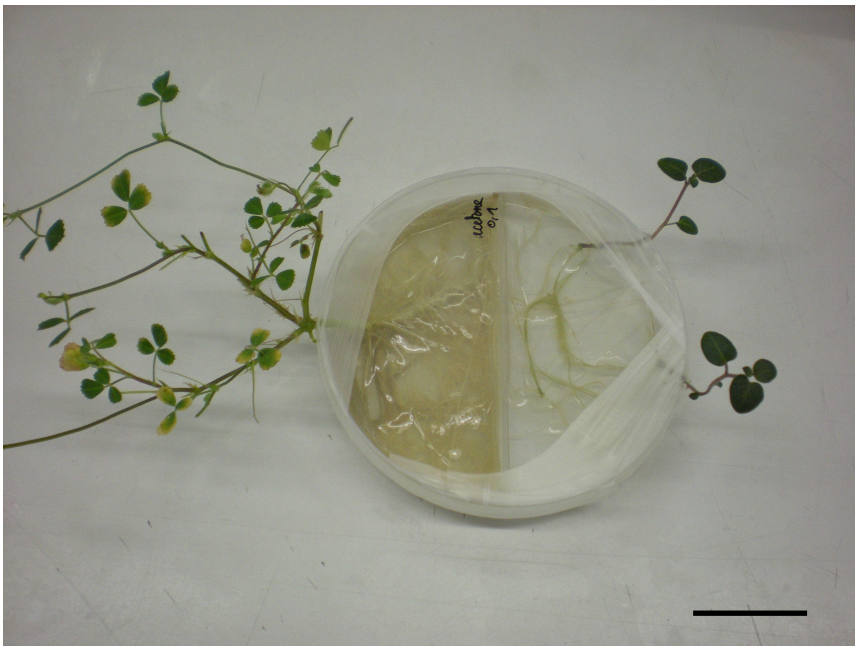

Figure 11. The mycelium donor Plant (MDP) *in vitro* culture system developed by Voets *et al.* (2009) for the fast and homogenous mycorrhization of young plantlets. Bar 26 mm.

The system consists of a bi-compartimented Petri plate separating a root compartment (RC) from a hyphal compartment (HC). In the RC, one four-days-old *Medicago truncatula* plantlet is associated with the AMF on MSR medium, lacking sucrose and vitamins. After eight weeks, an important fungal network develops in the RC and extends in the HC as a profuse extra-radical mycelium bearing hundreds of spores. Two four-days-old *M. truncatula* plantlets are subsequently inserted in the HC with their roots placed in contact with the extra-radical mycelium (Voets *et al*., 2009). Colonization starts within a few days and is optimal after 12 days.

3. Field experimental setups

Each field trial was made of different potato subplots representing different treatments. In the thesis, each potato subplot was arranged in a randomized complete block design replicated four times (example in Figure 12). Each potato subplot was separated by 2 meters from neighbouring subplot. Statistical analysis was perfomed with a linear mixed model where the factors such as “Disease management”, “Inoculation treatment” or “cultivar” are regarded as fixed factors, and block as random factor.

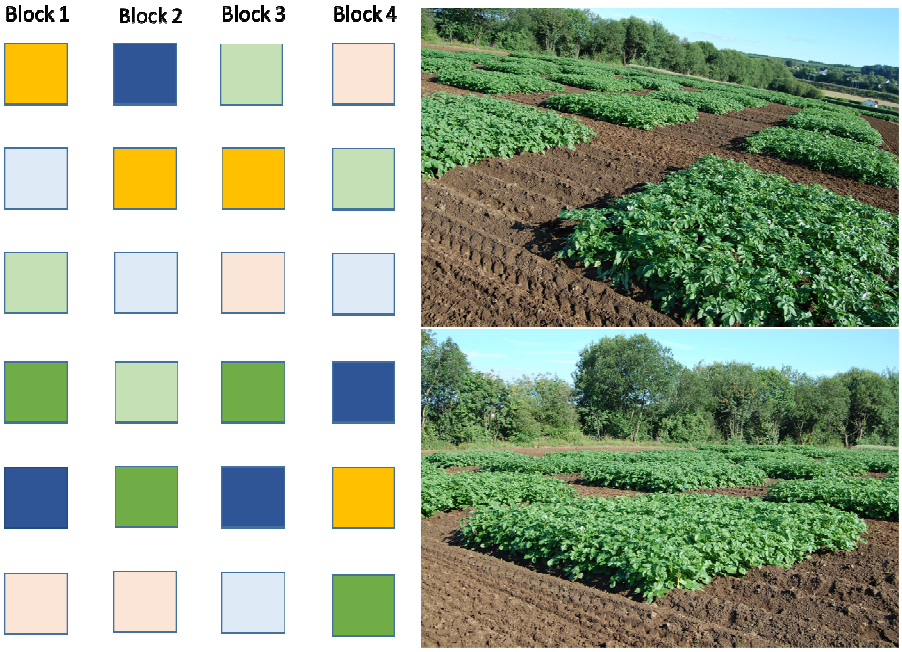

Figure 12. Experimental design and pictures of potato subplots of trial in Chapter 4. Different colors represent different cultivars/AMF inoculation treatments: orange (Charlotte AMF+), pink (Charlotte AMF-), light blue (Bintje AMF-), dark blue (Bintje AMF+), light green (Nicola AMF-), dark green (Nicola AMF+).

In the thesis several technical itineraries that valorisate beneficial fungal microorganisms have been tested to enhance potato yield (Figure 13). The first strategy consisted of a broad inoculation of *M. sativa* with beneficial fungal inoculants before potato plantation. The second strategy was to inoculate potato plants with beneficial fungal inoculants directly at potato plantation. The last strategy consisted to enhance native fungal microorganisms by sowing *M. sativa* before potato plantation.

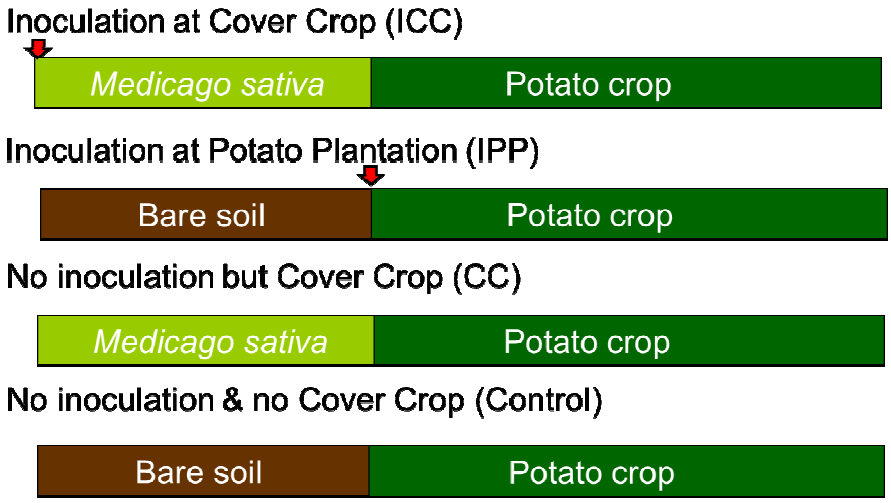

Figure 13. Technical itineraries tested to enhance abundance and efficacy of beneficial fungal microorganisms in order to increase potato yield

3. Evaluation of root colonization by AMF

The magnified intersections method developed by McGonigle *et al.* (1990) was used to assess the AMF root colonization. It is considered as the most adequate method to determine the abundance of arbuscules, spores/vesicles and hyphae

within roots. Stained roots are aligned in parallel to the long axis of a slide and observed under a compound microscope at magnification 200 x. The field of view of the microscope is moved using the stage graticule to make complete passes across each slide perpendicular to its axis. All intersections between roots and the vertical eyepiece crosshair are considered, except when the cortex is missing. In case throots are not totally parallel to the slide, rotation of the vertical crosshair ensures each intersection in at right angles to the axis of the root (Figure 14).

At each intersection, a mark is made if the vertical crosshair crosses an arbuscule, intraradical spore/vesicle or hyphae. Intersections are counted as followed: “negative” (i.e. no AMF observed in root), “hyphae only”, “arbuscules” and “spores/vesicles”. If the vertical crosshair crosses one or more arbuscules or spore/vesicle, the category is incremented by one, and similarly for intersections where only hyphae are observed. When both arbuscules and spores/vesicles are visualized at an intersection, the total number of intersections is only added by one.

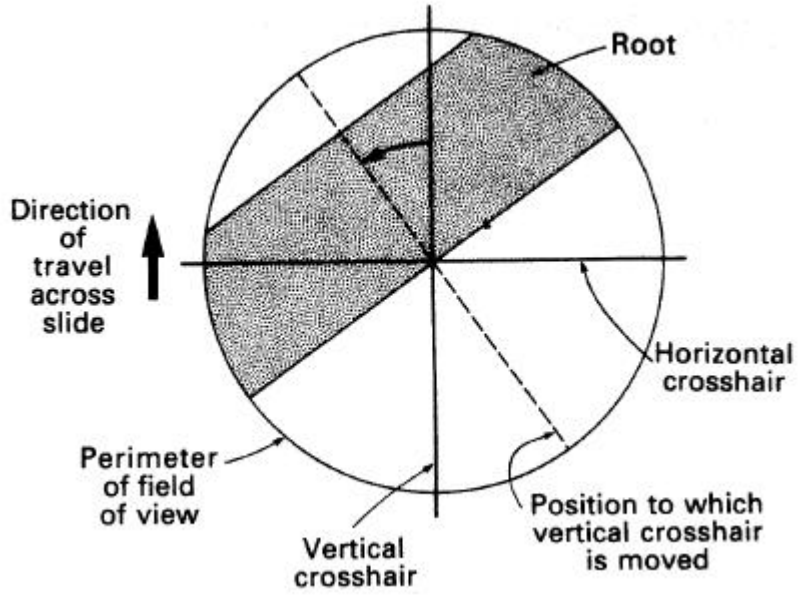

Figure 14. A diagram to show how a magnified intersection perpendicular to the long axis of the root can be made when the root is aligned with its long axis at an angle to the vertical crosshair. The stage is moved until the center of the crosshairs is contiguous with the first edge of the root reached. To make the perpendicular intersection, the vertical crosshair is then rotated as shown (From McGonigle *et al.* (1990) in New Phytologist).

The arbuscular colonization and spores/vesicles colonization are calculated by dividing the count for the “arbuscules” and “spores/vesicles” categories respectively by the total number of intersections examined. Total colonization is calculated as the proportion of non-negative intersections. The visualization of 200 intersections per subsample is recommended for this method (i.e. generally 2 slides are used).

4. Most Probable Number (MPN) of AMF in soil

The most probable number (MPN) method is widely used to estimate microbial populations in different matrices. The method, derived from the original work of (McCrady, 1915), consist of adding a volume of each of several dilutions of a sample to a number of replicate tubes of culture medium and, following incubation under appropriate conditions, recording the number of tubes showing growth at each dilution of inoculum. The estimation of population is based on the application of the theory of probability based on certain suppositions. The first supposition is that the inoculum contains a random distribution of microbial propagules. This means that each dilution is thoroughly mixed and well homogenized. Secondly, that each volume of inoculum containing at least one viable organism will show growth when inoculated in the culture medium. Many authors have made different approaches to calculate the MPN values (e.g., Halvorson and Ziegler (1933); Cochran (1950) and de Man (1983)). Recently, Jarvis *et al.* (2010) proposed a simple Excel spreadsheet to calculate automatically the MPN.

To determine AMF population density in soil, soil samples are first homogenized and spread on paper to remove plant material. They are subsequently air-dried, sieved with 5 mm mesh sieves, and stored at 4 °C until processing. *Medicago sativa* seeds are sown in a series of 12 soil dilutions (dilution factor 2) of each soil sample and replicated three times (Figure 15). The original soil is diluted with sterilized soil. Presence or absence of AMF in the roots of the trap plant is determined after 6 weeks of culture and the MPN then calculated as described by Jarvis *et al.* (2010).

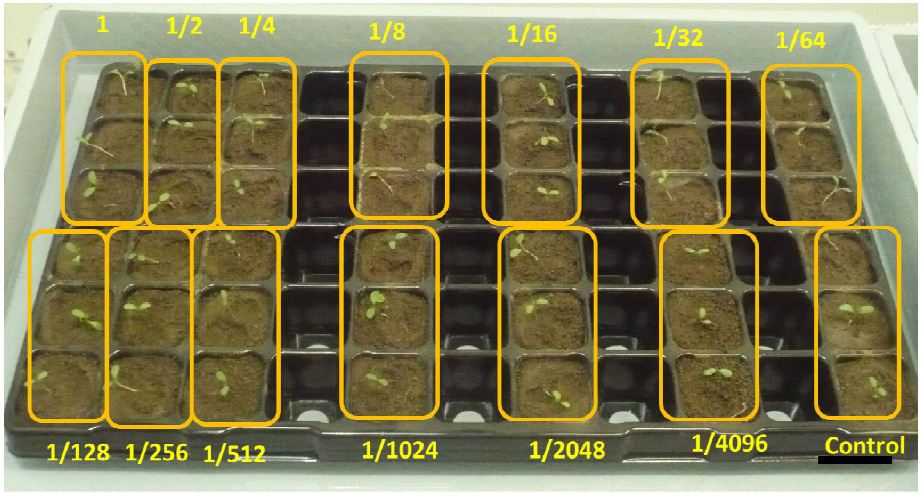

Figure 15. Design to evaluate AMF density in soil (number of propagules per gram of soil). *Medicago sativa* seedlings are grown in the original soil sample (1) and in a series of 12 soil dilutions (dilution factor 2) and replicated three times (three individual pots). Dilutions are performed with sterilized soil that is also used as negative control. Bar 5 cm.

5. Real-Time quantitative PCR (qPCR) of AMF Mitochondrial DNA

5.1 Molecular characterization of a *R. irregularis* strain

Fungal DNA from spores and mycelium of a strain of a pure *R. irregularis* strain produced *in vitro* is extracted with innuPREP Plant DNA kit (Analytikjena, Germany) and following the manufacturer's recommendations with slight modifications (homogenization step is adapted to fungal material and described further). Spores/mycelium of 2-month-old cultures are first extracted from MSR medium by citrate-buffer solubilization (Doner and Bécard, 1991), isolated with a micropipette under a dissecting microscope and placed in a 1.5 ml tube (Eppendorf, Germany). It is repeatedly centrifuged to eliminate water and air-dried. The tip of the tube is put in liquid nitrogen and

spores/mycelium are crushed with a piston before lysis with the buffer. Extracted DNA is then subjected to PCR amplification of mitochondrial large ribosomal subunit (mtLSU) RNA gene. The region is amplified by nested PCR using the primers RNL28a (Börstler *et al.*, 2008) and RNL5 (Raab *et al.*, 2005) in the first step and then RNL29 (Börstler *et al.*, 2008) and GImt4510R (Krak *et al.*, 2012) in the second step (Table 5). The PCRs are conducted using a total volume of 20 µl and containing 0.2 mM dNTP, 0.5 U of AmpliTaq® DNA polymerase (Applied Biosystems, USA), 1 × Taq buffer with KCL (Applied Biosystems, USA), 1 ng of genomic DNA and reaction-specific concentrations of primers and $MgCl_2$ (Table 5). An initial denaturation step at 94°C (4 min) is followed by 35 cycles of denaturation (94 °C, 30 s), annealing (59 °C in the first step and 53 °C in the second step, 30 s), and extension (72°C, 1.5 min), with a final extension step at 72 °C (10 min). The resulting PCR products are sequenced using the original PCR and additional internal primers (Table 5). One sequence type is then obtained and aligned together with mtLSU sequences of *R. irregularis* sensu lato as well as other species that are representative of all published haplotypes (www.ncbi.nlm.nih.gov/) using Mega6 software (http://www.megasoftware.net/) .

Table 5. PCR primers and conditions used for the characterization of *R. irregularis* strains in the large subunit mitochondrial DNA

Primer name(s)	Primer sequences (5' -> 3')	Application	Primer conc. (µM)	Annealing temp. (°C)	MgCl2 conc. (mM)
RNL28a/ RNL5	CCATGGCCAAGTGCTATTTA (Börstler et al. 2008) / GAGCTTCCTTTGCCATCCTA (Raab et al. 2005)	1st step of nested PCR	0.2 / 0.2	59	1.5
RNL29/ GImt4510R	TAATAAGACTGAACGGGTGT (Börstler et al. 2008) / CATCCACGCTAGTGTTAGC (Krak et al. 2012)	2nd step of nested PCR	0.1 / 0.1	53	2
RNL27	CCAACTATGCAACCGTAGG	Sequencing			
RNL24	GAGCATACTAAGGCGTAGAG	Sequencing			

To prepare the standards for the qPCR experiments, DNA extract from spores and mycelium of the *R. irregularis* strain grown *in vitro* is used. The concentration of DNA is measured with Quant-iT™ PicoGreen® dsDNA Assay kit (Life technologies, USA) using Fluoroscan Ascent FL instrument (Labsystems, USA) and diluted to 100 pg genomic DNA.

5.2 Design and optimization of qPCR assay

Primer pairs targeting to the mtLSU region are designed. Primer pairs designed to amplify species-specific isolates or haplotype-specific isolates can be designed. All primers are then tested for dimer formation using AmplifX software (by Nicolas Jullien; CNRS, Aix-Marseille Université – http://crn2m-univ-mrs.fr/pub/amplifx-dist).

The qPCR using LightCycler® Fast Start DNA Master SYBR Green I kit (Roche, Switzerland) is performed in 10 µl reaction mixtures on the LightCycler® 96 Real Time PCR instrument (Roche, Switzerland). The following cycling conditions are used: 10 min at 95°C, followed by 45 cycles of denaturation (95°C, 10 s), annealing (62°C, 10s), and extension (72°C, 15 s). The cycling is finalized by a standard melting curve analysis.

The efficiencies of the qPCR assays are estimated from standard calibration curves based on serial 5-fold dilutions of genomic DNA standards (10^{-1} to 10^{-5} ng μl^{-1}) and the mean efficiency is calculated from ten independent 5-fold dilutions of DNA template. The absolute quantification of the target sequences is performed based on the standard calibration curves using the LightCycler® 96 software, version SW1.1 (Roche, Switzerland). The resulting concentrations are

expressed as ng of *R. irregularis* DNA or ng of *R. irregularis* haplotype DNA/ ng template.

To ensure specificity of qPCR assays, cross-amplification tests are performed with a range of templates (DNA extracted from potato roots, DNA extracted from fungal spores/mycelium and DNA extracted from potato roots colonized by AMF from *in vitro* or *in vivo* cultures).

5.3 Traceability and/or quantification of AMF in roots with the mitochondrial markers

DNA is extracted using the innuPREP Plant DNA kit (Analytikjena, Germany) according to the manufacturer recommendations with slight modifications. For the homogenization step, frozen root samples are ground in liquid nitrogen and roots are lysed with 700 µl SLS buffer instead of 400 µl as described in the manufacturer recommendations. The concentration of DNA is measured with Quant-iT™ PicoGreen® dsDNA Assay kit (Life technologies, USA) using Fluoroscan Ascent FL instrument (Labsystems, USA), and 10 ng of total genomic DNA is used as the template in qPCR.

qPCR is performed as described above. Standard curves constructed from 5-fold dilutions of genomic standard are included in each run. The amplification of each dilution of genomic standard and experimental samples are performed in triplicate.

VI. RESEARCH RESULTS

PART I. In Vitro studies

CHAPTER 1

Do fungicides used to control *Rhizoctonia solani* impact the non-target arbuscular mycorrhizal fungus *Rhizophagus irregularis*?

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Catherine Buysens, Hervé Dupré de Boulois and Stéphane Declerck

Preface

In a recent study, Gallou *et al.* (2011) demonstrated that the AMF *R. irregularis* MUCL 41833 was able to increase the resistance of potato against *Rhizoctonia solani* MUCL 49235 at pre (before root colonization), early (first hyphae colonizing roots), and late (heavy root colonization with arbuscules and spores/vesicles formed) stage of potato root colonization by the fungal symbiont. This study was conducted under strict *in vitro* culture conditions and demonstrated the potential benefits of AMF to control this major potato pathogen. However, it is doubtful that AMF alone will replace the conventional control measures which are firstly based on fungicides. An integrated management of the disease combining the application of *R. irregularis* MUCL 41833 with fungicide at reduced doses may thus represent a more realistic option. To assess the potential of this strategy, the impact of fungicides on the non-target AMF should be evaluated first.

In **chapter I**, we assessed the impact of three different fungicides, widely used to control *R. solani,* on spore germination, root colonization, extraradical mycelium development and spore production of *R. irregularis* MUCL 41833. We determined the dose of application below which the fungicides were harmless to the AMF while still effective on the pathogen.

Abstract

There is growing evidence that the application of bio-control organisms (e.g. *Pseudomonas* and *Bacillus* spp., arbuscular mycorrhizal fungi – AMF) is a feasible option to reduce Rhizoctonia incidence in an integrated control strategy. However, the use of these microorganisms, in particular AMF, may be threatened by the application of fungicides, a widely-used measure to control *R. solani* in various crops among which potato. Prior to their application, it is thus important to determine the impact of fungicides on AMF. Here we investigated, under *in vitro* controlled conditions, the impact of azoxystrobin (a systemic broad-spectrum fungicide), flutolanil (a systemic Basidiomycota-specific fungicide) and pencycuron (a contact Rhizoctonia-specific fungicide) and their respective formulation (Amistar, Monarch and Monceren) on the growth and development of the AMF *R. irregularis* MUCL 41833 (spore germination, root colonization, extraradical mycelium development and spore production) at dosages used to control *R. solani in vitro*. We demonstrated that azoxystrobin and its formulation Amistar, at threshold value (estimated by the half maximal inhibitory concentration (IC_{50}) on dry weight of *R. solani*), did not affect spore germination and potato root colonization by the AMF, while the development of extra-radical mycelium and spores production was reduced at 10 times threshold value. Flutolanil and its formulation Monarch at threshold value did not affect spore germination and extra-radical development but decreased root colonization and arbuscules formation. At threshold value, pencycuron and its formulation Monceren, did not affect the spore germination and intra- or extra-radical structures of the AMF. These results suggest that azoxystrobin and pencycuron do not affect AMF at threshold

concentration to control *R. solani in vitro*, while flutolanil (as formulation) impact the intraradical phase of the fungus. These fungicides and AMF could thus potentially be used in parallel against Rhizoctonia disease in potato.

Keywords

Arbuscular mycorrhizal fungi; *Rhizophagus irregularis*; azoxystrobin, flutolanil, pencycuron, *Rhizoctonia solani.*

Introduction

Rhizoctonia solani Kühn (Basidiomycota) is a major soil-borne plant pathogen. In potato (*solanum tuberosum* L.) it causes stem canker or black scurf which leads to reduced plant vigor, tuber yield and quality (Banville, 1989). Control of this major pathogen can be managed via the use of a non-host preceding crop such as straw cereals (e.g. wheat, barley) and thus long rotations (> 5 years) (Bouchek B., 2014). Moreover, intermediate cover crops with sanitizer properties (e.g. mustard, radish) or properties that increase antagonistic microorganisms (e.g. clover, oat, rye, phacelia) can reduce the pathogen in the soil (Bouchek B., 2014). During the crop period it is recommended to select clean potato seeds (James and McKenzie, 1972), use resistant cultivars (Sedláková *et al*., 2013), have short crop cycles, have short defoliation-harvest periods, use germinated and heated potato tubers (Bouchek B., 2014) and to apply fungicides (El Bakali and Martín, 2006).

The three major fungicides used to control Rhizoctonia disease are azoxystrobin, flutolanil and pencycuron (Campion *et al*., 2003, Djébali and Belhassen, 2010) applied as seed or soil treatment. Azoxystrobin (trade name Amistar®) inhibits spore germination,

mycelial growth and sporulation. More specifically, it blocks mitochondrial respiration complex III via ATP synthesis disruption (Bartlett *et al.*, 2002). This systemic fungicide is characterized by a broad spectrum activity against species in the Oomycota, Ascomycota, Basidiomycota and Deuteromycota. Flutolanil (trade name Monarch®) inhibits hyphal growth and infection-cushion formation by the inhibition of the succinate dehydrogenase complex II in the respiratory electron transport chain, leading to the inhibition of aspartate and glutamate synthesis (Motoba *et al.*, 1988). This systemic fungicide is active essentially against the members of Basidiomycota (Motoba *et al.*, 1988). Pencycuron (trade name Monceren®) is a contact protective phenylurea fungicide originally developed for selective control of *Rhizoctonia* on rice and potato (Kuch *et al.*, 1988, Roberts and Stephens, 1984). This fungicide inhibits mycelial growth by blocking cell division and β-tubuline assembly during mitosis. Besides their mode of action, these three fungicides are thus characterized by an increased specificity towards *R. solani* (Azoxystrobin < Flutolanil < Pencycuron).

One promising alternative to replace fungicides or decrease their number and doses of application is to exploit bio-control microorganisms including plant growth-promoting bacteria (e.g. *Pseudomonas* and *Bacillus* spp.) and arbuscular mycorrhizal fungi (AMF) (Glomeromycota) (Alabouvette *et al.*, 2006, Haggag, 2010). These microorganisms can provide the plants with partial resistance to pathogens by several mechanisms such as induced systemic resistance (ISR) and mycorrhiza induced resistance (MIR) (Cameron *et al.*, 2013, Pozo and Azcón-Aguilar, 2007).

In the recent years, AMF have been reported to decrease the incidence of several fungal pathogens in primary agriculture crops like

the potato (Conrath *et al.*, 2006, Jung *et al.*, 2012). Yao *et al.* (2002) described a reduced severity of the disease in a greenhouse experiment with micropropagated potato cultivars pre-inoculated with either *Glomus etunicatum* or *Rhizophagus irregularis* and challenged three weeks later with *R. solani*. Under strict *in vitro* culture conditions, this increased resistance was observed at the pre (before root colonization), early (first hyphae colonizing roots) and late (heavy root colonization with arbuscules and spores/vesicles formed) stage of potato root colonization with *R. irregularis* MUCL 41833 (Gallou *et al.*, 2011). The authors suggested that the bio-protection of AMF during the pre-/early stages of symbiotic establishment may be associated to a priming of the *MAPK* genes and salicylic acid (SA)-dependent genes, while at the late stage of symbiotic establishment this resistance could be associated to a priming of SA-dependent genes and *PAL* gene.

Notwithstanding these promising results, it is doubtful that AMF may replace fungicides to control Rhizoctonia disease. They should rather be considered in an integrated management of the disease, favoring for instance a decrease in dose of fungicides application. It is thus of upmost importance to determine whether the fungicides used against Rhizoctonia may impact AMF at the dosage detrimental to the pathogen.

In the recent years, a number of studies have reported on the combination of bio-control fungi and fungicides. Van Den Boogert and Luttikholt (2004) demonstrated that azoxystrobin (a broad spectrum fungicide) was toxic to the bio-control agent *Verticillium biguttatum,* while pencycuron and flutolanil (two more Rhizoctonia-specific fungicides), co-applied with *V. biguttatum* showed additive control effects on black scurf. *Trichoderma harzianum*, another Rhizoctonia

bio-control fungus, was shown to tolerate over 100-fold higher doses of flutolanil than *R. solani,* which supports their combined use (Wilson *et al*., 2008). Several authors also evaluated the impact of fungicides on AMF in the field and in the greenhouse (e.g. Burrows and Ahmed (2007); Diedhiou *et al.* (2004); Ipsilantis *et al.* (2012); Jin *et al.* (2013)) and under *in vitro* conditions (e.g. Wan *et al.* (1998); Zocco *et al.* (2008); Calonne *et al.* (2012), Gong *et al.* (2014)). Systemic fungicides (e.g. metalaxyl, fludioxonil, carbathiin, thiabendazole, trifloxystrobin) applied as seed treatment restricted mycorrhizal colonization and P uptake (Jin *et al*., 2013), while those applied as soil drench (e.g. azoxystobin and kresoxim-methyl) reduced mycorrhizal activity (Diedhiou *et al*., 2004). Interestingly, no AMF inhibition was observed with azoxystrobin at recommended field dosage (1000 mgL^{-1} a.i.) when applied as leaf application, while strong impact on fungal activity was noticed when applied as soil drench (Diedhiou *et al*., 2004). To our knowledge, no studies reported on the impact of pencycuron or flutolanil on AMF.

In the recent years, root organ cultures (ROC) or whole plants grown *in vitro* have been used to investigate the impact of various fungicides on development (Gong *et al*., 2014, Zocco *et al*., 2008), physiology such as P transport (Zocco *et al*., 2011) and sterol biosynthesis (Calonne *et al*., 2012) of AMF. It is obvious that *in vitro* cultivation of AMF only partially reflect field situations. Microbes as well as chemical factors may degrade fungicides and reduce their activity with time. Moreover, the soil volume may result in a dilution effect. However, *in vitro* cultivation of AMF may also present some interesting advantages for screening pesticide compatibility with AMF. It allows to study the impact of fungicides on the pre-symbiotic and symbiotic phases of root colonization (Gong *et al*., 2014, Wan *et al*.,

1998, Zocco *et al.*, 2008, Zocco *et al.*, 2011) and on the metabolism (Calonne *et al.*, 2012) and transfer of nutrients (e.g. P) from fungus to plant (Zocco *et al.*, 2011). Several fungicides (fenpropimorph and fenhexamid – Campagnac *et al.* (2008); Zocco *et al.* (2011); Zocco *et al.* (2008), propiconazole – Calonne *et al.* (2012), chlorothalonil – Wan *et al.* (1998), carbendazim, chlorothalonil and thiram – Gong *et al.* (2014)) have been tested in the recent decade under reproducible *in vitro* cultural conditions avoiding any confounding effect with, for instance, other microbes. Several of these studies revealed closely-related results to those obtained in field or greenhouse experiments (fenpropimorph, fenhexamid and propiconazole – Dodd and Jeffries (1989); Kjøller and Rosendahl (2000), chlorothalonil – Zhang *et al.* (2006), carbendazim – Ipsilantis *et al.* (2012); Plenchette and Perrin (1992), thiram – Jin *et al.* (2013); Plenchette and Perrin (1992) supporting the reliability of the *in vitro* experimental approach.

The objective of the present study was to investigate, under *in vitro* controlled conditions, the impact of azoxystrobin, flutolanil and pencycuron and their respective formulation (Amistar, Monarch and Monceren) on the growth and development of the AMF *R. irregularis* MUCL 41833 (spore germination, root colonization, extraradical mycelium development and spore production) at dosages used to control *R. solani in vitro*. We challenged *R. irregularis* MUCL 41833 with the half maximal inhibitory concentration (IC_{50}) doses necessary to control *R. solani* and determined whether a broad-spectrum (azoxystrobin) or Basidiomycota-specific (flutolanil) systemic fungicide, blocking cell respiration, is more detrimental to AMF than a contact Rhizoctonia-specific fungicide (pencycuron) blocking cell division.

Materials and Methods

Biological material

Solanum tuberosum L. var. Bintje was supplied by the Station de Haute Belgique in Libramont (Belgium) on Murashige and Skoog (Duchefa, The Netherlands) medium. The plantlets were micropropagated every 5 weeks as described in Voets *et al.* (2005).

Seeds of *M. truncatula* Gaertn. cv. Jemalong A 17 (SARDI, Australia) were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 10 min and rinsed in sterilized (121 °C for 15 min) deionized water. Seeds were germinated in Petri plates (90 mm diameter) containing 35 ml of Modified Strullu-Romand (MSR) medium (Declerck *et al.*, 1998) without sucrose and vitamins, solidified with 3 g L^{-1} phytagel and adjusted to pH 5.5 before sterilization (121˚C for 15 min). The Petri plates were incubated at 27˚C in the dark. Seedlings were ready to use 4 days following germination.

The AMF *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler comb. nov. MUCL 41833 was supplied by the Glomeromycota *in vitro* collection (GINCO – www.mycorrhiza.be/ginco-bel) on MSR medium. The fungus was cultured for two months in the mycelium donor plant (MDP) *in vitro* culture system (Voets *et al.*, 2009).

Rhizoctonia solani Kühn (isolated from potato) MUCL 49235 was supplied by the Mycothèque de l'Université catholique de Louvain (MUCL – http://bccm.belspo.be/about/mucl.php) on Potato Dextrose Agar (PDA) medium. A plug of gel containing mycelium and

sclerotia was placed on PDA in Petri plates and subsequently incubated for two days at 27°C.

Fungicide active ingredients and formulations

Fungicides active ingredients (a.i.) that disrupt respiration (azoxystrobin and flutolanil) and cell division (pencycuron) and their respective formulations Amistar® (Syngenta Crop Protection N.V., (Seneffe, Belgium)), Monarch® (Belchim Crop Protection sa/nv (Londerzeel, Belgium)) and Monceren® (Bayer Crop Science SA-NA (Diegem, Belgium)), were used. Stock solution of each a.i. and formulation was prepared as follows: azoxystrobin, flutolanil and pencycuron were dissolved in acetone (10 % a.i. w/v), Monceren® in methanol (10 % a.i. w/v) into an ultrasonic bath for 10 minutes and Amistar® and Monarch® were diluted in water (10 % a.i. w/v).

Experimental design

Impact of fungicides on growth of R. solani MUCL 49235

Dilutions of each fungicide (a.i. or formulation) were prepared from the stock solutions to obtain final concentrations of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 mgL^{-1} a.i. The fungicides were added to sterilized (121 °C for 15 min) PDA cooled to 45°C. Controls, without fungicides, received a quantity of solvent (water, 0.1% acetone or methanol) equivalent to that used in the corresponding fungicide treatment. The medium (40 ml) supplemented or not with the fungicides (a.i. or formulation) was subsequently poured in 90 mm Petri plates.

One plug of mycelium (8 mm diam.) cut from the growing margin of a two-day-old culture of *R. solani* MUCL 49235 was placed

in the center of each control or fungicide-amended Petri plate. Each treatment was replicated five times.

After 72 hours of mycelial growth, PDA medium was melted at 121 °C and mycelium separated by filtration and subsequently air-dried to assess dry weight. The half maximal inhibitory concentration (IC_{50}) of each fungicide (a.i. or formulation), measured as the concentration of fungicide needed to decrease by half the dry weight of *R. solani*, was subsequently estimated via a non-linear dose-response curve using the model "Logistic 4p" with the software JMP version 10 (SAS Institute Inc. 2012).

Impact of fungicides on spore germination of R. irregularis MUCL 41833

Spores of a two month old culture of *R. irregularis* MUCL 41833 were extracted from the MSR medium by citrate-buffer solubilization (Doner and Bécard, 1991). They were subsequently isolated with a micropipette under a dissecting microscope and placed singly in each hole of a 24 Well Cell Culture Cluster (Corning Incorporated, USA) containing 2 ml of MSR medium lacking sucrose and vitamins and solidified with 4 gL^{-1} Phytagel (Sigma-Aldrich, Germany) without fungicides (i.e. the control) or supplemented with increasing concentrations (0.1, 1, 10, 100 mgL^{-1} a.i.) of the fungicides (a.i. or formulation). The 24 Well Cell Culture Clusters were incubated at 27°C in the dark and spore germination monitored under a dissecting microscope (10-40x magnifications) after 3, 6, 14 and 28 days. Forty-eight spores were considered per treatment.

The spores that did not germinate on the different concentrations of fungicides (a.i. or formulation) were washed-free from the fungicides in sterilized (121°C for 15 min) deionized water

and placed in holes of a 24 Well Cell Culture Cluster containing 2 ml of solidified MSR medium without fungicides. Germination was assessed after 6 days.

Impact of fungicides on root colonization of Solanum tuberosum L. var. Bintje, spore production and fungal regrowth

Spores (±100) of *R. irregularis* MUCL 41833 were associated with *M. truncatula* plantlets in the root compartment (RC) of a bi-compatmented MDP *in vitro* culture system (Voets *et al.*, 2009). After 8 weeks, the RC was covered with a dense root system and extensive extraradical mycelium network and hyphae extended profusely in the neighboring compartment (i.e. the hyphal compartment – HC). At that time, the MSR medium (25 ml) in the HC was cut 5 mm away from the plastic barrier separating the RC from the HC and removed from the Petri plate. One ml MSR medium was added in a thin layer to the HC. The AMF extended on this layer and covered the whole surface of the HC within twelve days.

Two holes (± 2 mm diam.), separated by 5 cm from each other, were made at the side of the HC in the base and the lid of the MDP *in vitro* culture system. Twelve-day-old micropropagated potato plantlets were plated on the extraradical mycelium network extending in the HC. The HC was then supplemented with 20 ml MSR medium lacking sucrose and vitamins without fungicide (i.e. the control) or with a selected concentration of fungicide (a.i. or formulation). Final concentration of fungicide in the HC was 0.1, 1, 10, 100 mgL^{-1} a.i. for azoxystrobin; 0.1, 1, 10 mgL^{-1} a.i. for flutolanil and 0.01, 0.5, 5 mgL^{-1} a.i. for pencycuron. The MDP *in vitro* culture systems were then incubated in a growth chamber set at 22/18 °C (day/night) with 70% relative humidity, a 16 h photoperiod and a photosynthetic photon flux

(PPF) of 300 µmol m^{-2} s^{-1}. The lamps used in the growth chamber covered a spectrum from λ = 400–700 nm.

After 14 days of contact with the extraradical mycelium, the potato plantlets were carefully collected from the HC. One potato plantlet per system was used for assessing root colonization while the other was used to evaluate spore production and mycelium regrowth (Voets *et al*., 2005) after plating on a fresh MSR medium without sugar or vitamins supplemented or not by fungicides (a.i. or formulation).

Root colonization was assessed following clearing with 10% KOH at 50°C for 90 min and staining with 5% blue ink (Parker®) diluted in vinegar (7° acidity) at 50°C for 60 min. The roots were then rinsed with deionized water and observed under a compound bright field microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 125-250 × magnifications. Root colonization was assessed by evaluating the percentage of total root colonization (% RC), arbuscules (% A) and spores/vesicles (% V) following the method of McGonigle *et al.* (1990). Two hundred intersections were observed per sample.

The second potato plantlet from each MDP *in vitro* culture system was plated in mono-compartmented Petri plates in absence (for the control) or presence of the same concentration of fungicide as for the colonization. After nine weeks, the number of newly produced spores was counted (Declerck *et al*., 1996) and extraradical hyphae length estimated under a stereo-microscope at 10-40 × magnifications. Hyphal length was estimated by counting the number of intersects between hyphae and a grid of lines (Voets *et al*., 2005) and then using the formula of Newman (1966).

Data on the percentage of germinated spores were analyzed using Chi-square test ($P < 0.05$). AMF root colonization was analyzed with the statistical software SAS Enterprise guide (version 4.3). Data expressed as percentage (%) were normalized by arcsine transformation and analyzed using a one-way ANOVA. The Tukey's honest significant difference test was used to identify the significant difference in root colonization ($P \leq 0.05$) between the fungicide treatments and the control. Non-linear dose-response curves of active ingredients and formulations were compared using the software JMP version 10 (SAS Institute Inc. 2012).

Results

Impact of fungicides on growth of R. solani MUCL 49235

The IC_{50} (mgL^{-1} a.i.) of azoxystrobin, flutolanil, pencycuron and their respective formulations (Amistar, Monarch and Monceren) was estimated on dry weight of *R. solani* MUCL 49235 (Table 6). All three selected fungicides (as a.i. or formulated) had a high activity ($IC_{50} \leq 0.1$ mgL^{-1} a.i.) against *R. solani* MUCL 49235. Dose-response curves of a.i. and formulation of the three selected fungicides were not significantly different (Table 6).

Table 6. IC_{50} (mgL^{-1} a.i.) of azoxystrobin, flutolanil and pencuryon and their respective formulations (Amistar, Monarch and Monceren) estimated on dry weight of *R. solani* (A). Comparison between dose-response curve of a.i. and formulation (B).

A)

	IC_{50} dry weight (mgL^{-1} a.i.)	Confidence limit at 95%
Azoxystrobin	0.015	0.008-0.022
Amistar	0.009	0.007-0.011
Flutolanil	0.13	0.13-0.13
Monarch	0.15	0.14-0.15
Pencycuron	0.010	0.009-0.011
Monceren	0.009	0.008-0.010

B)

	p-value
Azoxystrobin vs. *Amistar*	0.9419
Flutolanil vs. *Monarch*	0.9512
Pencycuron vs. *Monceren*	0.2665

Significant differences are noticed if $P < 0.05$.

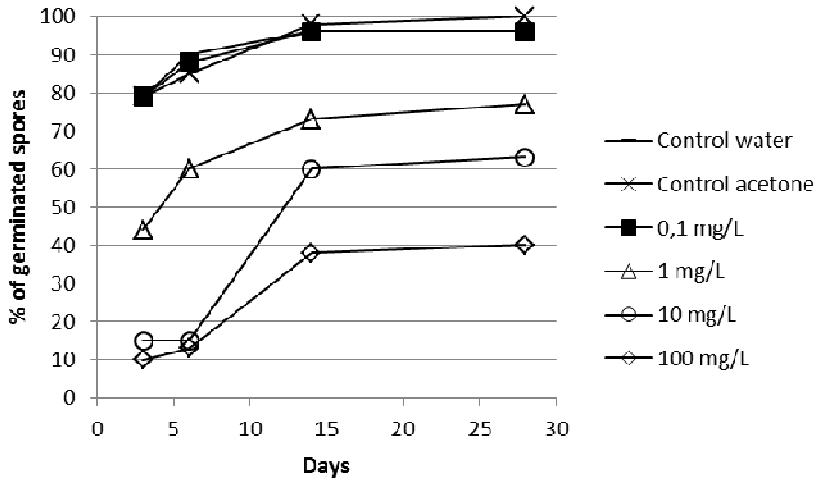
Impact of fungicides on spore germination of R. irregularis MUCL 41833

The impact of azoxystrobin, flutolanil and pencycuron and their respective formulations (Amistar, Monarch and Monceren), at

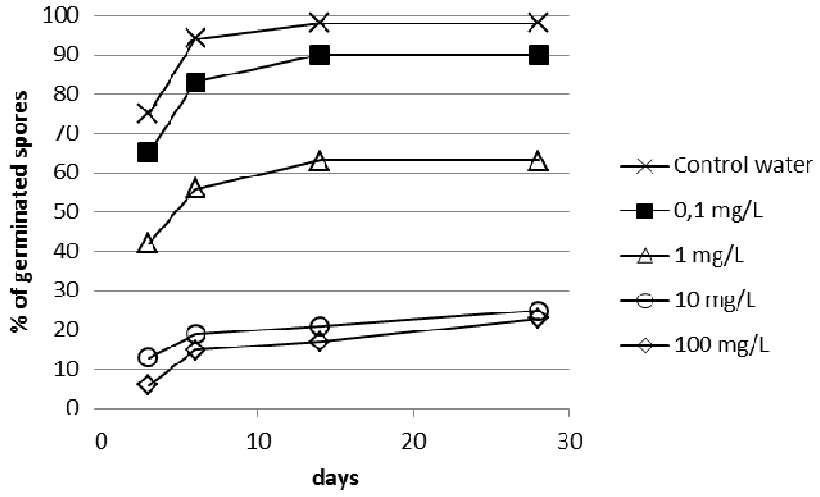
0.1, 1, 10 and 100 mgL^{-1} a.i., was evaluated on AMF spore germination after 3, 6, 14 and 28 days (Figure 16).

No significant differences were observed between the control treatments (water, acetone or methanol without fungicides). 75% to 88% of the spores germinated within 3 days and nearly all the spores within 28 days. Whatever the fungicide (a.i. or formulation) and time of observation, the percentage of germinated spores did not differ from the respective control at 0.1 mgL^{-1} a.i. (Figure 16).

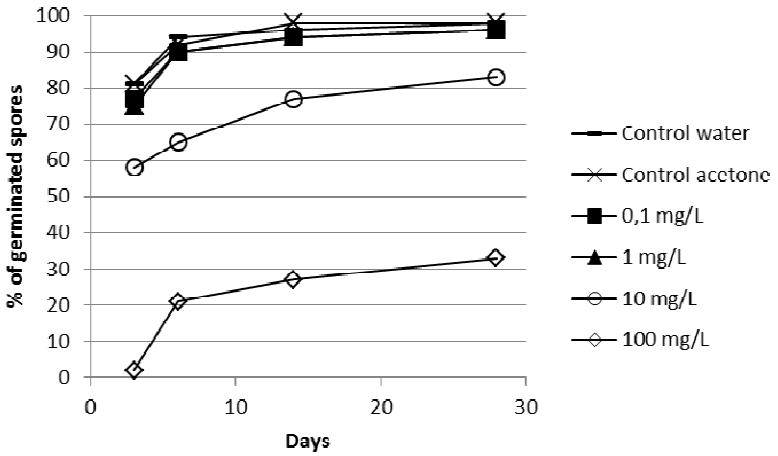
A



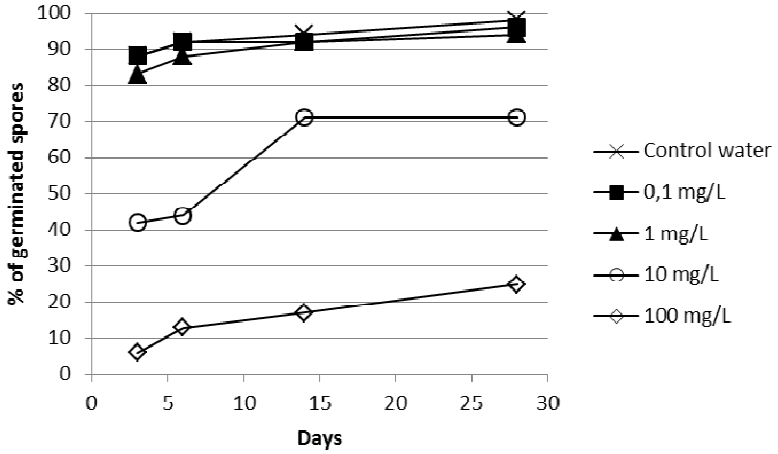
B


% of germinated spores
days
Control water
0,1 mg/L
1 mg/L
10 mg/L
100 mg/L

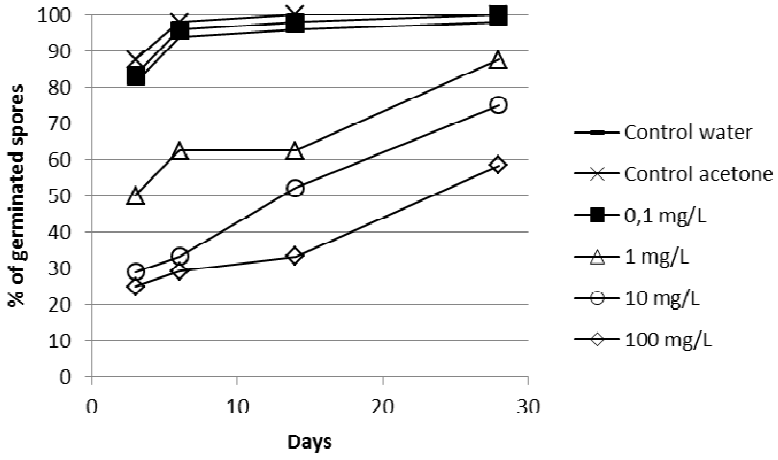
C


% of germinated spores
Days
Control water
Control acctone
0,1 mg/L
1 mg/L
10 mg/L
100 mg/L

D


% of germinated spores
Days
Control water
0,1 mg/L
1 mg/L
10 mg/L
100 mg/L

E


% of germinated spores
Days
Control water
Control acetone
0,1 mg/L
1 mg/L
10 mg/L
100 mg/L

F

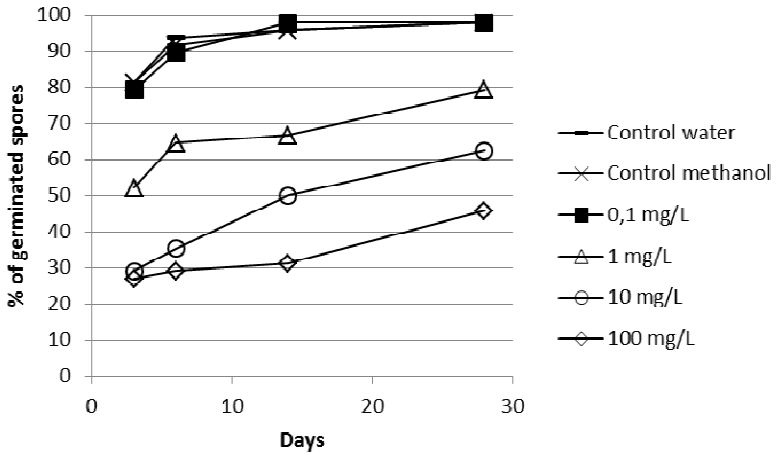


Figure 16: Dynamics of spore germination of *R. irregularis* MUCL 41833 in presence of azoxystrobin (A), Amistar (B), flutolanil (C), Monarch (D), pencycuron (E) and Monceren (F) at concentration 0.1 mgL^{-1} a.i. (□), 1 mgL^{-1} a.i. (Δ), 10 mgL^{-1} a.i. (○) and 100 mgL^{-1} a.i. (◊). Open symbols (□, Δ, ○, ◊) show a significant difference with control water (for Amistar and Monarch), methanol (for Monceren) or acetone (for azoxystrobin, flutolanil and pencycuron) (×). Closed symbols (■, ▲, ●, ♦) show no significant difference with the control (Chi-square test, $p < 0.05$; n=48). Control water for all molecules except Amistar and Monarch is marked with (–).

For azoxystrobin (a.i. or formulation), at 1, 10 and 100 mgL^{-1} a.i., the % germinated spores was significantly lower as compared to the control, whatever the time of observation (Figure 16A, B). At 1 mgL^{-1} a.i., the dynamics of spore germination was identical for azoxystrobin and Amistar. After 28 days, the % of germinated spores was 77% and 63%, respectively. For azoxystrobin at 10 and 100 mgL^{-1} a.i., the % germinated spores increased rapidly between day 6 and 14 and remained almost unchanged until the end of the experiment (day 28), with values of 63% and 40%, respectively. To the contrary, Amistar strongly impacted spore germination at 10 and 100 mgL^{-1} a.i.

The % germinated spores was around 20% at day 6 and remained unchanged until the end of the experiment.

Flutolanil (a.i or formulation) at 1 mgL^{-1} a.i. did not impact spore germination, while at 10 and 100 mgL^{-1} a.i., the % germinated spores was significantly lower as compared to the control (Figure 16C, D). At 10 mgL^{-1} a.i., 58 % of the spores germinated after 3 days and reached 83 % after 28 days. To the contrary, for Monarch, only 44% of spores germinated after 6 days and reached 71% after 28 days. At 100 mgL^{-1} a.i. spore germination was strongly impacted. After 28 days, the % germinated spores was 33% and 25% for flutolanil and Monarch, respectively.

For pencycuron (a.i. or formulation) at 1, 10 and 100 mgL^{-1} a.i., spore germination was significantly lower as compared to the control (Figure 16E, F). The dynamics of spore germination were identical for pencycuron and Monceren. After 28 days, at 1, 10 and 100 mgL^{-1} a.i., the % of germinated spores was 88% and 79%, 75% and 63%, 58% and 46%, respectively.

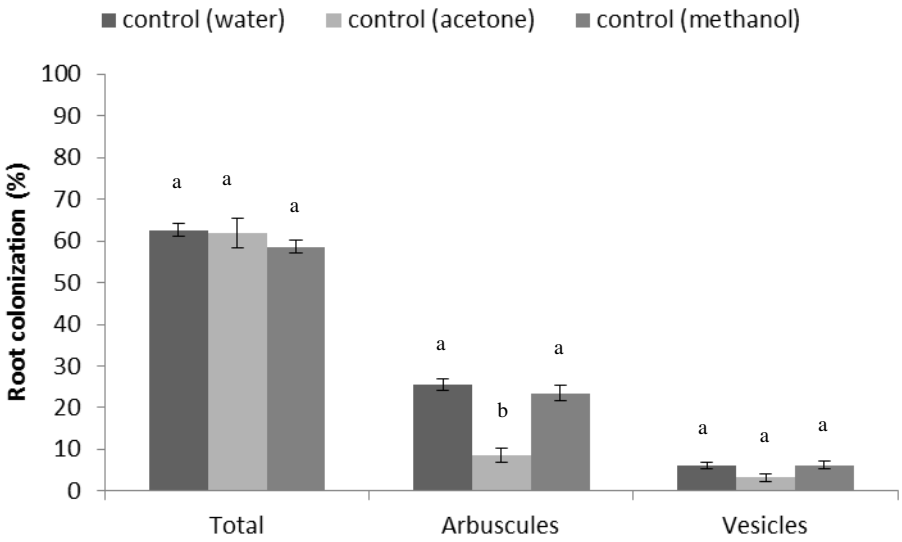
Interestingly, for all fungicides (as a.i. or formulation) and concentration tested, the complete inhibition of germination was never observed. Therefore, the non-germinated spores of the different treatments were washed-free from the fungicide and transferred on the MSR medium without fungicides. At 1, 10 and 100 mgL^{-1} a.i. of azoxystrobin, 91%, 56% and 68% (80%, 83% and 70%, for Amistar) of the non-germinated spores germinated on the new MSR medium within 6 days. At 1, 10 and 100 mgL^{-1} a.i. of flutolanil, 100%, 63% and 69% (67%, 83% and 65% for Monarch) of the non-germinated spores germinated on the new MSR medium and at the same concentrations of pencycuron, 67%, 58% and 60% (80%, 50%

and 50% for Monceren) of the non-germinated spores germinated on the new MSR medium.

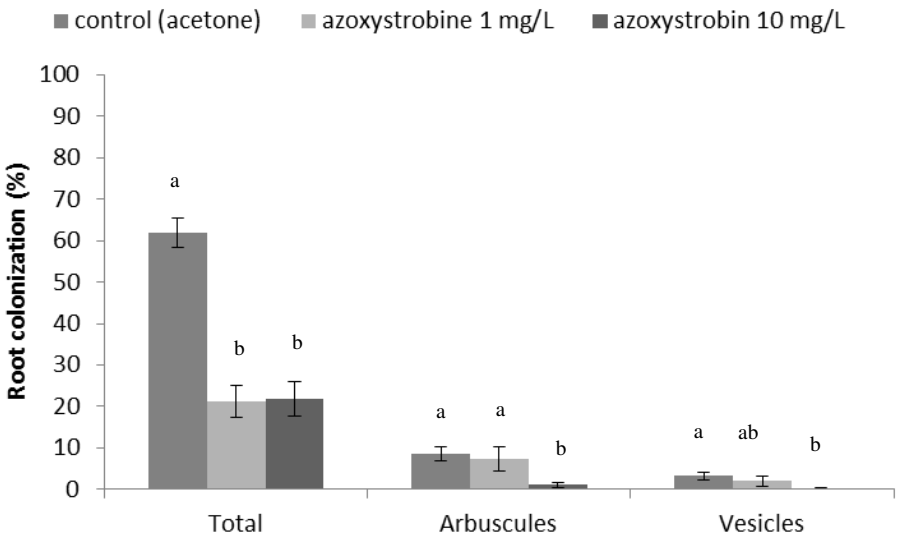
Impact of fungicides on Solanum tuberosum L. cv. Bintje root colonization, spore production and fungal regrowth

The impact of acetone and methanol, used as solvent for fungicides solubilization, was estimated on root colonization (Figure 17, A). For %RC and %V no significant differences were observed between the two solvents and as compared to the water control treatment. However, in the acetone control treatment the %A was significantly reduced by a factor 3 as compared to the water control treatment or the methanol control treatment. In contrast, the %A in the methanol control treatment did not differ from the water control treatment.

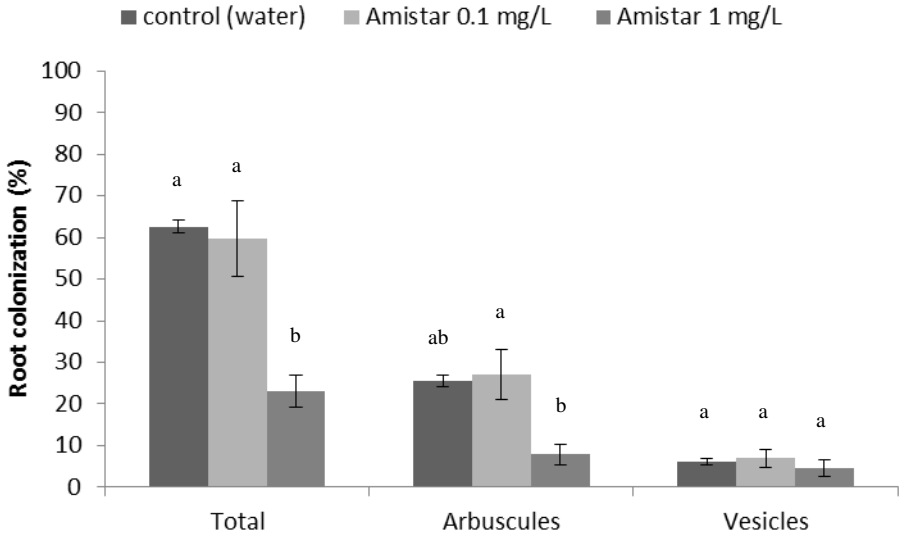
A



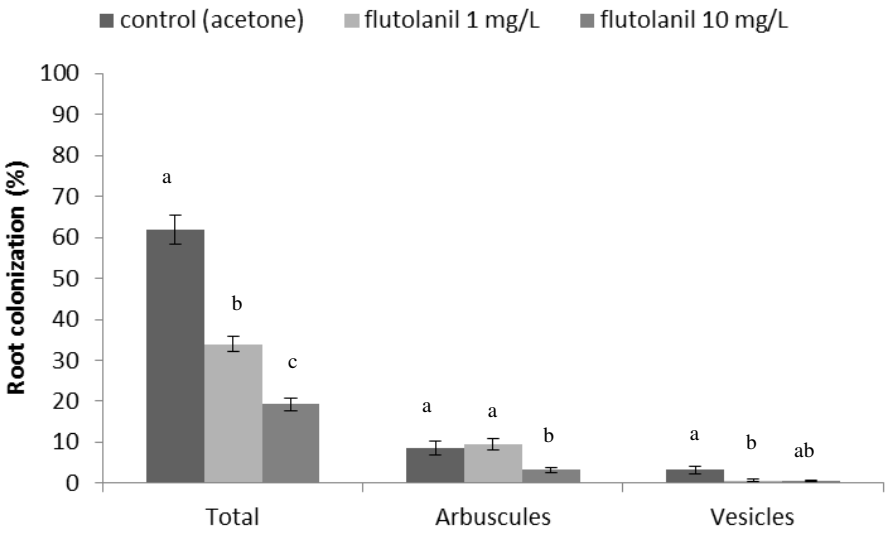
B


control (acetone)
azoxystrobine 1 mg/L
azoxystrobin 10 mg/L
Root colonization (%)
100
90
80
70
60
50
40
30
20
10
0
a
b
b
a
a
b
a
ab
b
Total
Arbuscules
Vesicles

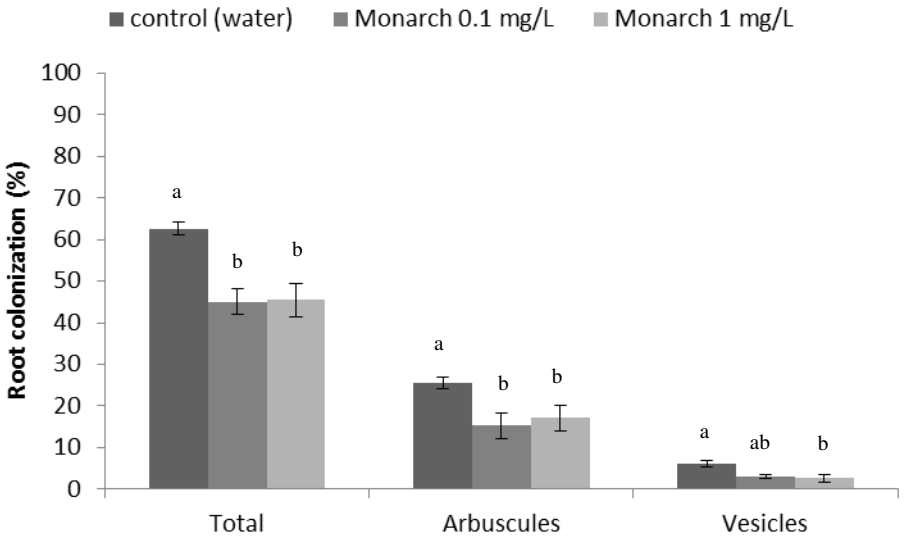
C


control (water)
Amistar 0.1 mg/L
Amistar 1 mg/L
Root colonization (%)
100
90
80
70
60
50
40
30
20
10
0
a
a
b
ab
a
b
a
a
a
Total
Arbuscules
Vesicles

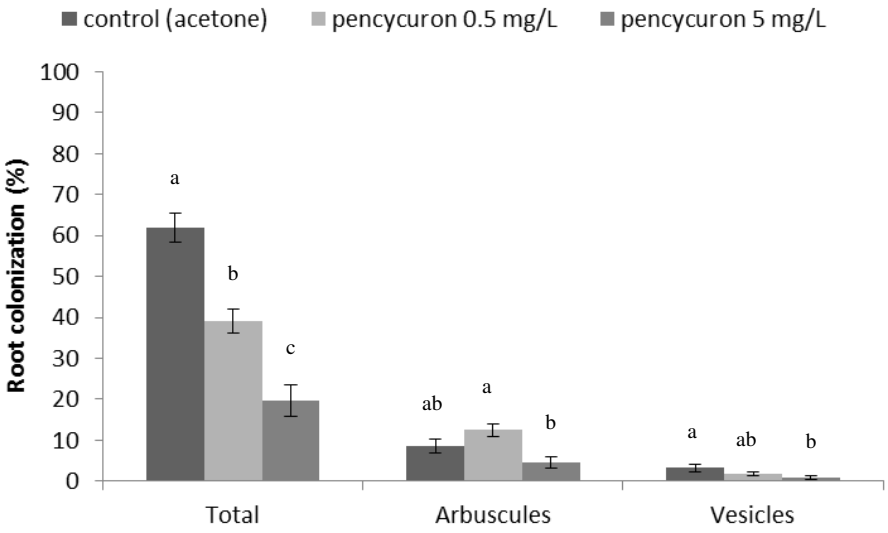
D


control (acetone)
flutolanil 1 mg/L
flutolanil 10 mg/L
Root colonization (%)
100
90
80
70
60
50
40
30
20
10
0
a
b
c
a
a
b
a
b
ab
Total
Arbuscules
Vesicles

E


control (water)
Monarch 0.1 mg/L
Monarch 1 mg/L
Root colonization (%)
100
90
80
70
60
50
40
30
20
10
0
a
b
b
a
b
b
a
ab
b
Total
Arbuscules
Vesicles

F



G

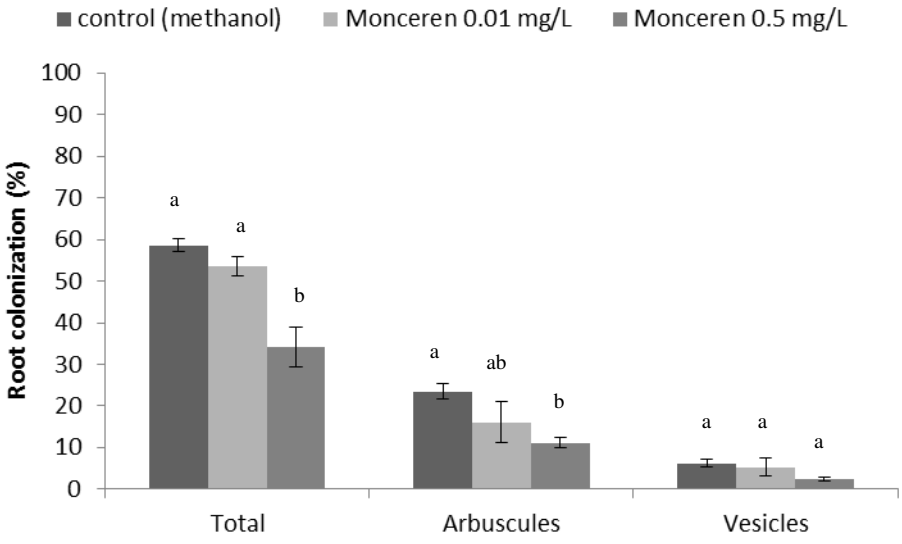


Figure 17. Percentage of root colonization (total, arbuscules and vesicles/spores) of potato by *R. irregularis* MUCL 41833 in absence (control water, acetone and methanol) (A) or presence of azoxystrobin (A), Amistar (B), flutolanil (C), Monarch (D) or

pencycuron (F), Monceren (G). Bars with the same letters are not significantly different (Tukey's test, $P < 0.05$; n= 7).

Root colonization in presence of azoxystrobin, flutolanil and pencycuron or their respective formulations (Amistar, Monarch and Monceren) at 0.1, 1, 10, 100 mgL^{-1} a.i. for azoxystrobin; 0.1, 1, 10 mgL^{-1} a.i. for flutolanil and 0.01, 0.5, 5 mgL^{-1} a.i. for pencycuron is presented in Figure 17, B-G.

Azoxystrobin at 1 and 10 mgL^{-1} a.i. significantly reduced %RC as compared to the acetone control treatment (Figure 17, B) and no root colonization was observed at 100 mgL^{-1} a.i. (data not shown). %A and %V did not differ from the acetone control treatment at 1 mgL^{-1} a.i. but significantly decreased at 10 mgL^{-1} a.i. (Figure 17, B). %A at 10 mgL^{-1} a.i. was significantly lower than %A at 1 mgL^{-1} a.i. (Figure 17, B). At 1 mgL^{-1} a.i. of Amistar the %RC, but not the %A or %V, was significantly reduced as compared to the water control treatment (Figure 17, C). To the contrary, at 0.1 mgL^{-1} a.i., no significant differences was observed for %RC, %A and %V with the water control treatment (Figure 17, C). %A at 1 mgL^{-1} a.i. was significantly lower than %A at 0.1 mgL^{-1} a.i. (Figure 17, C).

Flutolanil at 1 and 10 mgL^{-1} a.i. significantly reduced %RC as compared to the acetone control treatment (Figure 17, D). %RC at 10 mgL^{-1} a.i. was significantly lower than %RC at 1 mgL^{-1} a.i. (Figure 17, D). The %A did not differ from the acetone control treatment at 1 mgL^{-1} a.i. but was significantly lower at 10 mgL^{-1} a.i. (Figure 17, D). %A at 10 mgL^{-1} a.i. was significantly lower than %A at 1 mgL^{-1} a.i. (Figure 17, D). The %V was significantly lower from the acetone control treatment at 1 mgL^{-1} a.i. but not at 10 mgL^{-1} a.i. At 1 mgL^{-1} a.i. of Monarch the %RC, %A and %V was significantly reduced as

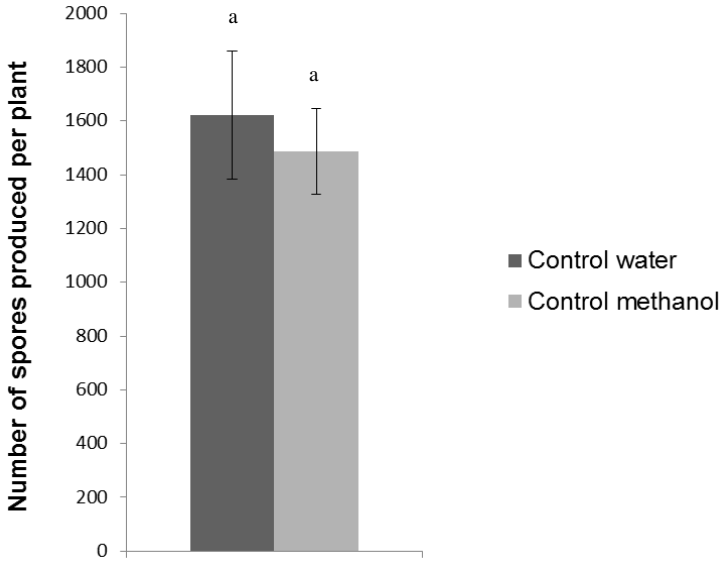
compared to the control water treatment (Figure 17, E). When concentration was reduced to 0.1 mg/L significant difference with control water treatment was found for %RC, %A but not for %V (Figure 17, E).

Pencycuron at 0.5 and 5 mgL^{-1} a.i. reduced significantly %RC as compared to the control acetone treatment (Figure 17, F). %RC at 5 mgL^{-1} a.i. was significantly lower than %RC at 0.5 mgL^{-1} a.i. (Figure 17, F). No significant difference with control acetone treatment was observed for %A at both concentrations, but %A at 5 mgL^{-1} a.i. was significantly lower than %A at 0.5 mgL^{-1} a.i. (Figure 17, F). %V at 5 mgL^{-1} a.i. was significantly lower than control acetone treatment but not at 0.5 mgL^{-1} a.i. At 0.5 mgL^{-1} a.i., of Monceren, the %RC and %A was significantly reduced as compared to control methanol treatment (Figure 17, G). No significant difference with control methanol treatment was observed for %V (Figure 17, G). When concentration was reduced to 0.01 mgL^{-1} a.i., no significant differences with the control methanol treatment were found for %RC, %A and %V (Figure 17, G). %RC at 0.5 mgL^{-1} a.i. was significantly lower than %RC at 0.01 mgL^{-1} a.i. (Figure 17, G).

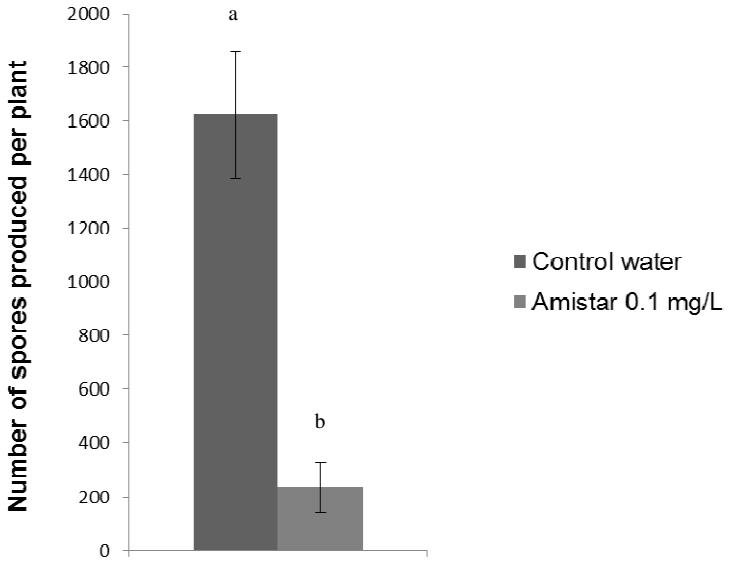
The number of new spores produced from plants colonized in the MDP *in vitro* culture system in absence (control water or methanol treatments) or in presence of Amistar, Monarch at 0.1 mgL^{-1} a.i. or Monceren at 0.01 mgL^{-1} a.i, following their transfer on MSR medium in mono-compartimented Petri plates under the same conditions as above, is presented in Figure 18. The number of new spores produced in the control methanol treatment was not significantly different as compared to the control water treatment (Figure 18, A). A significant reduction in spore production was observed with Amistar at 0.1 mgL^{-1} a.i. as compared to the water control treatment (Figure 18,

B), but no significant reduction was observed for Monarch at 0.1 mgL^{-1} a.i. (Figure 18, C). No significant reduction in spore production was observed for Monceren at 0.01 mgL^{-1} a.i. compared to the control methanol treatment (Figure 18, B).

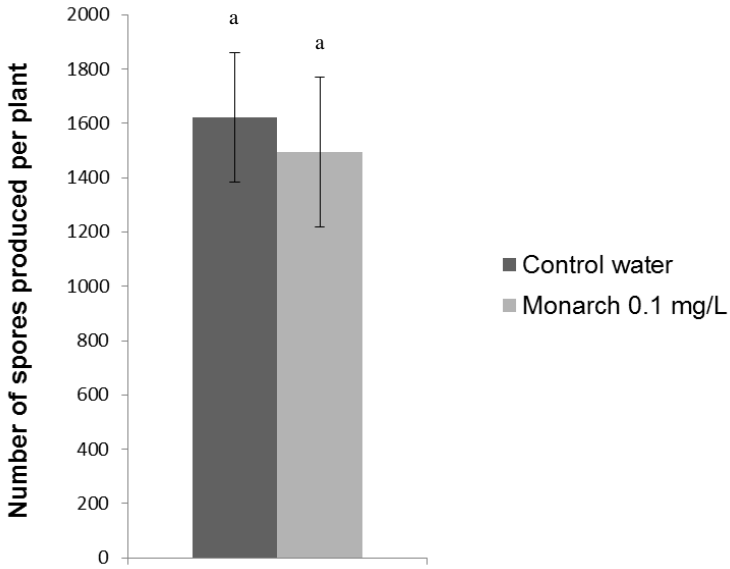
A



B


Number of spores produced per plant
2000
1800
1600
1400
1200
1000
800
600
400
200
0
a
b
Control water
Amistar 0.1 mg/L

C


Number of spores produced per plant
2000
1800
1600
1400
1200
1000
800
600
400
200
0
a
a
Control water
Monarch 0.1 mg/L

D

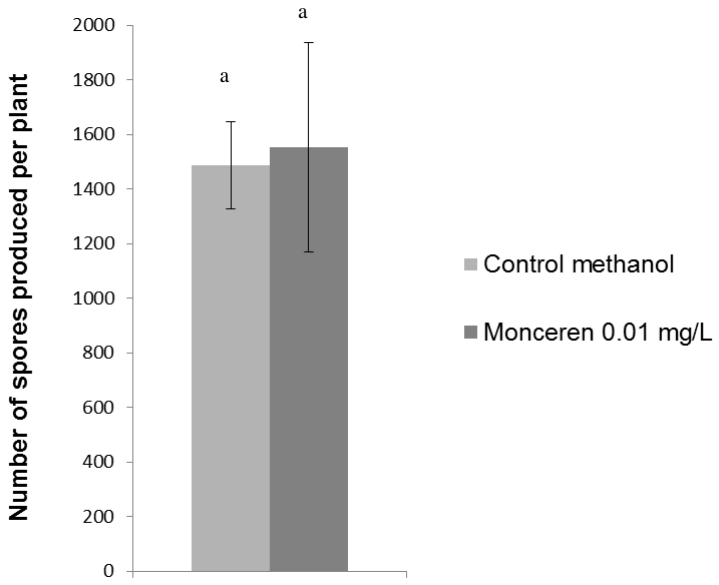
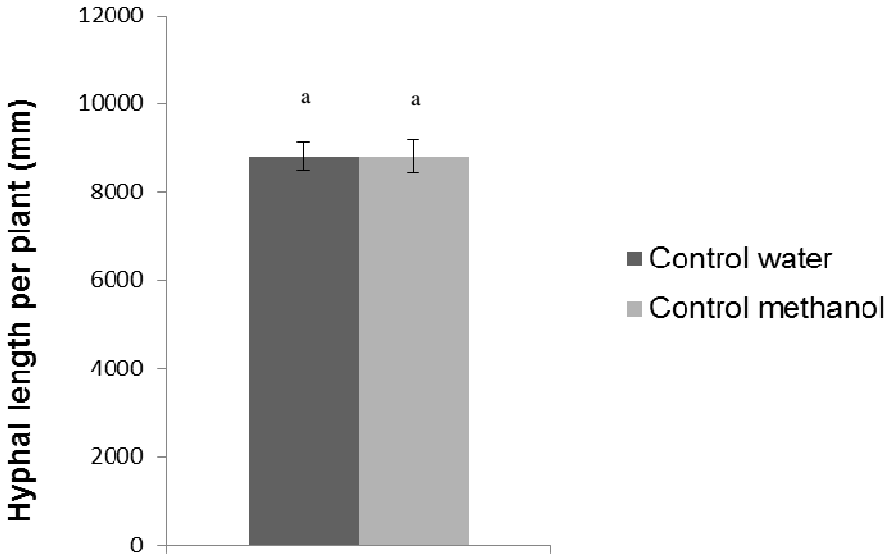


Figure 18. Spore production by *R. irregularis* MUCL 41833 after 9 weeks of culture in absence (control water or control methanol) (A), presence of Amistar at 0.1 mgL^{-1} a.i. (B), Monarch at 0.1 mgL^{-1} a.i. (C) and Monceren at 0.01 mgL^{-1} a.i. (D). Bars with the same letters are not significantly different (Tukey's test, $P < 0.05$; n= 5).

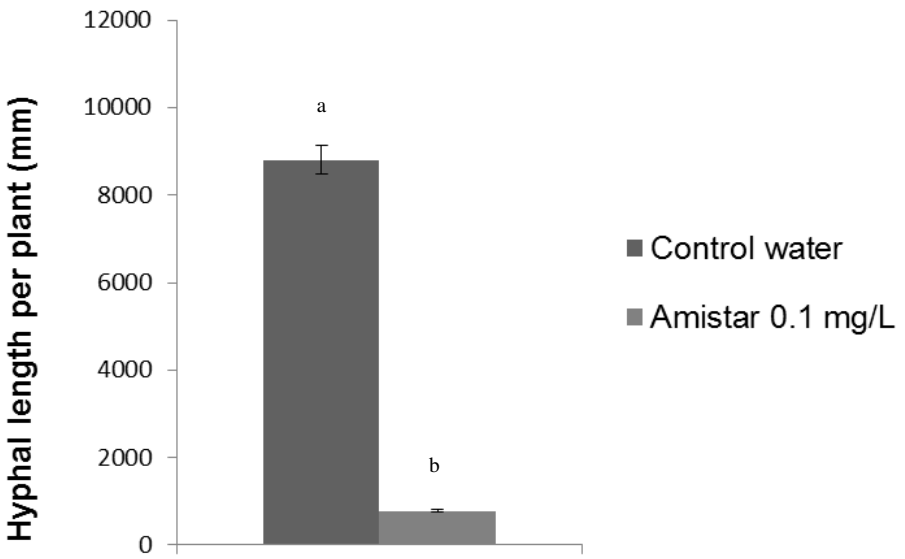
The extraradical mycelium regrowth from colonized plants under the MDP *in vitro* culture system in absence (control water or methanol) or in presence of Amistar or Monarch at 0.1 mgL^{-1} a.i. or Monceren at 0.01 mgL^{-1} a.i following their transfer on the MSR medium in mono-compartimented Petri plates under the same conditions as above, is presented in Figure 19. The extraradical mycelium length in the control methanol treatment was not significantly different as compared to the control water treatment (Figure 19, A). A significant reduction in mycelium growth with

Amistar at 0.1 mgL^{-1} a.i. compared to the water control treatment (Figure 19, B) was observed, while no significant reduction was observed for Monarch at 0.1 mgL^{-1} a.i. (Figure 19, C). No significant reduction was observed for Monceren at 0.01 mgL^{-1} a.i. as compared to the control methanol treatment (Figure 19, D).

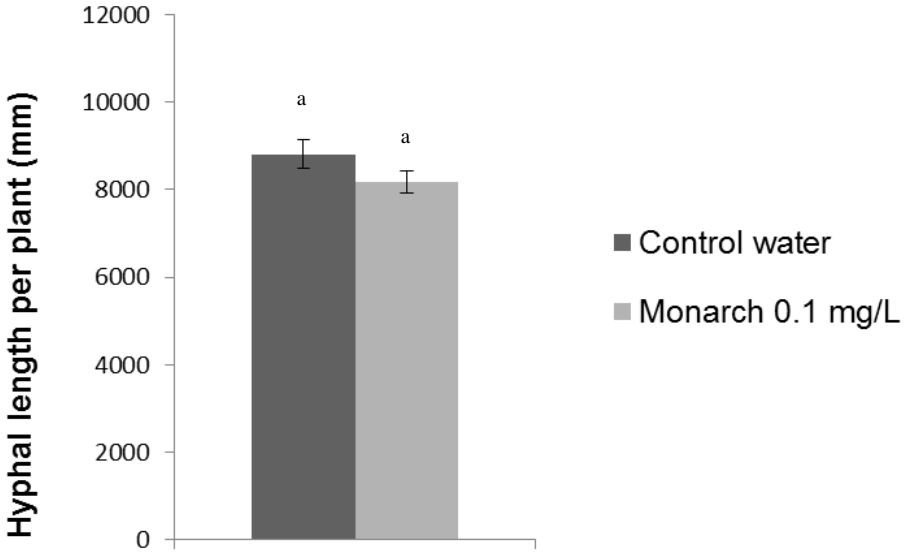
A



B


12000
10000
8000
6000
4000
2000
0
Hyphal length per plant (mm)
a
b
Control water
Amistar 0.1 mg/L

C


12000
10000
8000
6000
4000
2000
0
Hyphal length per plant (mm)
a
a
Control water
Monarch 0.1 mg/L

D

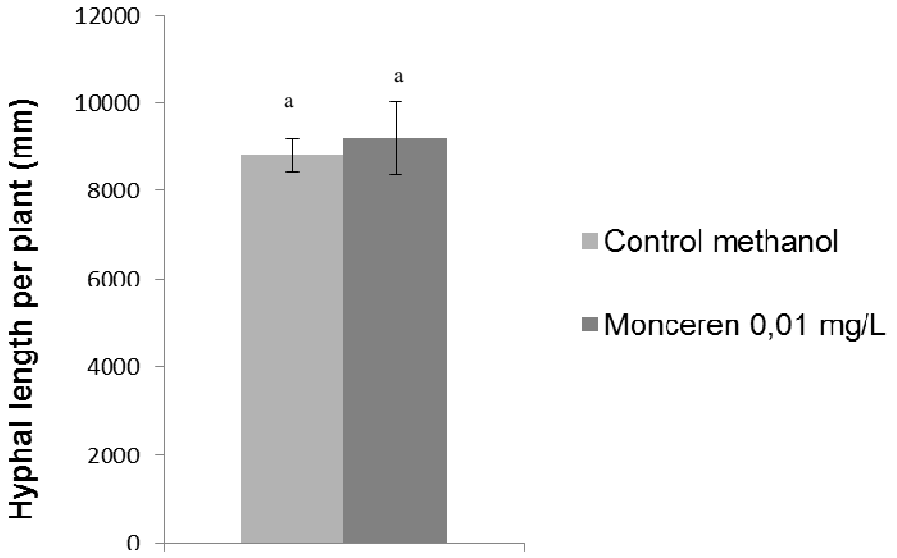


Figure 19. Hyphal length of *R. irregularis* MUCL 41833 per plant after 9 weeks of culture in absence (control water or control methanol) (A) or presence of Amistar at 0.1 mgL^{-1} a.i. (B), Monarch at 0.1 mgL^{-1} a.i. (C) and Monceren at 0.01 mgL^{-1} a.i. (D). Bars with the same letters are not significantly different (Tukey's test, $P < 0.05$; n= 5).

Discussion

Rhizoctonia stem canker and black scurf is an economically important disease of potatoes worldwide. Currently it is not possible to completely control this disease, but severity can be limited by a combination of cultural and crop protection factors. Seed or soil fungicide treatment is among the most widely used control strategy, that may also be harmful to non-target bio-control organisms (e.g. AMF), another strategy increasingly considered to control belowground diseases. Here, we tested the impact of a broad-spectrum (azoxystrobin) or Basidiomycota-specific (flutolanil)

systemic fungicide, blocking cell respiration and a contact Rhizoctonia-specific fungicide (pencycuron) blocking cell division and their respective formulations, Amistar, Monarch and Monceren, on AMF. IC_{50} was first established on dry weight of *R. solani* and data used as threshold concentrations to evaluate the impact of the fungicides on AMF. For the three selected fungicides, the IC_{50} did not differ significantly between a.i. and formulation. The values should be regarded as comparative rather than absolute and were approximately 0.01, 0.1 and 0.01 mgL^{-1} a.i. for azoxystrobin (Amistar), flutolanil (Monarch) and pencycuron (Monceren), respectively.

None of the parameters evaluated on *R. irregularis* MUCL 41833 AMF (i.e. spore germination, root colonization, spore production and hyphal length) was affected at IC_{50} threshold concentrations of *R. solani* MUCL 49235, except root colonization (%RC and %A) in presence of Monarch. Spore germination was impacted at concentrations above or equal to 1 mgL^{-1} a.i. for azoxystrobin (Amistar) and pencycuron (Monceren) and above or equal to 10 mgL^{-1} a.i. for flutolanil (Monarch). Interestingly, most of the non-germinated spores (50% to 100%), washed-free from the fungicides, were able to germinate on fungicide-free MSR medium. This suggested that the fungicides from 1 to 100 mgL^{-1} a.i. had a fungistatic effect on AMF spore germination rather than a fungitoxic effect. At concentrations above or equal to 1 mgL^{-1} a.i. for azoxystrobin (Amistar), above or equal to 0.1 mgL^{-1} a.i. for flutolanil (Monarch) or above or equal to 0.5 mgL^{-1} a.i. for pencycuron (Monceren), the fungicides significantly reduced or inhibited completely (100 mgL^{-1} a.i. azoxystrobin) root colonization of potato plant by AMF, while at concentrations of 0.1 mgL^{-1} a.i. of azoxystrobin (Amistar) and 0.01 mgL^{-1} a.i. of pencycuron (Monceren), root colonization was not affected. Monarch reduced root colonization at

1 mgL^{-1} a.i. at the same rate as at 0.1 mgL^{-1} a.i. New spores as well as hyphal growth was not affected by low concentrations of fungicides (0.1 mgL^{-1} a.i. of flutolanil (Monarch) or 0.01 mgL^{-1} a.i. of pencycuron (Monceren)), except azoxystrobin (Amistar) at 0.1 mgL^{-1} a.i. which is a dosis 10× higher than the *R. solani* IC_{50} threshold.

Azoxystrobin (Amistar) at 0.1 mgL^{-1} a.i. affected more extraradical structures (spores and hyphae) than intraradical structures (hyphae, spores/vesicles and arbuscules). This was earlier observed by Kjøller and Rosendahl (2000) in the greenhouse with propiconazole another broad-spectrum fungicide. The ALP[3] activity of the extraradical mycelium was decreased suggesting its higher sensitivity to this fungicide as compared to the intraradical mycelium which was not affected. This was confirmed *in vitro* by Calonne *et al.* (2012). These authors suggested that the target site of propiconazole was the same as phytopathogenic fungi. With azoxystrobin at 50 mg a.i. kg^{-1} soil, the enzymatic activity of AMF was inhibited demonstrating the fungicidal activity of this strobilurine on the respiratory electron transfer within mithochondria where SDH[4] is part of complex II (Diedhiou et al., 2004). However this concentration was probably 100 times higher than the actual rate of application of Amistar in potato crop (i.e. 1500 g a.i. ha^{-1}) which is about 0.5 mg a.i. kg^{-1} soil (if soil bulk density = 1.5 g cm^{-3} and depth of soil layer = 20 cm). In our *in vitro* experiment we observed that at 100 times higher concentrations than IC_{50} of *R. solani in vitro* (i.e. 1 mgL^{-1}) intra- and extraradical AMF was inhibited. Flutolanil at 0.1 mgL^{-1} a.i. affected

[3] AMP alkaline phosphatases are hydrolase enzymes involved in intracellular P transport and transformation, and fungal nutrition. AMF ALP activity is mainly present in the vacuolar compartment of both arbuscules and intraradical hyphae

[4] Succinate dehydrogenase is a enzyme complex who bound to the inner mitochondrial membrane of many fungal cells. The enzyme is one of the key enzymes in the Krebs cycle and may closely related to respiratory activity

more intraradical structures (hyphae and arbuscules) than extraradical structures (spores and hyphae). This is possibly because of its high systemic activity and inhibition of SDH activity. Pencycuron at 0.01 mgL^{-1} a.i. did not affect intra- or extraradical structures but at higher concentration inhibited AMF growth. Even if fungicides at high doses seem to impact AMF enzymatic pathways, the enzymes of the respiration pathway or cell division pathway in AMF are probably less sensitive to azoxystrobin, flutolanil and pencycuron than those of phytopathogenic fungi. Zocco *et al.* (2008) suggested this hypothesis on the sterol biosynthesis pathway for fenpropimorph and fenhexamid. Moreover Glomeromycota differ from Basidiomycota on the coenocytic nature of their hyphae (i.e. living hyphae lacking septa and thereby nuclei, mitochondria and other organelles sharing a common cytoplasm) involving other enzymatic metabolisms.

Some fungicides can impact root colonization by impacting root growth. Calonne *et al.* (2012) suggested that propiconazol, a Sterol Biosynthesis Inhibitor (SBI) fungicide (Class I: Δ14 demethylation) inhibited both AMF symbiotic partners development by their sterol metabolism alteration. Campagnac *et al.* (2008) evaluated two other SBI fungicides, fenpropimorph (Class II: Δ14-reduction and/or Δ8 → Δ7 isomerisation) and fenhexamid (Class III: C4 demethylation). Fenpropimorph altered root growth, root colonization and extraradical structures at increased concentrations (Campagnac *et al.*, 2008, Zocco *et al.*, 2008). To the contrary fenhexamid did not modify plant sterol profiles and had limited impact on mycorrhizal colonization (Campagnac *et al.*, 2008).

In this work, we used an *in vitro* easy-to-use, reproducible methodology (Zocco *et al.*, 2011) to evaluate the toxicity of fungicides broadly used to control *Rhizoctonia* disease, on the AMF *R.*

irregularis MUCL 41833. We demonstrated that azoxystrobin, flutolanil and pencycuron at concentrations equal to the IC_{50} for *R. solani*, did not affect the extra- and/or intra-radical phase of AMF. Only root colonization (%RC and %A) was significantly reduced with flutolanil (as formulation) at threshold value. This method was similarly applied to fenpropimorph and fenhexamid (Zocco et al., 2011; Campagnac et al., 2010), propiconazole (Calonne et al., 2012), chlorothalonil, carbendazin and thiram (Gong et al., 2014) and could thus represent a standardized approach to evaluate compounds and formulations. Indeed, screening the impact of fungicides on AMF under *in vitro* conditions offer several advantages, among which the easy non-destructive observations of the mycelium architecture and sporulation that is nearly impossible to achieve *in vivo* and the microbial-free experimental conditions avoiding any confounding effects with unwanted microorganisms. Our results seem to confirm earlier greenhouse experiments with fungicides applied on soil or seeds (Burrows and Ahmed, 2007, Diedhiou *et al.*, 2004, Jin *et al.*, 2013). These authors demonstrated that systemic fungicides impact more AMF than contact fungicides and hypothesized that it was because compounds of systemic fungicides accumulate in or on the roots in contrast to contact fungicides. In our study, we also noticed that systemic fungicides flutolanil and azoxystrobin affected more intra- or extraradical structures of AMF than the contact fungicide pencycuron at threshold concentration. Unfortunately, with the exception of azoxystrobin (Diedhiou *et al.*, 2004), no other greenhouse studies were conducted with flutolanil or pencycuron, the two other fungicides used in our study.

The three fungicides used in our experiment are characterized by an increased specificity towards *R. solani* (Azoxystrobin - broad-

spectrum < Flutolanil - Basidiomycota-specific < Pencycuron - *Rhizoctonia* specific) and different mode of action. Contact and most specific fungicide pencycuron looks compatible with AMF at the IC_{50} of *R. solani*. Systemic fungicide flutolanil seem to have a more pronounced effect on root colonization probably because of accumulation of the fungicide in the root zone. Azoxystrobin is known to have a lower systemic activity than flutolanil and this could explain why it does not affect intraradical AMF at 10× IC_{50} for *R. solani.* A broad parallel of field dosages with the quantities used in our experiment (i.e. in Petri plates) can be made if we hypothesize that soil bulk density is 1.5 g cm^{-3}, that fungicide penetrate until 20 cm under the soil layer and that potato tubers are planted at a density of 20 deciton (dt) ha^{-1}. We also should consider that *in vitro* the compounds are not degraded at the same speed as in the field. At field application, Amistar is applied at 1500 g ha^{-1} a.i., Monarch at 9,2 g dt^{-1} a.i. and Monarch at 25 g dt^{-1} a.i. what correspond to 0.75, 0.09 and 0.25 mgL^{-1} a.i, respectively. These values are equal or higher than the threshold values measured in our *in vitro* experiment. Indeed, under our *in vitro* culture conditions azoxystrobin and pencycuron did not affect AMF at threshold concentration to control *R. solani in vitro*, while flutolanil (as formulation) impacted the intraradical phase of the fungus. These fungicides and AMF could thus potentially be used in parallel against Rhizoctonia disease in potato. Further experiments are now required to evaluate, under semi-controlled greenhouse and microcosm field trials, the impact of fungicides on AMF at dosages detrimental to *R. solani* and at dosages where the pressure by *R. solani* is decreased to a level compatible with its control by the AMF itself.

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CHAPTER 2

Glyphosate impacts sporulation dynamics of *Rhizophagus irregularis* and *Rhizophagus intraradices* more severely than chemical or mechanical defoliation

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Preface

In **chapter I**, we evaluated the direct impact of three fungicides, used to control *R. solani* in potato crops, on *R. irregularis* MUCL 41833. It is obvious that other agricultural factors such as the application of herbicides may impact the non-target AMF. Herbicides may impact the AMF indirectly (via the plant) by inhibiting the production of photosynthates (e.g. carbon resources) and their subsequent allocation to the AMF.

In **chapter II**, we compared the impact of different agricultural defoliation treatments (mechanical defoliation or chemical defoliation with a contact defoliant (diquat-formulation)) and a systemic herbicide application (glyphosate- formulation), on the dynamics of spore production of *R. irregularis* and *R. intraradices* associated to potato or barrel medic. We discussed the strategies to apply in order to attenuate the impact of defoliation or herbicide application on *R. irregularis* and *R. intraradices* in agro-ecosystems.

Abstract

Arbuscular mycorrhizal fungi (AMF) are obligate root symbionts which are totally dependent on the plant for their carbon nutrition. In return, the mineral uptake of the plants is facilitated by the fungus. If the impact of grazing or mechanical defoliation of shoot and leaves on the plant-to-fungus pathway of C is well documented, no information, to our knowledge, is available on the impact of chemical defoliation or systemic herbicides on AMF sporulation dynamics. Here, we evaluated under *in vitro* conditions, the impact of two defoliation treatments (mechanical defoliation or chemical defoliation (diquat-formulation)) and one systemic herbicide application (glyphosate-formulation) on the dynamics of spore production of *Rhizophagus irregularis* and *Rhizophagus intraradices* associated to *Solanum tuberosum* and/or *Medicago truncatula*. The systemic herbicide affected the spore production rate more rapidly and severely than chemical and mechanical defoliation. We hypothesized that glyphosate had a faster and stronger effect than the chemical or mechanical defoliation by disruption of the C metabolism in the whole plant (roots included) combined with a possible direct effect on the fungus via the release of the systemic herbicide in the roots and medium. This hypothesis should, however, be further analysed by tracing C metabolism in the plant after herbicide or defoliation treatment. The application of systemic herbicides that could impact AMF via the plant should thus be limited as far as possible. Moreover, defoliation or systemic herbicides applied in the plateau phase of AMF sporulation instead of in the lag- or log-phase should be the preferred in agricultural itineraries.

Keywords

Arbuscular mycorrhizal fungi; *Rhizophagus irregularis*; *Rhizophagus intraradices*; glyphosate; diquat; systemic herbicide; contact defoliant

Introduction

Arbuscular mycorrhizal fungi (AMF) colonize the roots of most agricultural crops and weeds indiscriminately (Yamato, 2004). They provide the plants with nutrients (e.g. N and P) in exchange of carbohydrates. Up to 20% of photosynthetically fixed carbon may be allocated to the fungus (Nottingham *et al.*, 2010), representing a significant cost to the host plant (Douglas, 2008, Kiers and Van Der Heijden, 2006). These fungi, which are fully dependent on host-derived carbohydrates, are thus unable to survive long periods without a living host (Bécard *et al.*, 2004). Chemical/mechanical defoliations and herbicide applications, three common agricultural factors used to facilitate crop harvest, suppress cover crops or control weeds, are thus likely to impact AMF.

Mechanical defoliation (Saravesi *et al.*, 2014) or severe grazing by herbivores (Gehring and Whitham, 1994) have been reported to affect AMF root colonization, suggesting that colonization is limited by host carbon availability. Even though a number of studies have reported increased root colonization following grazing (see meta-analysis of Barto and Rillig (2010)). The altered allocation of C resources to the AMF immediately following defoliation has been observed on spores production (IJdo *et al.*, 2010), although stimulation has also been mentioned (Klironomos *et al.*, 2004). The differences in sporulation or root colonization response after defoliation between studies may be related to various factors (as reviewed by Barto and Rillig (2010)) among which the fungal species.

For instance, IJdo *et al.* (2010) have demonstrated *in vitro*, that repeated defoliation of *Medicago truncatula* affected *Rhizophagus intraradices* and *Scutellospora reticulata* differently. The rate of spore production of *R. intraradices* was decreased immediately after defoliation and re-established shortly thereafter, at regrowth of leaves, while *S. reticulata* presented a higher resistance to the temporally applied disturbance (IJdo *et al.*, 2010). These authors have attributed the differences in sporulation response to the life history strategy of both organisms by observing the sporulation dynamics after defoliation. Klironomos *et al.* (2004) have found also that the effects of defoliation differed between AMF species and was dependent on the frequency of defoliation. It has been suggested that species may differ in C demand (Saikkonen *et al.*, 1999) or sensitivity to photosynthates stress (Bethlenfalvay *et al.*, 1984).

The impact of herbicides on AMF has been reported in several studies. Most have described a direct impact of herbicides on spore viability, germination or plant colonization (Druille *et al.*, 2013, Karpouzas *et al.*, 2014, Li *et al.*, 2013, Pasaribu *et al.*, 2011, Wan *et al.*, 1998), while a few have mentioned an indirect impact via the plant (Zaller *et al.*, 2014). However, to the best of our knowledge, no study have reported on chemical defoliants and on the effects of herbicides on the dynamics of spores' production.

Glyphosate (Monsanto) is the world's most widely used systemic non selective herbicide to control weeds or suppress cover crops (Woodburn, 2000). It kills plants by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase), a key enzyme in the shikimate biosynthetic pathway (Helander *et al.*, 2012). Over 30% of the carbon fixed by the plants passes through this pathway (Shaner, 2006). Glyphosate is translocated from the treated

leaves to the shoots and roots within 48 to 72h. Neumann *et al.* (2006) have shown that glyphosate was subsequently released in the soil via root decay and have suggested that it could be taken up by non-target microorganisms (e.g. fungi or bacteria), impacting their shikimate pathway. Indeed, EPSP synthase is not only found in plants but also in bacteria and fungi (Padgette *et al.*, 1995) and can thus modify microbial communities in the soil which can have a positive or negative impact on ecosystem functions. The persistence and transport of glyphosate in soil is dependent on soil composition, climatic conditions and microbial activity (Carlisle and Trevors, 1988), as well as agricultural management (Helander *et al.*, 2012). *In vitro* (Wan *et al.*, 1998) and *in vivo* (Druille *et al.*, 2013) studies on AMF have shown that glyphosate and aminomethylphosphonic acid (AMPA), a glyphosate secondary metabolite, were relatively toxic to AMF (IC_{50} of 0.5 ± 0.3 ppm and 3.8 ± 2.8 ppm, respectively) and reduced arbuscules when glyphosate was applied on plant leaves.

Diquat (Syngenta Crop Protection) is a contact defoliant inhibiting photosynthesis by causing a deviation of electron flow from photosystem I with subsequent inhibition of $NADP^+$ reduction and the production of peroxide radicals leading to the destruction of cell membranes (Zweig *et al.*, 1965). It is used to dry many crops to facilitate seed harvest and is not translocated to the roots and is thus not directly lethal to the plant. To our knowledge, no study on the effects of diquat on AMF has been conducted so far. It is probable that under natural conditions diquat does not impact AMF directly because it is inactivated and immobilized in the soil (Tucker *et al.*, 1967). Conversely, it is possible that it may reduce AMF spores production by impacting plant photosynthesis and thus carbohydrate production.

In the present study, we evaluated and compared the effects of two different defoliation treatments (mechanical defoliation and chemical defoliation with a contact defoliant (diquat-formulation)), with the application of a systemic herbicide (glyphosate), on the dynamics of spores production of *Rhizophagus irregularis* and *Rhizophagus intraradices.* Spores were considered because their production is strongly linked to mycelium production (Declerck *et al*., 2001) and have been considered as C storage structures in the mycelium as evidenced by radioactive tracers (Bago *et al*., 2003). Spores are also the more easy fungal structures to monitor non-destructively and to model *in vitro* (Declerck *et al*., 2001, IJdo *et al*., 2010). Finally, spores production in *R. intraradices* has been shown to be influenced instantaneously by mechanical defoliation (IJdo *et al*., 2010) and represent thus an adequate parameter to evaluate and compare the short-term effects of mechanical defoliation, chemical defoliation and the application of a systemic herbicide on AMF. The study has been conducted with two AMF (*Rhizophagus irregularis* MUCL 41833 and *Rhizophagus intraradices* MUCL 49410) and two plants (*Solanum tuberosum* and *Medicago truncatula*) as model plants/AMF couples grown together *in vitro*.

Materials and methods

Biological material

Solanum tuberosum L., var. Bintje was supplied by the Station de Haute Belgique in Libramont (Belgium) and micro-propagated every 5 weeks on Murashige and Skoog medium (Duchefa, The Netherlands) as described in Voets *et al.* (2005).

Seeds of *Medicago truncatula* Gaertn. cv. Jemalong A 17 (SARDI, Australia) were surface-disinfected by immersion in sodium

hypochlorite (8% active chloride) for 10 min and rinsed in sterilized (121 °C for 15 min) deionized water. Seeds were germinated in Petri plates (90 mm diameter) containing 35 ml of Modified Strullu-Romand (MSR) medium (Declerck *et al.*, 1998) without sucrose and vitamins, solidified with 3 g L^{-1} phytagel and adjusted to pH 5.5 before sterilization (121˚C for 15 min). The Petri plates were incubated at 27˚C in the dark. Seedlings were ready to use 4 days after germination.

Both plants have been used as models in the autotrophic culture system developed by Voets *et al.* (2005) for the *in vitro* mycorrhization of potato plantlets, and by Voets *et al.* (2009) for barrel medic seedlings. Sporulation dynamics of *Rhizophagus irregularis* and *R. intraradices* were established with both plants, under non-perturbed (Voets *et al.*, 2005) and mechanical defoliation (IJdo *et al.*, 2010) conditions, for potato and barrel medic, respectively.

Rhizophagus irregularis (Błaszk., Wubet, Renker & Buscot) C. Walker & Schuessler (2010) [as 'irregulare'] MUCL 41833 and *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & Schuessler (2010) MUCL 49410 were supplied by the Glomeromycota *in vitro* collection (GINCO) (http://www.mycorrhiza.be/ginco-bel/index.php) and maintained in the mycelium donor plant (MDP) *in vitro* culture system (Voets *et al.*, 2009) until thousands of spores were produced. Both strains (formerly classified as *Glomus* species) have similar spore production dynamics with lag, log and plateau phases (Declerck *et al.*, 2001, Declerck *et al.*, 1996).

Herbicides

We used a systemic herbicide (RoundUp® Max – Monsanto, Belgium) containing 450 g L^{-1} glyphosate, diluted in 250 mL of water (8 mL L^{-1}) which is the recommended field dosis, and a contact defoliant (Reglone® – Syngenta Crop Protection N.V., Belgium) containing 200 g L^{-1} diquat, diluted in 250 mL of water (1.5 mL L^{-1}) which is less than the recommended field dosis but was determined in a preliminary experiment as the minimal dosis necessary to desiccate leaves of *in vitro* plants completely after 96 h.

Autotrophic culture systems

We used the autotrophic culture system developed by Voets *et al.* (2005) for the *in vitro* mycorrhization of potato plantlets, and adapted by Voets *et al.* (2009) for barrel medic seedlings. Briefly, an opening (± 2 mm diameter) was made in the base and the lid of mono-compartmented Petri plates (90 mm diameter) containing 40 ml of MSR medium (Declerck *et al.*, 1998) without sucrose and vitamins, solidified with 3 g L^{-1} phytagel and adjusted to pH 5.5 before sterilization (121˚C for 15 min). Ten-day old micropropagated potato plantlets or four-day old barrel medic seedlings were transferred in the Petri plates with their roots plated on the surface of the MSR medium and shoots developing under open air conditions. An approximate of 100 spores of *R. irregularis* MUCL 41833 or *R. intraradices* MUCL 49410 was inoculated in the vicinity of the roots. Each Petri plate was then closed and sealed with Parafilm M® (Bemis Company, Inc.) and the opening carefully plastered with sterilized (121°C for 15 min) silicon grease (VWR International, Belgium) to avoid contaminations. The Petri plates were covered with opaque plastic bags to keep the AMF and plant roots in the dark. They were further incubated in a growth chamber under controlled conditions (i.e. 22/18°C (day/night), 70% relative humidity, a photoperiod of 16 h

day^{-1} and an average photosynthetic photon flux density of 225 µmol m^{-2} s^{-1}). MSR medium was added twice a month to the potato and once a week to the barrel medic plant systems to supply the plantlets with renewed nutrients. After defoliation, MSR medium was added only to the "Control" treatments because defoliated plants did not need extra medium.

Experimental design

After a few weeks (five for potato, eight or nine for barrel medic) of association in the autotrophic culture systems, a profuse mycelium bearing numerous spores was obtained. For potato associated to *R. irregularis* MUCL 41833 and barrel medic associated to *R. irregularis* MUCL 41833 or *R. intraradices* MUCL 49410, the systems were divided into four groups (see Table 7) to which defoliation treatment was applied, in such a way that the average number of spores at the time of treatment was similar for all groups (Table 8). The first group of plants was not defoliated or killed with an herbicide ("Control"). The three other groups were defoliated either mechanically with scissors ("Mechanic") or chemically (diquat-formulation – "Diquat-F") or killed with an herbicide (glyphosate-formulation – "Glyphosate-F").

Table 7. Number of replicated autothrophic *in vitro* culture systems considered within each treatment (Control, Mechanic, Glyphosate-F or Diquat-F) and for each plant-AMF association (Potato- *R. irregularis*, Barrel medic – *R. irregularis*, Barrel medic – *R. intraradices*) (contaminations reduced number of replicates between treatments.)

Plant		Potato	Barrel medic	
Isolate		*R. irregularis*		*R. intraradices*
Treatments	**Control**	11	5	6
	Mechanic	11		8
	Glyphosate-F	11	5	
	Diquat-F	11		7

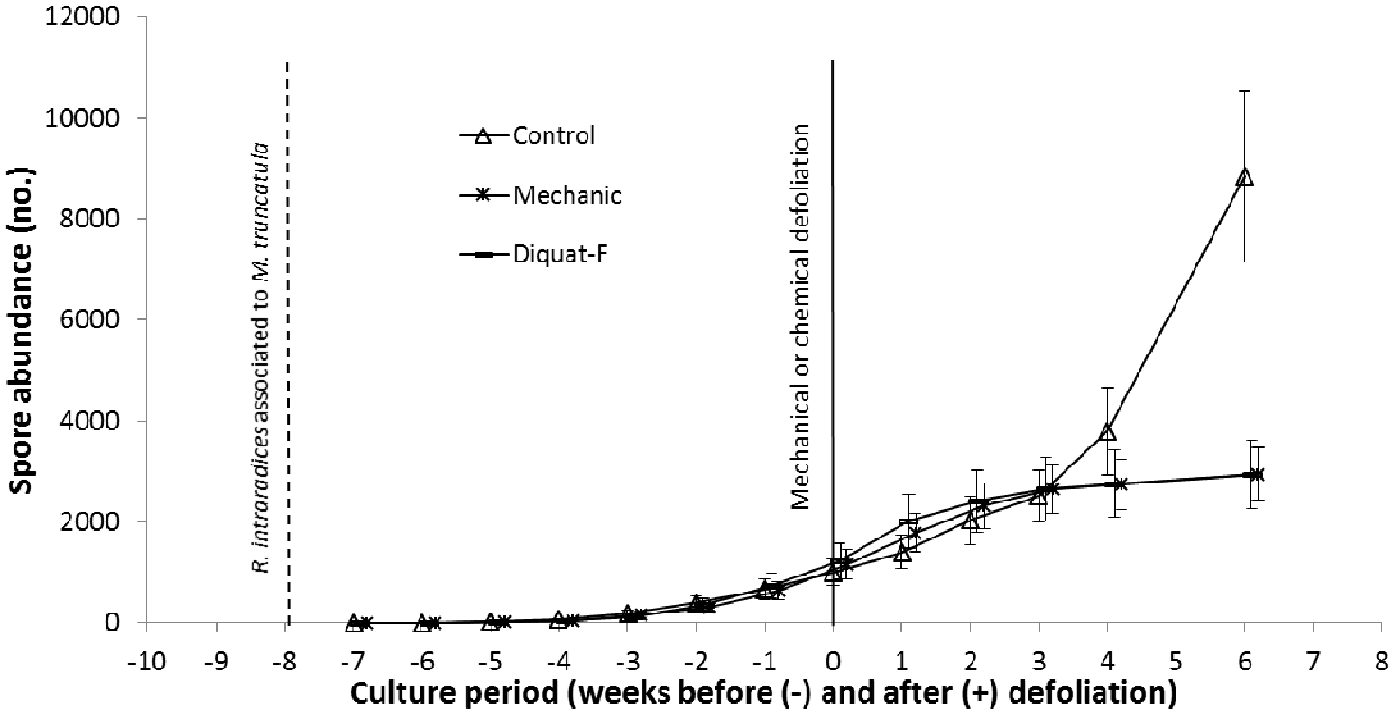
Table 8. AMF growth descriptors measured in autotrophic culture systems according to three treatment groups (Mechanic, Diquat-F and Glyphosate-F) and a non-defoliated control with potato and barrel medic plants: number of replicate systems followed through time (n), mean number of spores at defoliation (time 0), root dry weight (DW) and intra-radical colonization levels at harvest (presented as Total%, Arbuscules% and Spores/Vesicles%).

Experimental design			At defoliation (time 0)		At harvest		
					Root colonization (%)		
Plant	Treatment	n	Number of spores	Root DW (g)	Total	Arbuscules	Spores/ Vesicles
Potato	Control	11	311 ± 68[a]	0.079 ± 0.007[a]	41.2 ± 2.0[a]	9.4 ± 1.1[a]	3.8 ± 0.5[a]
	Mechanic	11	300 ± 81[a]	0.054 ± 0.003[b]	16.5 ± 2.3[b]	2.6 ± 0.7[b]	1.1 ± 0.4[b]
	Diquat-F	11	292 ± 69[a]	0.060 ± 0.003[b]	19.8 ± 2.0[b]	3.1 ± 0.6[b]	1.0 ± 0.3[b]
	Glyphosate-F	11	304 ± 78[a]	0.055 ± 0.003[b]	19.0 ± 2.2[b]	2.1 ± 0.3[b]	0.8 ± 0.2[b]
Barrel medic	Control	11	929 ± 218[a]	0.132 ± 0.002[a]	64.8 ± 1.6[a]	12.4 ± 0.3[a]	6.7 ± 0.3[a]
	Mechanic	8	1157 ± 305[a]	0.086 ± 0.002[b]	35.4 ± 2.3[b]	5.8 ± 0.2[c]	3.0 ± 0.2[b]
	Diquat-F	7	1226 ± 355[a]	0.088 ± 0.002[b]	35.7 ± 1.8[b]	7.2 ± 0.2[b]	3.4 ± 0.2[b]
	Glyphosate-F	5	840 ± 284[a]	0.074 ± 0.004[c]	35.1 ± 0.8[b]	5.4 ± 0.3[c]	1.5 ± 0.3[c]

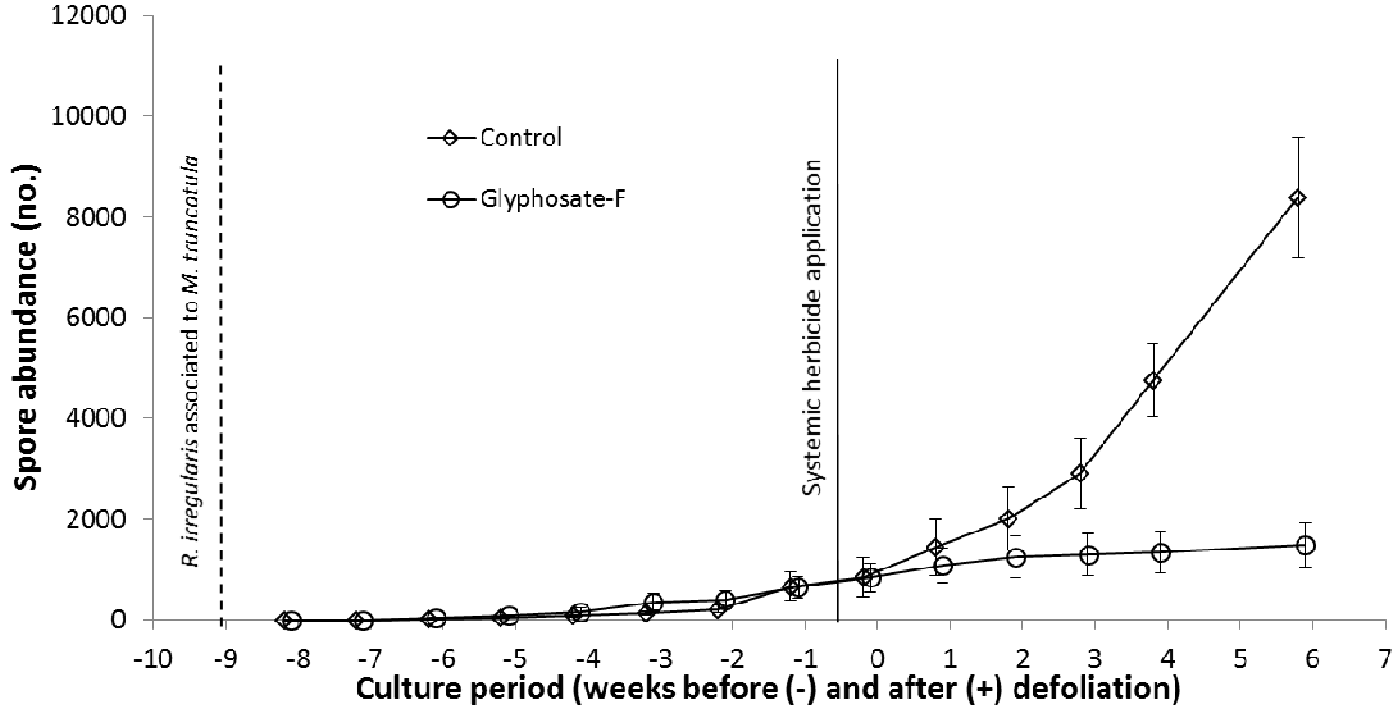
All data are means ± SE. Different letters indicate significant differences (Tukey's HSD, $\alpha = 0.05$).

For potato, the four groups (each with 11 systems) were used in a single experiment with *R. irregularis* MUCL 41833. For barrel medic, a first experiment was performed with the Control and Glyphosate-F groups (with 5 systems for each group), with *R. irregularis* MUCL 41833 strain. The second experiment was performed with the Control (6 systems), Diquat-F (7 systems), and Mechanic (8 systems) groups with *R. intraradices* strain MUCL 49410 (Table 7). The dynamics of spore production over the first 8 weeks of association, i.e. before defoliation treatment, was similar for plants inoculated in the two experiments, i.e. with *R. irregularis* MUCL 41833 and with *R. intraradices* MUCL 49410 (Figure 20). No significant difference in spore abundance eight weeks after association was observed between *R. irregularis* and *R. intraradices* (one way ANOVA, $F_{1,29} = 3.00$, $p = 0.094$), allowing the results of the two experiments to be pooled for the statistical analysis of sporulation dynamics. Mechanical defoliation was performed with scissors at the crown of the plant. Diquat-F and Glyphosate-F were applied on the shoots and leaves of each plant with a sprayer. It was performed once and no regrowth was observed.

a)


Spore abundance (no.)
12000
10000
8000
6000
4000
2000
0
Control
Mechanic
Diquat-F
R. intraradices associated to M. truncatula
Mechanical or chemical defoliation
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8
Culture period (weeks before (-) and after (+) defoliation)

b)


12000
10000
8000
6000
4000
2000
0
Spore abundance (no.)
Control
Glyphosate-F
R. irregularis associated to M. truncatula
Systemic herbicide application
-10
-9
-8
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
Culture period (weeks before (-) and after (+) defoliation)

c)

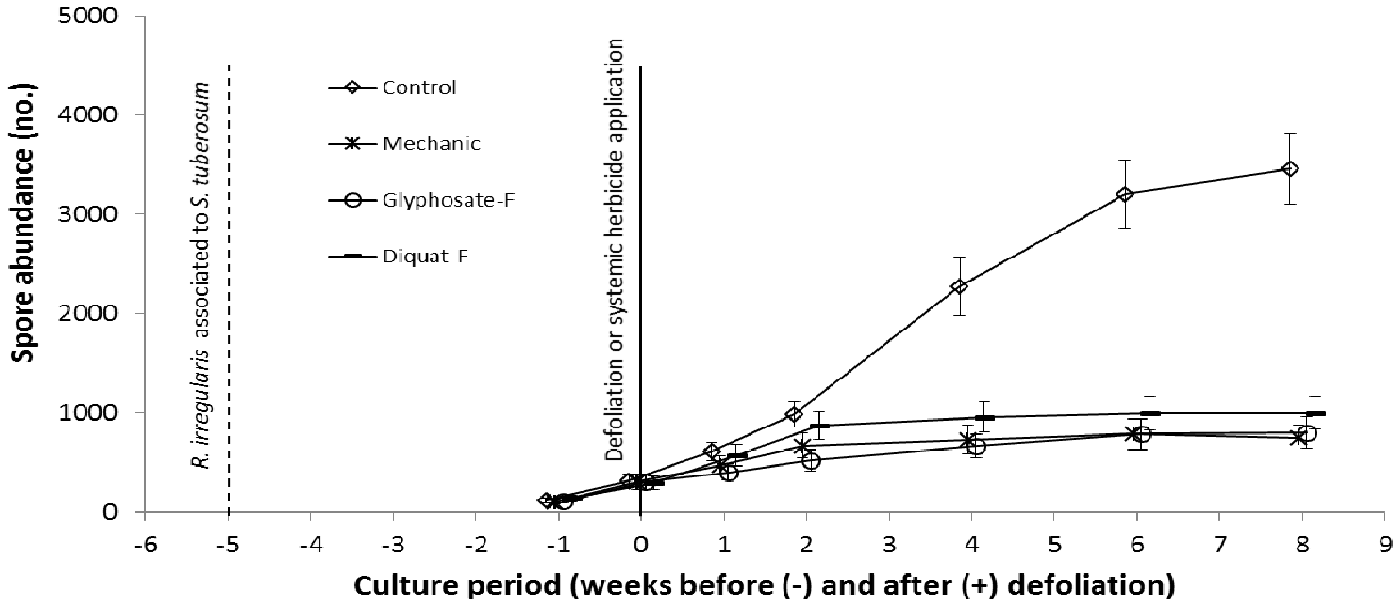


Figure 20. Spore abundance of a) *Rhizophagus intraradices* MUCL 49410 associated with *in vitro* grown *Medicago truncatula*, b) *Rhizophagus irregularis* MUCL 41833 associated with *in vitro* grown *Medicago truncatula* and c) *Solanum tuberosum*. Control plants were not defoliated. The other plants were defoliated mechanically (Mechanic) or chemically with a systemic herbicide (Glyphosate-F) or a contact herbicide (Diquat-F). Data are means ± SE. Points were slightly staggered for easier visualization.

Sporulation dynamics

The dynamics of spore production was followed weekly under a stereo-microscope (Olympus SZ40, Olympus Optical GmbH, Germany) at ×10-40 magnification. A grid of lines was marked on the bottom of each autotrophic culture system to facilitate counting (Declerck *et al.*, 1996). Quantification was regularly performed before and after the application of the defoliation treatment (Figure 20).

Spore production dynamics were evaluated by quantifying the average weekly rate of spore production from defoliation (R_t) according to the following formula:

$$R_t = \sqrt[t]{\frac{N_t}{N_0}}$$

with N_t being the abundance of spore at week t, and N_0 the abundance of spores when the defoliation treatment was applied (week 0). Density dependence is known to affect spore production rate (Declerck *et al.*, 2001), but the autotrophic culture systems were distributed among the four treatment groups in such a way that they had a similar average abundance of spores at the week of defoliation (Table 8). We decided therefore not to implement any correction for density dependence, our analysis being conservative because the effect of density-dependence, if any, would be randomized among the four treatment groups and increase the residual variation, i.e. making the effects less significant statistically.

The spore production rate was ln-transformed and analyzed, for each plant species separately, using a three-way ANOVA model (time and defoliation as fixed crossed factors, replicate as random factor crossed with time but nested within defoliation). Differences

between defoliation types were subsequently tested week by week using contrasts to establish the rapidity of impact of each defoliation treatment. Number of replicates per defoliation being different in the experiment with barrel medic (Table 7), we checked that the results of the ANOVA analysis were robust to this imbalance. To do so, we randomly down-sampled the data to produce a series of 1000 perfectly balanced datasets (with 5 replicate systems per defoliation), and recomputed the ANOVA tests.

Plant root weight and AMF root colonization

At the end of the experiment, roots were harvested and colonization estimated. Roots were first cleaned from MSR medium and then dried at 60°C during 24 hours to estimate dry weight. The roots were subsequently cleared with 10% KOH at 50°C for 90 min and stained with 5% blue ink (Parker®) diluted in vinegar (7° acidity) at 50°C. The roots were rinsed with deionized water and observed under a compound microscope (Olympus BH2, Olympus Optical GmbH, Germany) at x 200 magnification. Root colonization was assessed by evaluating the proportion of total root colonization, arbuscules and vesicles/spores in 200 microscopic intersects, following the method of McGonigle *et al.* (1990). These variables were normalized by arcsine transformation and the effect of defoliation tested using a one-way ANOVA, completed with Tukey's honest significant difference tests to compare individual defoliations.

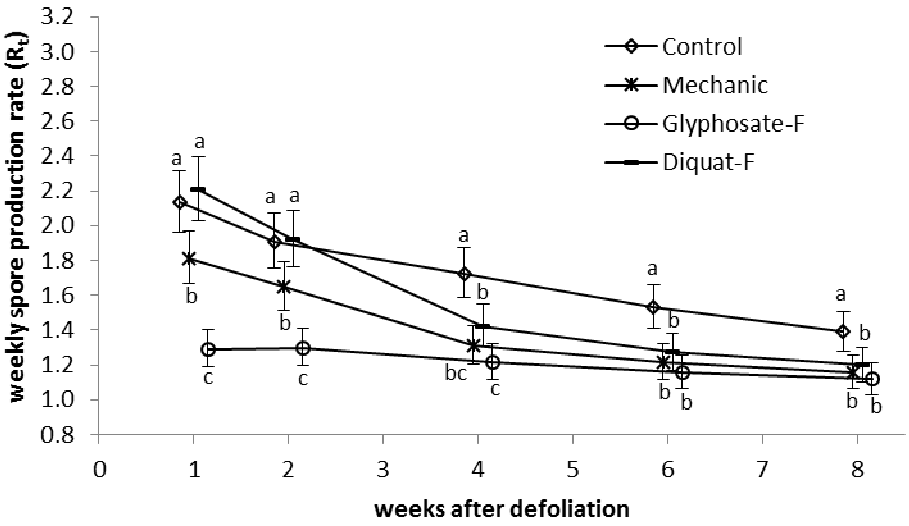
Results

Sporulation dynamics

Sporulation dynamics are presented in Figure 20. The first daughter spores were observed 3 weeks after plant-fungus

association. Spore production of *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410 increased gradually on both plants. A clear and general decrease of spore production rate, as quantified by the average weekly spore production rate from defoliation (R_t), was observed after plant defoliation (Figure 21). The magnitude of the effect differed between defoliation treatments and these differences changed as time passes from defoliation (Table 9). Overall, the results were quite similar for potato and barrel medic. Defoliation via Glyphosate-F had the strongest and fastest effect, decreasing weekly spore production rate already by 40% and 19% after 1 week, for potato and barrel medic, respectively. Mechanic defoliation and defoliation with Diquat-F affected spore production nearly as severely from six weeks after defoliation, but at a slower and more progressive pace. For potato, mechanic defoliation significantly affected spore production from the first week, faster than defoliation with Diquat-F; at week 4, the impact of both defoliations became similar. For barrel medic, both mechanic defoliation and defoliation with Diquat-F affected spore production from week 2 only, and their effects never significantly differed until the end of the experiment. Conclusions were robust to statistical imbalance: the tests of the treatment, week and treatment*week effects, and the contrasts comparing the four defoliations on a week per week basis, were similar when tested on randomly downsampled balanced datasets (with 5 replicates per defoliation level) (Table 9).

a)


weekly spore production rate (R_t)
weeks after defoliation
Control
Mechanic
Glyphosate-F
Diquat-F

b)

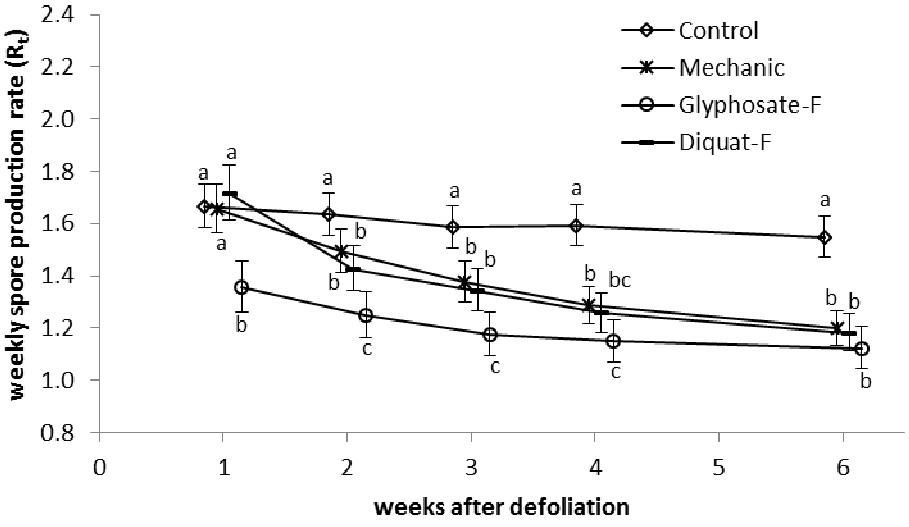

weekly spore production rate (R_t)
weeks after defoliation
Control
Mechanic
Glyphosate-F
Diquat-F

Figure 21. Weekly rate of spore production (R_t) of *Rhizophagus irregularis* MUCL 41833 or *Rhizophagus intraradices* MUCL 49410 associated with *in vitro* grown a) potato and b) barrel medic plants, after defoliation treatment applied at week 0. R_t was computed as the average rate over the period from week 0 to week *t*; it is therefore a cumulative measure. Data are least-squares means ± 95% confidence limits; means with the same letter were not statistically different at that week (3 way ANOVA with contrasts, see text for details). Points were slightly staggered for easier visualization.

Table 9. Statistical analysis of average weekly spore production rates (R_t) using a three-way ANOVA (time and defoliation as fixed crossed factors, replicate as random factor crossed with time but nested within defoliation).

Source	Degrees of freedom	Sum of squares	Mean square	F-test	P-Value	
Potato						
Time	4	5.2767	1.3192	68.18	**<0.0001**	
Defoliation	3	3.6325	1.2108	7.56	**0.0004**	
Replicate (Defoliation)	40	6.4092	0.1602	.	.	
Time × Defoliation	12	1.0055	0.0838	4.33	**<0.0001**	
Time × Replicate (Defoliation)	160	3.0959	0.0193	.	.	
Error	0	0	.	.	.	
Corrected total	219	19.4197				
Barrel medic						**% significant**
Time	4	1.0275	0.2569	31.61	**<0.0001**	100.00
Defoliation	3	1.5982	0.53274	4.39	**0.0122**	60.00
Replicate (Defoliation)	27	3.2751	0.1213	.	.	
Time × Defoliation	12	0.2805	0.0234	2.88	**0.0018**	61.20
Time × Replicate (Defoliation)	108	0.8778	0.0081	.	.	
Error	0	0	.	.	.	
Corrected total	154	7.0040				

"." denotes statistics that could not be computed given the ANOVA model used. Tests that revealed significant differences at the 0.05 α level are indicated in bold. "% significant": proportion of the 1000 perfectly balanced datasets with 5 replicates per defoliation group (produced from randomly downsampled data) for which the effect was significant at the 0.05 α level, as a way to confirm that the results of the full analysis were only slightly impacted by statistical imbalance of the original data.

Plant root growth and AMF root colonization

After eight weeks of defoliation for potato and six weeks for barrel medic, dry weight of roots of both plants was evaluated (Table 8). Defoliation, whatever the treatment, had a clear negative impact on both root dry weight and root colonization, and this was observed similarly with potato and barrel medic (Table 8). On potato, no significant difference between the three defoliant or herbicide treatments was observed. However, on barrel medic, the negative effect of Glyphosate-F on dry weight and spores/vesicles abundance was significantly larger than after mechanical defoliation or defoliation with Diquat-F, and the negative effect of Glyphosate-F or mechanical defoliation on the arbuscules abundance was significantly larger than defoliation with Diquat-F (Table 8).

Discussion

Chemical/mechanical defoliations and herbicide applications, three common agricultural factors used to facilitate crop harvest, suppress cover crops or control weeds, are likely to impact AMF. Here we used a reproducible *in vitro* culture system to evaluate the impact of two defoliation treatments (i.e. mechanical defoliation and chemical defoliation with a contact defoliant – diquat-formulation) and a systemic herbicide application (glyphosate-formulation) on the sporulation dynamics of *R. irregularis* and *R. intraradices* associated to potato or barrel medic. We hypothesized that the systemic herbicide had a faster and stronger effect than the contact defoliant or mechanical defoliation, because of its disruption of C metabolism in the whole plant (roots included) combined with a possible supplementary effect on AMF by the translocation of the herbicide within the roots in close contact with the IRM or release in the

medium via root decay and contact with the ERM. The contact defoliant or mechanical defoliation in contrast should have a slower and less marked impact on AMF as plants might have kept their capacity to mobilize C from reserves and ensure the transfer of these resources towards the AMF and because of the absence of contact with AMF structures.

In our study, we observed a clear and general decrease of spore production rate of *R. irregularis* and *R. intraradices* after plant defoliation, as in the study of IJdo *et al.* (2010). These authors suggested that the production of novel spores was directly dependent on the C flow from the plant and that this AMF was able to modulate spore production according to C availability. Our study supports this observation but revealed a difference of effect between the defoliant and herbicide treatment that confirmed our hypothesis: The systemic herbicide (Glyphosate-F) had a faster and stronger effect than the mechanical or chemical defoliation (Diquat-F). Glyphosate-F impacts directly the root part of the plant because of its rapid translocation to the roots (Shaner, 2006). The C flow from the root to the AMF was thus possibly directly interrupted, but this need to be further studied. This was corroborated by Druille *et al.* (2013) who found that spore viability was not reduced when glyphosate was applied on leaves (indirect pathway) but only when it was applied on the soil (direct pathway). However, in this study only spore viability was evaluated and not the production of new spores.

Mechanical defoliation and chemical defoliation using diquat seem to have a slower and less pronounced impact on AMF spore production. Both defoliation treatments inhibit plant photosynthetic production (by eliminating or drying foliar part of the plant) but do not impact directly the roots. Because C present in the roots could still be

translocated to the AMF, the impact of these defoliation treatments was slower and less pronounced. However, the contact defoliant (Diquat-F) and mechanical defoliation affected spore production nearly as severely as with the systemic herbicide (Glyphosate-F) six weeks after defoliation.

At harvest, 13 weeks after association of potato with *R. irregularis* MUCL 41833, 15 or 14 weeks after association of barrel medic with *R. irregularis* MUCL 41833 or *R. intraradices* MUCL 49410, mean root colonization for the control plants (i.e. without defoliation or herbicide application) was relatively high. All types of treatments and especially the systemic herbicide (Glyphosate-F) reduced colonization by *R. irregularis* and *R. intraradices* and root dry weight. Druille *et al.* (2013) have observed also a significantly reduction of root colonization (in particular arbuscules) after glyphosate application. They have explained that arbuscules were sensitive to the reduction in supply of carbohydrates caused by the herbicide. However, from our study a direct effect of glyphosate on the arbuscules via its translocation to the roots cannot be excluded. The contact defoliant (Diquat-F) in contrast does not move to the roots. The impact of defoliation on arbuscules or intraradical spores/vesicles formation is thus probably only caused by photosynthesis disruption in the shoots and reduction/interruption of carbon flow to the roots. The impact of mechanical defoliation on arbuscules or intraradical spores/vesicles is comparable to the contact defoliant because only the foliar part of the plant is affected.

As a conclusion, mechanical/chemical defoliation or herbicides strongly impact spore production of *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410, and thus may probably hinder the increase of propagules within agro-ecosystems. Systemic herbicides such as

glyphosate are probably more detrimental as they may also impact the AMF via translocation within roots and thus contact with the IRM and ERM via release into the soil. The time of defoliation may also play an important role on AMF spores populations. For instance, AMF sporulation dynamics follow a lag log and plateau phase (Declerck *et al.*, 2001, Declerck *et al.*, 1996). Defoliation of plants in the plateau phase of spore production should presumably be less detrimental to the AMF and spores production than defoliation in the lag and log phases. Techniques such as cover cropping with or without inoculation of AMF may, before defoliation, help to restore or increase AMF population suitable for cash crop. Other itineraries such as intercropping perennial and annual plants seem also a good agricultural strategy to avoid the negative impact of defoliation or herbicides on AMF populations by maintaining the beneficial mycorrhizal networks for faster colonization of crops. Systemic herbicide applications that could impact AMF via the plant should be avoided ad far as possible. Moreover, defoliation and systemic herbicides applied in the plateau phase of AMF sporulation instead of in the lag- or log-phase should be the preferred in agricultural itineraries.

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and acknowledges its financial support. Thanks are also to Tankoano Yentema Priscile who conducted part of the experiment.

PART II. Field studies

CHAPTER 3

Cover crop inoculation with *Rhizophagus irregularis* and *Trichoderma harzianum* increases potato yield

Adapted from the research article submitted to

Applied soil ecology

Catherine Buysens, Vincent César, François Ferrais,

Hervé Dupré de Boulois and Stéphane Declerck

Preface

The studies in **chapters I** and **II** were conducted under strict *in vitro* culture conditions in order to understand how the application of chemicals (i.e. fungicides and herbicides) could impact AMF. Results in **chapter I** suggested that azoxystrobin and pencycuron do not affect the AMF at field dosages to control *R. solani*, while flutolanil (as formulation) impacts the intraradical phase of the fungus. In **chapter II** we observed that a systemic herbicide (glyphosate) affects the rate of spore production more rapidly and severely than a contact defoliant (diquat) or a mechanical defoliation during the lag and log phase of spore production. Appropriate and timely defoliation could thus probably attenuate the impact of defoliation on spore production.

In **chapter III**, we aimed (1) to investigate the effects of *R. irregularis* MUCL 41833 and *T. harzianum* MUCL 29707 inoculated jointly on potato yield, under organic as well as conventional agricultural management and (2) to evaluate some agricultural factors on the population density, root colonization and plant growth promotion of AMF.

Abstract

Arbuscular mycorrhizal fungi and *Trichoderma* spp. are soil microorganisms that increase growth and improve a/biotic stress resistance of numerous crop plants. So far, most studies on potato were conducted under greenhouse or *in vitro* conditions and few reported on results within the field. We aimed to investigate the impact of co-inoculation of *R. irregularis* MUCL 41833 and *T. harzianum* MUCL 29707 at cover crop or at potato plantation, on potato yield. Two macroplot trials were set up over two growing seasons with the cultivars Sarpo Mira and Bionta, respectively. In both trials, individual subplots were arranged in a randomized complete block design replicated four times. Microbial inoculants were entrapped in alginate beads and inoculated at cover crop or potato plantation. Potato yield (i.e. tuber weight, size and number) was estimated. In both trials, inoculating the cover crop significantly increased tubers weight (37 % for cultivar Sarpo Mira and 13 % for cultivar Bionta) as compared to the control (i.e. absence of inoculation and cover crop). For cultivar Bionta, inoculating the cover crop or the potato tuber at plantation increased significantly ($P < 0.05$ and $P < 0.01$, respectively) the percentage of potatoes with calibre > 30 mm as compared to the control. With Sarpo Mira, inoculating the cover crop increased significantly ($P < 0.0001$) the number of tubers as compared to the control but no effect was observed with inoculation at potato plantation. Our results suggested that the application of beneficial microorganisms at cover crop sowing prior to potato plantation is an adequate, easy-to-apply cropping itinerary to increase potato production.

Keywords

Arbuscular mycorrhizal fungi; *Rhizophagus irregularis*; *Trichoderma harzianum*; *Solanum tuberosum*; cover crop; inoculation

Introduction

The application of plant-growth promoting microorganisms or plant resistance enhancers (e.g. arbuscular mycorrhizal fungi (AMF) or *Trichoderma* spp.) has become an option increasingly considered for improving crop production and plant health (Chaparro *et al.*, 2012, Conrath *et al.*, 2006, Gianinazzi *et al.*, 2010), paired or in replacement to the use of fertilizers and pesticides (Brimner and Boland, 2003).

AMF are ubiquitous soil microorganisms that form symbiotic associations with the vast majority of terrestrial plants (Smith and Read, 2008), including many agricultural crops (e.g. Douds *et al.* (2007)). These fungi provide the plants with minerals (in particular phosphorus) in exchange for carbohydrates (Smith and Read, 2008). As a result plant growth is improved. They also increase the resistance of plants against abiotic (Boomsma and Vyn, 2008, Evelin *et al.*, 2009) and biotic stresses (Pozo and Azcón-Aguilar, 2007). *Trichoderma* spp. are free-living fungi present in the soil and rhizosphere of plants. They are opportunistic avirulent plant symbionts increasing plant growth and resistance against biotic stresses (Harman *et al.*, 2004).

A number of studies have reported the synergism between AMF and *Trichoderma* spp. (Calvet *et al.*, 1993, Chandanie *et al.*, 2009). Co-inoculation was shown to increase the growth of numerous plants including citrus, asparagus and cucumber (Arriola *et al.*, 2000, Camprubí *et al.*, 1995, Chandanie *et al.*, 2009). A few studies also

mentioned mycoparasitism of *T. harzianum* on the extraradical (Rousseau *et al.*, 1996) and intraradical mycelium (De Jaeger *et al.*, 2010) of the AMF, although the vast majority reported that AMF development was not suppressed in presence of the antagonistic fungus but rather stimulated (Green *et al.*, 1999, Mar Vázquez *et al.*, 2000).

Within soils, the plant growth promotion effects of AMF and/or *Trichoderma* spp. are dependent on various factors. Among these, the presence and abundance of the microorganisms within the soil, their capacity to colonize plants and efficacy to promote growth of the target crop are determinant. Two distinct but complementary strategies may be followed to increase the population/efficacy of AMF and *Trichoderma* spp. within the soil. The first aims to promote agricultural itineraries favoring the survival or increase of the indigenous population of beneficial microorganisms (Gosling *et al.*, 2006). The second aims at introducing selected microorganisms into the field (Pellegrino *et al.*, 2011).

Agricultural factors may be beneficial or detrimental to soil beneficial microorganism. For instance, some pesticides (e.g. benomyl, propiconazol, fenpropimorph, azoxystrobin, carbendazim, prochloraz, glyphosate, flutolanil) are most often detrimental to AMF (Buysens *et al.*, 2014, Diedhiou *et al.*, 2004, Druille *et al.*, 2013, Ipsilantis *et al.*, 2012, Kjøller and Rosendahl, 2000, Zocco *et al.*, 2008) and *Trichoderma* spp. (Roberti *et al.*, 2006), while cover cropping was reported beneficial to soil microbial populations (Reddy *et al.*, 2003) and community structure (Elfstrand *et al.*, 2007).

Similarly, some key factors have been identified for the success of inoculation and survival of AMF and *Trichoderma* spp. in

soils: species compatibility, field carrying capacity and priority effect (Leandro *et al*., 2007, Verbruggen *et al*., 2013). Introduced species should, indeed, be compatible with the agricultural factors, environmental conditions (e.g. soil type and pH (Oehl *et al*., 2010), and host plant (Öpik and Moora, 2012)). The soil environment should contain a habitat niche that supports the introduced microorganisms. Agricultural soils have sometimes reduced "carrying capacities" caused by the absence of a suitable host plant, high nutrient levels or inappropriate temperature. The timing and competition with other AMF, *Trichoderma* spp. and microbes for the establishment of communities also influence the success of inoculation (Dickie *et al*., 2012).

Finally, the quality of inoculum, i.e. number of viable propagules, absence of contaminants, and proper formulation, is central to the success of inoculation whatever the microorganism considered (IJdo *et al*., 2011). Among the formulations frequently reported, the immobilization of microorganisms within beads offers an excellent protection of the inoculum against biotic and abiotic stresses (Bashan, 1998). Encapsulation of beneficial microorganisms such as *Glomus versiforme* and *Trichoderma viride* (Fravel *et al*., 1985) have been successfully reported. De Jaeger *et al.* (2011) further demonstrated the capacity of the AMF *R. irregularis* MUCL 41833 and *T. harzianum* MUCL 29707 to be co-entrapped into alginate beads.

Potato is ranked fourth among staple crops worldwide (FAOSTAT, 2012). This crop is characterized by heavy mechanization and high levels of pesticide applications. Few studies are available on the association of potato plants with AMF and/or *Trichoderma* spp. The effect of a direct inoculation of AMF on potato

was investigated by Douds *et al.* (2007) under different fertilization treatments. These authors noticed that inoculation with *Glomus intraradices* significantly increased potato yield and root colonization. Increased resistance against potato root and shoot diseases was also reported with AMF (Duffy and Cassells, 2000, Gallou *et al.*, 2011) and *Trichoderma* spp. (Gallou *et al.*, 2009).

Among the few studies conducted in potato fields (Bayrami *et al.*, 2012, Douds *et al.*, 2007) none specifically addressed the effect of agricultural factors, such as cover crop inoculation, on AMF and *Trichoderma* spp. and subsequent effects on potato yield. In the present study, two independent macroplot trials were conducted over two growing seasons to compare two cropping itineraries (cover crop inoculation prior to tuber plantation and direct tuber inoculation at potato planting with *R. irregularis* MUCL 41833 and *T. harzianum* MUCL 29707) on potato yield (i.e. tuber weight, size and number). We hypothesized that inoculating both microorganisms at cover crop sowing would result in a higher potato production than inoculation of potato at planting or the use of a cover crop without inoculation of beneficial microorganisms. The first trial was conducted without herbicide and fungicide treatments with cultivar Sarpo Mira and the second trial under organic or conventional weed and disease management with cultivar Bionta.

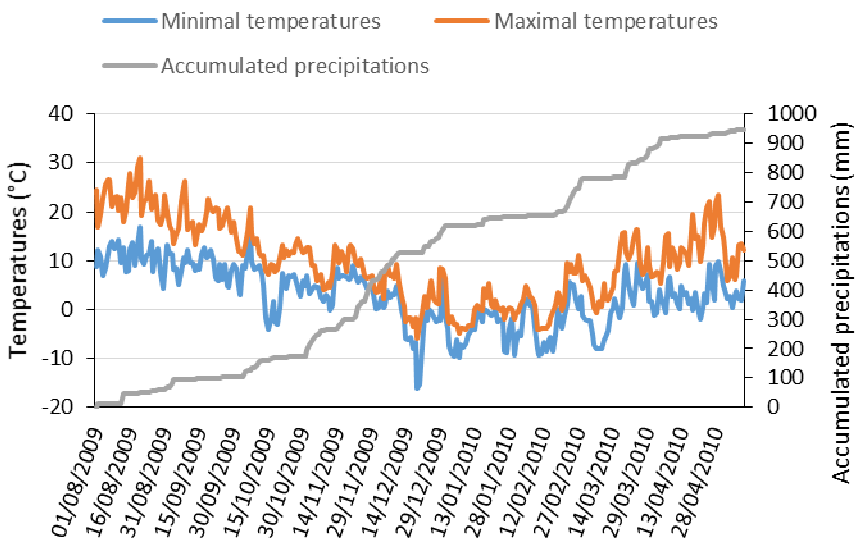
Material and methods

Experimental sites

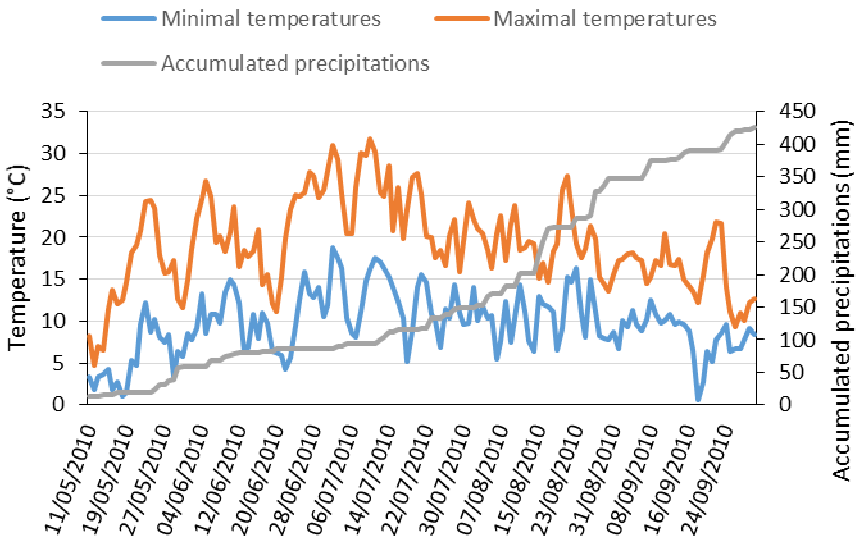
The two trials were conducted in Libramont (Belgium) at the Walloon Agricultural Research Centre, Life Sciences Department, Breeding and Biodiversity Unit, on two independent plots located next to each other (49°55'N, 5°22'E). The first trial was conducted from

August 2009 to September 2010 and the second from September 2011 to October 2012. The climate of the region is temperate. Maximal temperatures, minimal temperatures and cumulative rainfall of both trials are presented in Figure 22. The soil was silty stony with a pH of 6.3 ± 0.1 and 5.2 ± 0.0 in H_2O and KCl, respectively. Soil organic matter and C content were 48 ± 3 g/kg and 28 ± 2 g/kg, respectively.

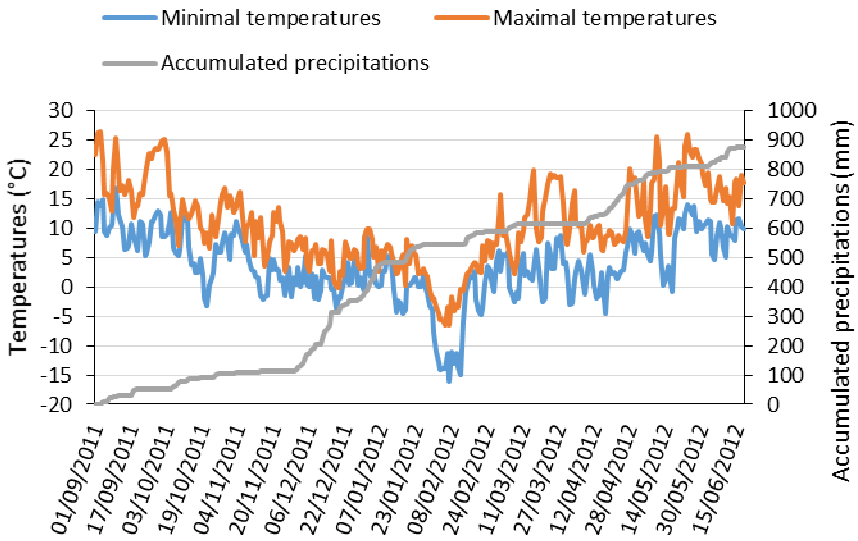
a)



b)


Minimal temperatures
Maximal temperatures
Accumulated precipitations
Temperature (°C)
Accumulated precipitations (mm)
35
30
25
20
15
10
5
0
450
400
350
300
250
200
150
100
50
0
11/05/2010
19/05/2010
27/05/2010
04/06/2010
12/06/2010
20/06/2010
28/06/2010
06/07/2010
14/07/2010
22/07/2010
30/07/2010
07/08/2010
15/08/2010
23/08/2010
31/08/2010
08/09/2010
16/09/2010
24/09/2010

c)


Minimal temperatures
Maximal temperatures
Accumulated precipitations
Temperatures (°C)
Accumulated precipitations (mm)
30
25
20
15
10
5
0
-5
-10
-15
-20
1000
900
800
700
600
500
400
300
200
100
0
01/09/2011
17/09/2011
03/10/2011
19/10/2011
04/11/2011
20/11/2011
06/12/2011
22/12/2011
07/01/2012
23/01/2012
08/02/2012
24/02/2012
11/03/2012
27/03/2012
12/04/2012
28/04/2012
14/05/2012
30/05/2012
15/06/2012

d)

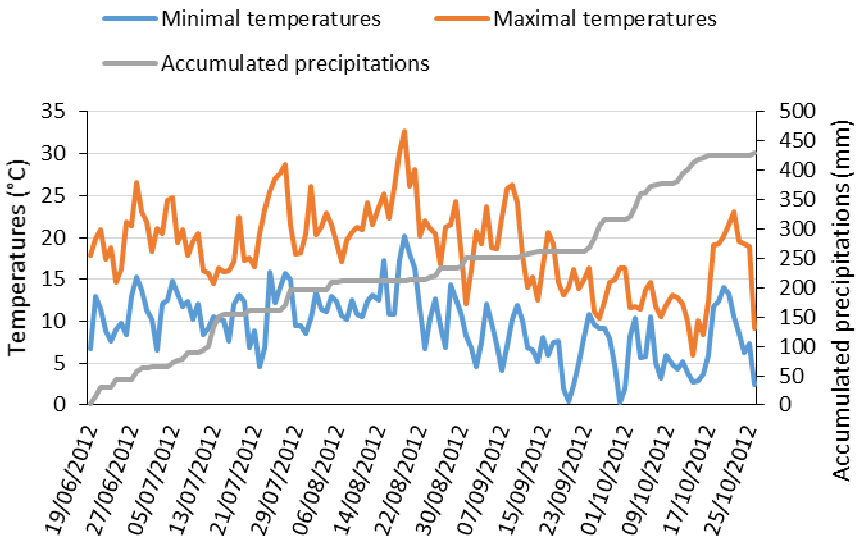


Figure 22. Climatic data (i.e. precipitations, minimal temperatures and maximal temperatures) a) before potato crop in trial 1 (2009/2010), b) during potato crop in trial 1 (2010), c) before potato crop in trial 2 (2011/2012) and d) during potato crop in trial 2 (2012) (source Pameseb asbl).

In the plot of the first trial, the soil was covered with grassland before experiment, while in the second trial, the plot was cultivated with *Triticum spelta* var. Cosmos under conventional farming before the trial.

Biological material

Solanum tuberosum L. var. Sarpo Mira (Danespo, Danemark) and var. Bionta (NÖS, Austria) were used in the 1st and 2nd trial, respectively. Sarpo Mira is highly resistant and Bionta moderately resistant to late blight (Rietman *et al.*, 2012). After storage at low

temperature (3-4 °C), the potato tubers were placed at 10-15 °C for three weeks before planting.

Medicago sativa (Philip-Seeds, Belgium) was used as overwintering cover crop before potato planting.

The AMF *Rhizophagus irregularis* MUCL 41833 was supplied by the Glomeromycota *in vitro* collection (GINCO – http://www.mycorrhiza.be/ginco-bel). The fungus was grown *in vitro* as detailed in Cranenbrouck *et al.* (2005) and subsequently mass-produced during 6 months on maize grown under greenhouse conditions in lava (DCM, Belgium) supplemented with Osmocote®.

Trichoderma harzianum Rifai MUCL 29707 was supplied by the Mycothèque de l'Université catholique de Louvain (MUCL – http://bccm.belspo.be/about/mucl.php) on Malt Agar 2 % (Duchefa Biochemie, Haarlem, The Netherlands). A plug of gel containing several conidia and mycelium was placed into a sterile tube (1.5 ml) filled with 0.4 ml of 1 % sterile distilled water-peptone (SDWP) (Duchefa Biochemie, Haarlem, The Netherlands). The plug was mixed in the SDWP with a vortex mixer (8000 rpm) for 15 s and 50 µl of the suspension was spread on PDA (Oxoid LTD, England). The Petri plates were incubated 4 days in the dark at 25°C and subsequently maintained at 4 °C until needed.

Beneficial microorganisms (BM), *R. irregularis* and *T. harzianum*, were entrapped together in alginate beads following the method described in De Jaeger *et al.* (2011) slightly modified with a filler to maintain the shape of the beads after drying. Each gelled bead had a volume of approx. 34 µl and contained an approximate of 20 AMF propagules (i.e. root fragments and isolated spores/vesicles)

and/or 5 conidia of *T. harzianum*. Beads were then air-dried to 35 % of their initial weight.

Experimental design and field management

In both trials, individual subplots were arranged in a randomized complete block design replicated four times. Time line of both trials is presented in Figure 23. Throughout the trials, no organic or chemical fertilizers were used.

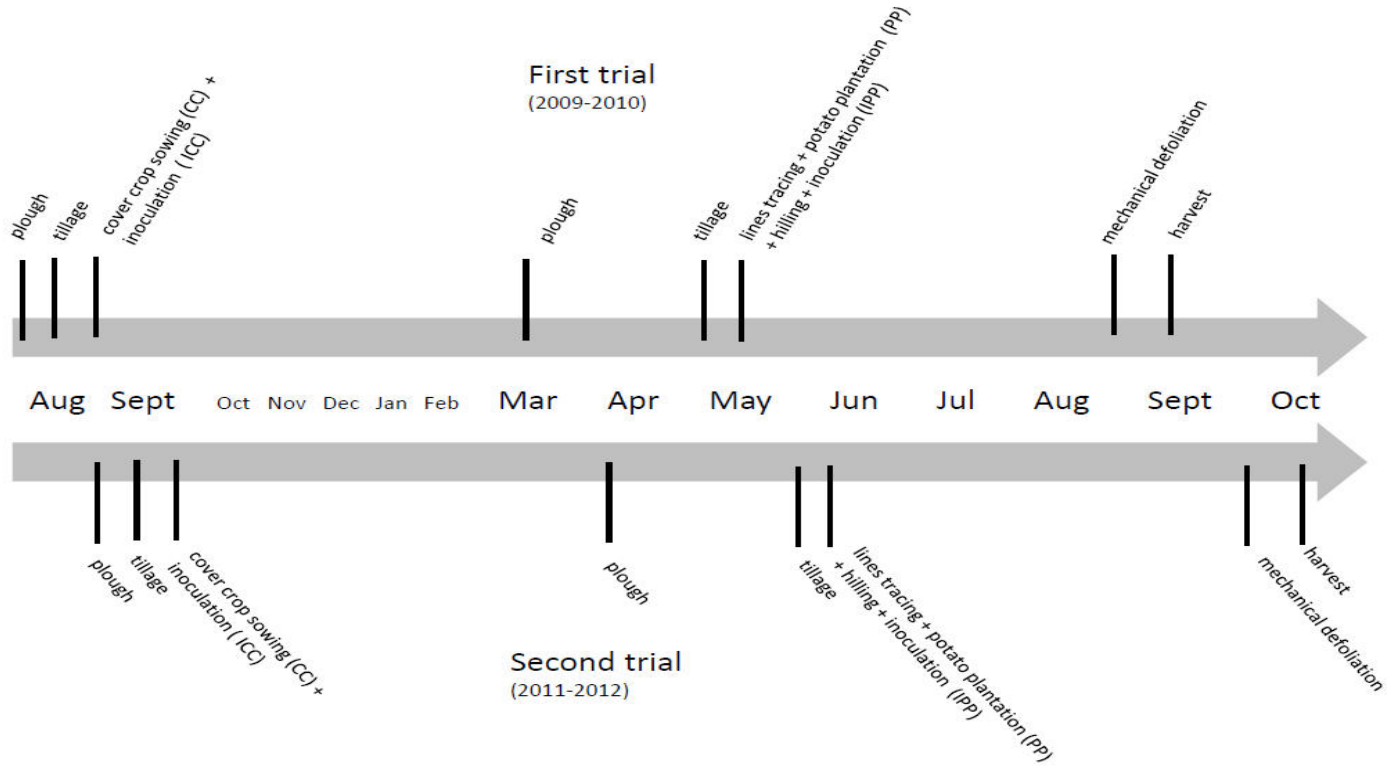


Figure 23. Time line of the first and the second field trial.

First trial

This trial was conducted with Sarpo Mira. Four different cropping itineraries (treatments) were considered: inoculation at cover crop of both BM prior to potato planting (ICC), direct inoculation at potato planting (IPP), cover crop sowing without BM inoculation (CC) or absence of cover crop and BM inoculation (Control).

The trial was conducted without herbicides or fungicides (weeds were eliminated mechanically). Each individual subplot measured ± 21 m^2 (3 m × 7 m) with 60 plants and was separated from a neighboring subplot by a distance of 2 m. *Medicago sativa* was sown on the 20th of August 2009 in the ICC and CC treatments, using a cereal sower at a density of 30 kg/ha. In the ICC treatment, the seeds were sown concomitantly with the BM. Beads containing the BM were mixed with the seeds at an approximate density of 4 million beads/ha. Untreated but certified potato tubers were planted on the 11th of May 2010. In the IPP treatment, beads containing the BM were inoculated in the planting holes close to the tubers (± 30 beads per tuber which represented an approximated density of 1 million beads/ha). Hilling was then performed in all the plots. At the end of the trial, all subplots were mechanically defoliated. Harvest was done on the 22th of September 2010.

Second trial

This trial was conducted with Bionta. Eight different cropping itineraries (i.e. treatments) were considered. Half of them were treated with synthetic fungicides and herbicides (termed “synthetic” management system (Synth)), while the other half were treated with non-synthetic fungicides and without herbicides (termed “non-synthetic” management system (Non-Synth)) (Table 10). Within both

management systems, similar treatments as in trial 1 were considered, i.e. ICC, IPP, CC and Control. The weed and disease management is detailed in Table 10. Each individual subplot had a surface of ± 36 m² (4.5 m × 8 m) with 132 plants and was separated from a neighboring subplot by a distance of 2 m. Seeds of *Medicago sativa* were sown on the 13th of September 2011 and potato tubers planted on the 19th of June 2012 without or concomitantly with the BM as in the first trial and in the same amounts. At the end of the experiment the non-synth subplots were mechanically defoliated, while herbicides were used in the "Synth" subplots. Harvest was done on the 23th of October 2012.

Table 10. Management systems applied in the second trial: "synthetic" weed and disease management system (Synth) and "non-synthetic" weed and disease management system (NonSynth).

	Synth	Non-synth
15th of March	RoundUp® Max (Monsanto Europe N.V.) 5 l/ha	/
18th of June	RoundUp® Max (Monsanto Europe N.V.) 5 l/ha	/
19th of June	Amistar® (Syngenta Crop Protection N.V.) 6 l/ha	/
26th of June	Artist® (Bayer Crop Science SA NV) 2 kg/ha Centium® (FMC Chemicals SPRL) 250 ml/ha	/
July – August	Tattoo® C (Bayer Crop Science SA NV) 4 x 2.7 l/ha Infintito® (Bayer Crop Science SA NV) 2 x 1.6 l/ha Belchim Cymoxanil-M® (Belchim Crop Protection NV/SA) 2 kg/ha Shirlan® (Syngenta Crop Protection N.V.) 7 x 0.4 l/ha	Naturen Bouillie Bordelaise (Scotts Benelux B.V.B.A) 8 x 10 kg/ha
1st of October	Reglone® (Syngenta Crop Protection N.V.) 5 l/ha	/

Estimation of Potato tuber weight, size and number

At harvest, tubers (two central lines in each plot, i.e. 40 plants per subplot in trial 1 and 44 plants per subplot in trial 2) were collected. Tubers were counted, weighted and their size distribution (< 30 mm or > 30 mm) determined.

Estimation of AMF and *T. harzianum* field density

The field abundance of AMF and *T. harzianum* was estimated only in the second trial. Soil samples were collected on the 13th of September 2011, the 19th of June 2012 at a depth of 0-20 cm and the 23th of October 2012 within the potato hills. Dates corresponded to the period just before *M. sativa* sowing, to potato planting and to potato harvest, respectively. The soil samples were homogenized and spread on paper to remove plant material. They were subsequently air-dried, sieved with 5 mm (AMF) or 2 mm (*T. harzianum*) mesh sieves, and stored at 4 °C until processing. For the AMF density determination, soils of the 4 blocks of each treatment were mixed together to perform a single MPN per treatment. *M. sativa* seeds were sown in a series of 13 soil dilutions (dilution factor 2) of each soil sample and replicated three times. Presence or absence of AMF in the roots of the trap plant was determined after 6 weeks of culture. Most Probable Number (MPN) was then calculated as described by Jarvis *et al.* (2010). The abundance of AMF propagules was performed as described by Porter (1979). For *T. harzianum*, the abundance was estimated only at harvest on the same soil samples as above. Number of colony forming units (cfu) per gram of soil was determined on *T. harzianum* selective medium (THSM) as described by Williams *et al.* (2003).

AMF root colonization

Root colonization by AMF was estimated only in the second trial. Eight randomly-selected plants per treatment (i.e. two potato plants per plot over the 4 blocks) were analyzed. Roots were cleared (10 % KOH at 50 °C for 90 min) and stained (5 % blue ink (Parker®) diluted in vinegar (7 ° acidity) at 50 °C for 60 min). Root colonization

was subsequently assessed following the method of McGonigle *et al.* (1990). Two hundred intersections were observed under a compound microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 20-40× magnifications. Total root colonization (% RC), abundance of arbuscules (% A) and intraradical spores/vesicles (% V) were determined.

Statistical analyses

Data analysis was performed with the SAS statistical software version 9.3 (SAS Inc., Cary, NC). Data for tuber weight and number was analyzed using a Linear Mixed Model (PROC MIXED) where "Management" and "BM itinerary" were regarded as fixed factors, and block as random factors. Data on proportion of tubers > 30 mm and AMF root colonization, expressed as percentage (%), were normalised by arcsine transformation and analyzed using a Linear Mixed Model. Dunnett-Hsu significant difference test was used to identify the significant differences ($P \leq 0.05$) with reference to the Control treatment.

Results

Potato tuber weight, size and number

First trial

The average tubers weight per plant, proportion of tubers > 30 mm and number of tubers per treatment are presented in Figure 24 and statistics are described in Table 11. A significant effect of the factor "BM itinerary" was observed on potato tubers weight ($P < 0.001$). Taking the Control treatment as reference, potato tubers weight was significantly ($P < 0.001$) higher (i.e. by 37 %) in the ICC treatment.

Table 11. Statistics of tuber weight and characteristics (percentage tubers > 30 mm, number of tubers/plant – N°/plant) with following "BM itinerary" (beneficial microorganisms inoculated at cover crop sowing (ICC) prior to potato plantation or directly to potato at planting (IPP) or no inoculation of beneficial microorganisms at cover crop (CC) or potato planting (Control).

Cropping scenarios	Weight (g/plant)	Tubers	
		Size > 30 mm (%)	N°/plant
ICC	1420 ± 80	62 ± 1	11 ± 1
IPP	1040 ± 20	62 ± 3	8 ± 0
CC	990 ± 80	67 ± 1	7 ± 1
Control	1040 ± 20	61 ± 2	8 ± 0
Effects (p-values)			
BM itinerary	**0.0005**	0.1080	**0.0006**
Dunnett-Hsu test			
ICC vs Control	**0.0001**	0.9925	**<0.0001**
IPP vs Control	0.9937	0.9867	0.9699
CC vs Control	0.9785	0.1429	0.1069

Data are means ± SE of 4 blocks (40 plants per block). Main effect "BM itinerary" factor is presented. Tests that revealed significant differences at the 0.05 α level are indicated in bold. The Dunnett-Hsu test compares means against the mean of Control.

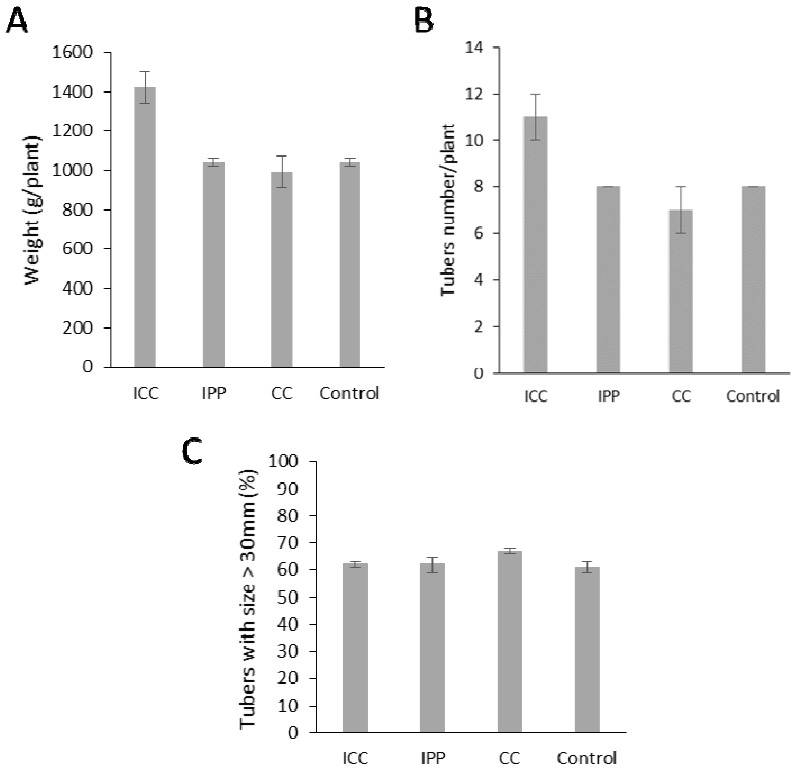


Figure 24. Tuber weight and characteristics (percentage tubers > 30 mm, number of tubers/plant – N°/plant) with following "BM itinerary" (beneficial microorganisms inoculated at cover crop sowing (ICC) prior to potato plantation or directly to potato at planting (IPP) or no inoculation of beneficial microorganisms at cover crop (CC) or potato planting (Control).

The % tubers > 30 mm ranged from 61 ± 2 % to 67 ± 1 % among the different treatments (Table 11). However, no significant effect on % tubers > 30 mm was noticed for the factors "BM itinerary".

The number of tubers per plant ranged from 7 ± 1 to 11 ± 1 among the different treatments (Table 11). A significant effect ($P < 0.001$) of the factor "BM itinerary" was observed. The number of tubers was significantly ($P < 0.0001$) higher in the treatment with

plants inoculated with BM at cover crop (ICC) as compared to the Control treatment (Table 11).

Second trial

The average tubers weight per plant, proportion of tubers > 30 mm and number of tubers per treatment are presented in Figure 25 and statistics are described in Table 12. A significant effect of the factor “BM itinerary” was noticed on potato tubers weight ($P < 0.05$). To the contrary, no significant effect on tubers weight was observed for the factor “Management” and there was no interaction between both factors. As the factor “Management” was not significant, average tubers weight per plant was calculated for the “Non-Synth” and “Synth” management system combined. Taking the Control treatment as reference, potato tubers weight was significantly ($P < 0.01$) higher (i.e. by 13 %) in the ICC treatment with BM.

Table 12. Tuber weight and characteristics (percentage tubers > 30mm, number of tubers/plant – N°/plant) in the different cropping scenarios that are a combination of a "Management" system ("synthetic" management system (Synth) or "non-synthetic" management system (NonSynth)) and a "BM itinerary" (beneficial microorganisms inoculated at cover crop sowing (ICC - prior to potato plantation) or directly to potato at planting (IPP) or no beneficial microorganisms inoculation at cover crop (CC) or potato planting (Control).

Cropping scenarios	**Weight (g/plant)**	**Tubers**	
		Size > 30 mm (%)	**N°/plant**
Synth/ICC	1420 ± 50	76 ± 1	15 ± 1
NonSynth/ICC	1370 ± 50	73 ± 2	17 ± 1
Synth/IPP	1380 ± 60	76 ± 1	15 ± 0
NonSynth/IPP	1260 ± 50	74 ± 1	16 ± 1
Synth/CC	1330 ± 40	74 ± 1	15 ± 1
NonSynth/CC	1270 ± 70	72 ± 2	15 ± 1
Synth/Control	1240 ± 80	69 ± 1	15 ± 1
NonSynth/Control	1240 ± 40	72 ± 2	15 ± 1
Effects (p-values)			
Management	0.0934	0.2891	0.5039
BM itinerary	**0.0200**	**0.0157**	0.2279
Management × BM itineray	0.6178	0.1800	0.6838
Dunnett-Hsu test			
ICC	1400 ± 40	74 ± 1	
IPP	1320 ± 40	75 ± 1	
CC	1300 ± 40	73 ± 1	
Control	1240 ± 40	70 ± 1	
ICC vs Control	**0.0063**	**0.0356**	
IPP vs Control	0.2316	**0.0069**	
CC vs Control	0.3857	0.1276	

Data are means ± SE of 4 blocks (44 plants per block). Main effects and interaction between the factors: "Management" and "BM itinerary" are presented. Tests that revealed significant differences at the 0.05 α level are indicated in bold. The Dunnett-Hsu test compares means against the mean of Control.

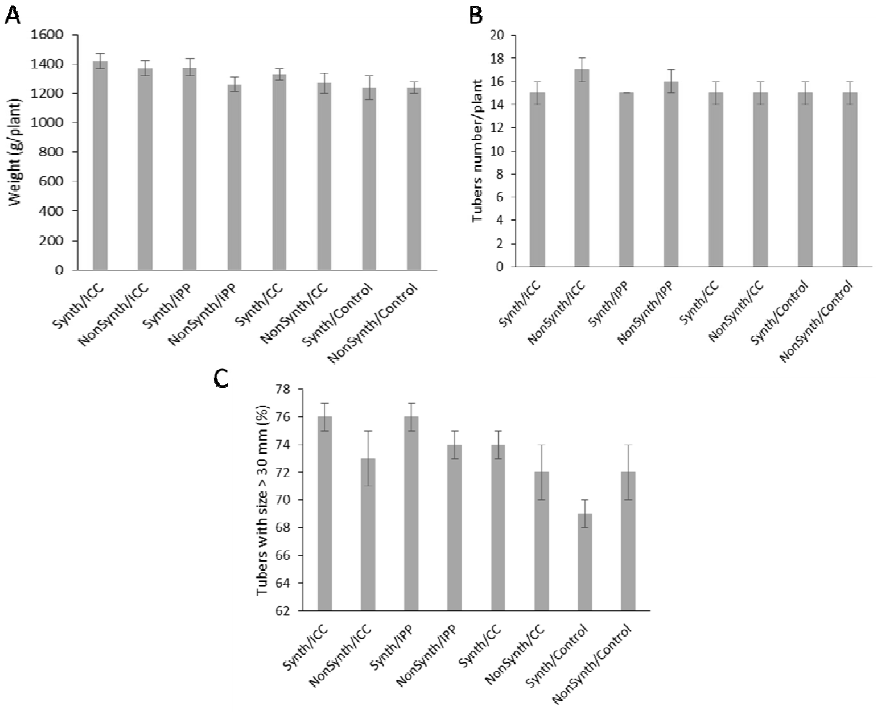


Figure 25. Tuber weight and characteristics (percentage tubers > 30mm, number of tubers/plant – N°/plant) in the different cropping scenarios that are a combination of a "Management" system ("synthetic" management system (Synth) or "non-synthetic" management system (NonSynth)) and a "BM itinerary" (beneficial microorganisms inoculated at cover crop sowing (ICC - prior to potato plantation) or directly to potato at planting (IPP) or no beneficial microorganisms inoculation at cover crop (CC) or potato planting (Control).

The % tubers > 30 mm ranged from 69 ± 1 % to 76 ± 1 % (Table 12) among the different treatments. A significant effect ($P < 0.05$) of the factor "BM itinerary" was noticed on this parameter. To the contrary, no significant effect on % tubers > 30 mm was observed for the factor "Management" and there was no interaction between both factors. As previously, average % tubers > 30 mm was

calculated for the “Non-Synth” and “Synth” management systems combined (Table 12). The % tubers > 30 mm were significantly higher when plants were inoculated with BM (ICC ($P < 0.05$) and IPP ($P < 0.01$)) than when neither inoculation of BM nor cover crop was used (Control) (Table 12).

The number of tubers per plant among the different treatments ranged from 15 ± 1 to 17 ± 1 (Table 12). No significant effect of the factors “Management” or “BM itinerary” and no interaction between both factors was observed (Table 12).

Estimation of AMF and *T. harzianum* field density

Second trial

In September 2011 (i.e. before the setup of the second trial), the experimental field contained on average 15 [6.3 - 34] propagules of AMF per gram of soil. In June 2012 (i.e. at potato planting) it ranged from 17 [7.5 - 38] to 99 [34 - 290] propagules of AMF per gram of soil and in October 2012 (i.e. at harvest) it ranged from 18 [7.9 - 42] to ∞ [42 - ∞] propagules of AMF per gram of soil (Table 13). Infinity (∞) means that for all tested dilutions the results were positive. No statistical analysis could be conducted with the ∞ values. Moreover, at each time of observation, 95 % confidence limits of each treatment were bridging 95 % confidence limits of the other treatment which means that statistically we cannot conclude anything.

Table 13. Density of AMF and *Trichoderma harzianum* in soil at potato planting (only AMF) and at harvesting for the different cropping scenarios that are a combination of a "Management" system ("synthetic" management system (Synth) or "non-synthetic" management system (NonSynth)) and a "BM itinerary" (beneficial microorganisms inoculated at cover crop sowing (ICC - prior to potato plantation) or directly to potato at planting (IPP) or no beneficial microorganisms inoculation at cover crop (CC) or potato planting (Control)).

	Potato Planting	Harvest	
Cropping scenarios	**AMF (propagules/ g dry soil)**	**AMF (propagules/ g dry soil)**	***Trichoderma harzianum* (10^3 cfu/ g dry soil)**
Synth/ICC	34 [15 – 77]	18 [7.9 – 42]	4 ± 1
NonSynth/ICC	99 [34 – 290]	∞ [42 - ∞]	5 ± 2
Synth/IPP	17 [7.5 – 38]	∞ [42 - ∞]	5 ± 3
NonSynth/IPP	99 [34 – 290]	99 [34 – 290]	3 ± 1
Synth/CC	64 [25 – 170]	22 [9.7 – 50]	2 ± 1
NonSynth/CC	21 [9.3 – 46]	∞ [42 - ∞]	2 ± 0
Synth/Control	47 [18 – 120]	28 [12 – 64]	4 ± 1
NonSynth/Control	99 [34 – 290]	∞ [42 - ∞]	3 ± 1
Effects (p-values)			
Management			0.6176
BM itinerary			0.3336
Management × BM itinerary			0.6916

Data of AMF density are means with 95 % confidence limits (soils of 4 blocks were mixed together to perform a single MPN per treatment) and data of *T. harzianum* are means ± SE of 4 blocks (soil of 2 plant hills were sampled per block and pooled together). Main effects and interactions between the factors "Management" and "BM itinerary" are presented for *T. harzianum*. Tests that revealed significant differences at the 0.05 α level are indicated in bold.

Density of *T. harzianum* estimated in soil of each treatment at the end of the trial (i.e. at harvest) is presented in Table 13. *T. harzianum* abundance ranged from 2 ± 0 to 5 ± 2 × 10^3 cfu/g of dry soil. Neither "BM itinerary" nor "Management" factor had an effect on *T. harzianum* abundance. Similarly, no interaction was noticed between the two factors.

AMF root colonization

Second trial

Average potato AMF root colonization for each treatment is presented in Table 14 The % of total root colonization (% RC) ranged from 21 ± 2 % to 65 ± 6 % and the % of arbuscules (% A) from 7 ± 1 % to 35 ± 7 %. The % of spores/vesicles (% V) remained low (from absence of spores/vesicles to 1.5 ± 1.5 %). Neither “BM itinerary” nor “Management” factor had an effect on % V. To the contrary, a significant effect of the factor “BM itinerary” was noticed on the % RC ($P < 0.0001$) and % A ($P < 0.0001$). The factor “Management” had no significant effect on % RC or % A. No interaction was noticed between the two factors for % RC, % A or % V. As above, average % RC, % A and % V were calculated for the “Non-Synth” and “Synth” management systems combined (Table 14). Significant difference for % RC and % A were noticed between the Control and the ICC and IPP treatments, while none was observed with the CC treatment (Table 14). The % RC of the plants in the ICC and IPP treatments was on average more than twice that of the Control treatment (respectively 62 ± 5 % and 55 ± 4 % compared to 26 ± 3 %). The % A was more than three times higher for the plants in the ICC treatment and nearly three times higher for the plants in the IPP treatment as compared to the Control treatment (respectively 31 ± 5 % and 25 ± 3 % compared to 9 ± 1 %).

Table 14. Total (% RC), arbuscular (% A) and spore/vesicular (% V) colonization of the roots of potato at harvesting for the different cropping scenarios that are a combination of a "Management" system ("synthetic" management system (Synth) or "non-synthetic" management system (NonSynth)) and a "BM itinerary" (beneficial microorganisms inoculated at cover crop sowing (ICC - prior to potato plantation) or directly to potato at planting (IPP) or no beneficial microorganisms inoculation at cover crop (CC) or potato planting (Control)).

Cropping scenarios	**% RC**	**% A**	**% V**
Synth/ICC	65 ± 6	35 ± 7	0.4 ± 0.2
NonSynth/ICC	58 ± 9	27 ± 7	0.5 ± 0.2
Synth/IPP	52 ± 4	27 ± 3	1.5 ± 1.5
NonSynth/IPP	58 ± 6	24 ± 6	0.2 ± 0.2
Synth/CC	24 ± 5	12 ± 3	0.0 ± 0.0
NonSynth/CC	30 ± 5	11 ± 3	0.2 ± 0.1
Synth/Control	31 ± 4	12 ± 2	0.3 ± 0.1
NonSynth/Control	21 ± 2	7 ± 1	0.2 ± 0.1
Effects (p-values)			
Management	0.7900	0.1241	0.7136
BM itinerary	**< 0.0001**	**< 0.0001**	0.6330
Management × BM itinerary	0.3083	0.7539	0.6524
Dunnett-Hsu test			
ICC	62 ± 5	31 ± 5	
IPP	55 ± 4	25 ± 3	
CC	27 ± 4	11 ± 2	
Control	26 ± 3	9 ± 1	
ICC vs Control	**< 0.0001**	**< 0.0001**	
IPP vs Control	**< 0.0001**	**0.0010**	
CC vs Control	0.9978	0.8402	

Data are means ± SE of 4 blocks (2 plants per block). Main effects and interactions between the factors "Management" and "BM itinerary" are presented. Tests that revealed significant differences at the 0.05 α level are indicated in bold. The Dunnett-Hsu test compares means against the mean of Control.

Discussion

Arbuscular mycorrhizal fungi and *Trichoderma* spp. are key soil microorganisms repeatedly reported to increase plant growth and health. These soil inhabitants, in particular AMF, are impacted by

agricultural factors such as ploughing, over-fertilization, pesticides application and monoculture, resulting in the erosion of diversity, modification of community structure and decrease of population densities (Gosling *et al.*, 2006). Their application or management in cropping systems thus necessitates the development of agricultural itineraries that stimulate their functions as plant growth promotors and plant resistance enhancers. Basically, two strategies can be followed to increase the density/efficacy of these beneficial microorganisms: (1) the application of adequate agricultural itineraries (e.g. use of mycotrophic cover crops) favoring/stimulating the indigenous populations or (2) the inoculation of selected beneficial microorganisms with known impact on the target crop. A third option, tested in our study, is the combination of the two above-mentioned strategies. We evaluated the impact of inoculated beneficial microorganisms combined in alginate beads on potato tuber production when inoculum is applied at cover crop sowing (i.e. before potato plantation) or at tuber plantation.

Among the strategies to increase the natural or inoculated populations of beneficial microorganisms, is the use of cover crops. This management itinerary is basically used to control weeds (Moonen and Bàrberi, 2004, Samarajeewa *et al.*, 2006) or pathogens (Larkin, 2008), mobilize nitrogen and reduce N leaching or produce organic matter (Andraski and Bundy, 2005, Hansen *et al.*, 2000, Kuo and Sainju, 1998). It also impacts the soil microbial populations (Reddy *et al.*, 2003) and community structure (Elfstrand *et al.*, 2007). For instance, Boswell *et al.* (1998), Kabir and Koide (2000), Ramos-Zapata *et al.* (2012) and Lehman *et al.* (2012) demonstrated that the use of mycotrophic cover crops (e.g. *Mucuna deeringiana* (Bort.) Merr. – tropical legume) before cash crop or as weed control strategy

during cash crop, increased the natural population of AMF in soil and subsequent root colonization of cash crop. In our experiment (second trial), we observed densities of *T. harzianum* of 10^3 cfu/ g dry soil, what is mostly found in agricultural soils (from 10^2 - 10^4 cfu/ g dry soil) (Leandro *et al.*, 2007). This density did not increase significantly in presence of cover crop (CC) as compared to the control (i.e. absence of cover crop). Similarly, the population density of AMF in soil at potato planting or harvest seemed not to increase due to the prior growing of the cover crop, even though statistical analysis could not be conducted. Potato root colonization by AMF did not increase significantly in presence of cover crop as compared to the control (i.e. absence of cover crop). This may suggest that the low density of indigenous BM populations might not have increased significantly in presence of the cover crop. Even if some cover crops are known to increase beneficial microorganism, no studies demonstrated to our knowledge that *M. sativa*, the cover crop used in our experiments, increased the indigenous population of *T. harzianum* or AMF. Furthermore, we did not observe a higher biomass of potato in the treatment under cover crop as compared to the treatment without cover crop (first and second trial). This therefore indicates that potential advantages of the cover crop used (e.g. on N availability and beneficial microorganism populations) were not significant to increase potato production in our trials.

Under low population densities as estimated in the second trial, the inoculation of beneficial microorganisms is another option to increase rhizosphere/plant colonization and subsequent benefits to plant growth and health (Douds *et al.*, 2007, Pellegrino *et al.*, 2011). Here two inoculation strategies were used: (1) a broad application at cover crop sowing to increase the number of propagules in soil before

potato plantation or (2) a targeted application on the seed tuber at potato planting.

Root colonization by AMF, estimated at harvest (second trial), was significantly higher in treatments with cover crop or direct tuber inoculation as compared to the non-inoculated treatments. This may suggest that the inoculated AMF was efficient in colonizing the potato plants as compared to the indigenous AMF. Curiously, the population density of AMF in soil seemed not to increase with inoculation but this could not be confirmed statistically. Differences have been reported among AMF communities from potato rhizosphere soil and roots (Cesaro *et al.*, 2008). For instance, *R. irregularis* is known to be more dominant in potato roots as compared to soil (Cesaro *et al.*, 2008, Senés-Guerrero *et al.*, 2013) and could thus explain the increased root colonization without increased soil population in our experiment. In contrast, the population density of *T. harzianum* did not increase with inoculation. Inoculation densities were probably too low to increase *T. harzianum* population. Although we cannot conclude that inoculation did not affect the overall population of *T. harzianum*, its density may not have been sufficient to induce beneficial effects on potato. Indeed, Leandro *et al.* (2007) estimated that Trichoderma population must reach 10^5-10^7 cfu/g dry soil before significant effect on plant growth can be detected. But, the presence of *T. harzanium* in close contact with AMF (i.e. in the beads) may have stimulated the latter (De Jaeger *et al.*, 2011).

In both trials we observed that, compared to the control treatment (i.e. no BM inoculation and absence of cover crop), the most advantageous agricultural itinerary to increase potato yield was the inoculation of cover crop with both microorganisms. Interestingly, for cultivar Bionta inoculating the cover crop increased potato yield

and the size of the tubers. For cultivar Sarpo Mira inoculating the cover crop increased potato yield and the number of tubers per plant. Inoculation at potato planting also increased potato tuber production for cultivar Bionta, but only significantly on the % of potatoes > 30 mm.

Between organic and conventional agriculture management systems (tested in the second trial), no difference was observed in root colonization by AMF (difference in abundance of AMF in soil could statistically not be determined) or in population density of *T. harzianum* in soil at harvest. This suggested that the fungicides/herbicides applied in both management systems had similar effects (or absence of effects) on the inoculated microorganisms. It is well-known from the literature that the impact of pesticides on AMF or *T. harzianum* largely varies with the type of compound, dosage (Buysens *et al.*, 2014, Sarkar *et al.*, 2010, Wan *et al.*, 1998), mode of application (Diedhiou *et al.*, 2004) and fungal species (Fontanet *et al.*, 1998, Jin *et al.*, 2013, Kjøller and Rosendahl, 2000). The apparent lack of effect of the pesticides used in our study could also be linked to the experimental and environmental conditions such as the presence of microorganisms having the capacity to degrade pesticides, adsorption to soil particles, dilution and availability of pesticides. The effects of pesticides on AMF and *Trichoderma* spp. should be considered over the long term. Indeed, repeated and chronic exposure (due to soil build-up) could affect these microorganisms (Mäder *et al.*, 2000). It has also been observed that application of pesticides could favor beneficial microorganisms by reducing antagonistic and competitive pressures (Schweiger *et al.*, 2001).

Soil inoculation should be considered, preferably but not exclusively, under conditions where indigenous populations are low and inefficient to induce benefits to plants (e.g. yield increase). Our study demonstrated that management of indigenous BM population by the use of a mycotrophic cover crop did not result in higher potato production and that it was ineffective to promote potato production compared to the inoculation with *R. irregularis* MUCL 41833 and *T. harzanium* MUCL 29707. Indigenous population densities probably did not increase sufficiently, due to the low initial level of population, or were not efficient to stimulate potato growth and production. Inoculation of BM in both trials, increased significantly potato tuber weight as compared to the non-inoculated treatments without cover crop. This was mainly attributed to improved AMF colonization of the potato plants. The inoculation of BM via cover crop seems a more efficient strategy as compared to the direct inoculation at potato plantation. However, difference between these strategies on potato production may not be solely attributed to AMF colonization rates. Our field trial also suggested that BM application is not hindered by the fungicides and/or herbicides used in organic or conventional agriculture (e.g. synthetic vs. non-synthetic) used, but long-term experiments should be conducted. It further highlights the necessity to trace the inoculated microorganisms within the potato roots and rhizosphere throughout the cultivation season and in comparison to the local microbial population to comprehend the impact of agricultural factors and/or local microbes on the population dynamics of the inoculants introduced.

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CHAPTER 4

Tracing native and inoculated *Rhizophagus irregularis* in three potato cultivars (Charlotte, Nicola and Bintje) grown under field conditions

Adapted from the research article in preparation for

Mycorrhiza

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Preface

The study conducted in **chapter III** showed that inoculating a cover crop with *R. irregularis* MUCL 41833 and *T. harzianum* MUCL 29707 increased potato yield. However, in this study it was not possible to distinguish the inoculated AMF strain from native ones. Therefore, the effects on potato growth could not be indiscutably linked to the inoculant.

In **chapter IV**, a molecular technique was proposed to distinguish inoculated strains from native strains. The evaluation was made on three different potato cultivars (Bintje, Nicola, Charlotte) grown under field conditions.

Abstract

The use of arbuscular mycorrhizal fungi (AMF) to improve plant growth and resistance/tolerance to abiotic and biotic stresses is receiving an increased interest in research programs as well as by farmers. Evaluating the efficiency of AMF inoculants to increase crop performance under field conditions is essential if the objective is to convince farmers to apply inoculum and concomitantly to decrease the application of fertilizers and pesticides. In this study *Rhizophagus irregularis* MUCL 41833 was inoculated on three potato cultivars under field conditions. Mitochondrial Large SubUnit (mtLSU) of inoculated *R. irregularis* MUCL 41833 was characterized in order to design haplotype- (inoculated *R. irregularis* haplotype) and species-specific (native or inoculated *R. irregularis*) markers. The markers were validated on inoculated potato cv. Bintje. roots grown under controlled greenhouse conditions. The magnitude of detection was determined by Real-Time quantitative PCR and linked to the stage of root colonization. The lowest concentrations (i.e. $3.9\ 10^{-6} \pm 2.1\ 10^{-6}$ ng/ ng total DNA) were found in the early stage of root colonization (first hyphae colonizing roots), while the highest concentrations ($7.4\ 10^{-3} \pm 5.1\ 10^{-3}$ ng/ ng total DNA) were found in the late stage (heavy root colonization with arbuscules and spores/vesicles formed) of root colonization. These markers allowed the quantification and tracability of native and inoculated *R. irregularis* in the field. Inoculated *R. irregularis* MUCL 41833 was detected at a very low level (between 10^{-5} and 10^{-7} ng/ ng total DNA) in a minority of plants, in contrast to native *R. irregularis* that was detected at higher levels (between 10^{-4} and 10^{-6} ng/ ng total DNA) in all plants. In conclusion, Real-Time quantitative PCR of mtLSU allows the traceability of inoculated and

native *R. irregularis* strains in the field and the determination of their colonization importance that vary from one potato cultivar to another.

Keywords

Arbuscular mycorrhizal fungi; *Rhizophagus irregularis*; Real-Time quantitative PCR; mitochondrial LSU; traceability

Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil microorganisms that form symbiotic associations with the majority of land plants (Smith and Read, 2008), including most agricultural crops (e.g. Douds *et al.* (2007)). The benefit of AMF to plants is mainly attributed to increased uptake of nutrients (e.g. P, N) (Smith and Read, 2008). They are also of recognized importance in nutrient cycling, soil aggregation and water retention, and improve the tolerance/resistance of plants to biotic and abiotic stresses (Gianinazzi *et al.*, 2010).

Despite their clear economic and ecological significance, the number of studies that indubitably report their potential benefits in the field remains scarce (e.g. Douds *et al.* (2007)) as compared to the numerous results obtained in greenhouse or growth chamber conditions (e.g. Duffy and Cassells (2000), Yao *et al.* (2002), Vosátka and Gryndler (2000). It is obvious that under field conditions inoculated AMF have to compete with native AMF communities (Verbruggen *et al.*, 2013) and numerous agricultural as well as environmental factors that influence the AMF association (see Gosling et al., 2006).

Rhizophagus irregularis is a worldwide distributed AMF, widely used for commercial applications owing to its easy mass-production

(IJdo *et al.*, 2010) and life history strategy adapted to agricultural soils (Chagnon *et al.*, 2013). However, field experiments that undoubtedly demonstrate the effect of this AMF on crops are rare. This is because *R. irregularis* is a ubiquitous AMF species and crops naturally become colonized by native AMF species making it difficult to distinguish the effects of applied inoculum versus indigenous AMF species, in particular if the local communities harbor similar species to the inoculated ones.

Evaluating the efficiency of inoculated AMF to increase crop performance under field conditions is necessary if the objective is to convince farmers to apply inoculum and concomitantly to decrease the application of fertilizers and pesticides. Microscopically, it is not possible to distinguish different AMF species nor different AMF isolates of the same species based on structures formed in the roots. Molecular tools have been developed to differentiate AMF at family and species level (e.g. Gollotte *et al.* (2004); Helgason *et al.* (1999); Krüger *et al.* (2009); Redecker (2000)). Intraspecific markers were also developed to detect different isolates of *R. irregularis* (Croll *et al.*, 2008, Koch *et al.*, 2004, Raab *et al.*, 2005) by amplifying the mitochondrial large subunit (mtLSU). This allowed the discrimination of different haplotypes of this specific fungus in the field (Börstler *et al.*, 2008, Börstler *et al.*, 2010). The mtLSU was shown to be homogeneous within the same isolate (Raab *et al.*, 2005) in contrast to the nuclear Large SubUnit (nrLSU). Börstler *et al.* (2008) revealed with a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach and sequencing that the diversity of mtLSU haplotypes of *R. irregularis* was very high in field populations as well as in isolates collected worldwide. Real-time PCR in the large subunit of mitochondrial DNA (mtDNA) was recently used

by Krak *et al.* (2012) to study the dynamics of two coexisting isolates of *R. irregularis*. To our knowledge this technique was not yet applied on field samples in order to distinguish inoculated from native *R. irregularis* isolates and for their quantification.

Potato (*Solanum tuberosum*) is the most important tuber crop worldwide (FAO, 2013). This crop is characterized by heavy mechanization and applications of fertilizers and pesticides. The effect of a direct inoculation of AMF on potato was investigated by Douds *et al.* (2007) and Bayrami *et al.* (2012) under different fertilization treatments. These authors noticed that inoculation with *R. intraradices* or *Funneliformis mosseae* significantly increased potato yield and root colonization under low fertilization treatments. However, none of these studies could firmly demonstrate that the increased yield was strictly related to the inoculated isolate. Indeed, the authors could not distinguish the contribution of native and inoculated AMF to root colonization.

The aims of the present study were (1) to characterize the mtLSU of the AMF isolate *R. irregularis* MUCL 41833, as inoculant used in the field; (2) to validate the markers on inoculated potato plants under greenhouse conditions (3) to evaluate field inoculation success at potato planting by quantifying native versus the introduced *R. irregularis* MUCL 41833 isolate; and (4) to compare AMF root colonization between different potato cultivars and in particular by *R. irregularis*.

Materials and methods

Biological material

Solanum tuberosum L. cv. Bintje, cv. Nicola and cv. Charlotte (Euroseeds, Belgium) were used in this study. These cultivars are amongst the most widely cultivated in Belgium and Europe (www.fiwap.be). After storage at low temperature (3-4 °C), the potato tubers were placed three weeks at 10-15 °C before planting.

Medicago truncatula Gaertn. cv. Jemalong A 17 (Sardi, Australia) was used as mycorrhizal donor plant and in the inoculum infectivity test described below.

The AMF *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler comb. nov. MUCL 41833 was supplied by the Glomeromycota *in vitro* collection (BCCM/MUCL/GINCO – http://www.mycorrhiza.be/ginco-bel). The fungus was cultured *in vitro* as detailed in Cranenbrouck *et al.* (2005) and subsequently mass-produced during 6 months on *Zea mays* L.cv. ES. Ballade (Euralis, France) grown under temperate greenhouse conditions in pots containing lava (DCM, Belgium) supplemented with Osmocote®.

Entrapment of AMF and inoculum infectivity

R. irregularis was entrapped in alginate beads following the method described in De Jaeger *et al.* (2011) slightly modified with a filler to maintain the shape of the beads after drying. Each gelled bead had a volume of 34 µl and contained an approximate of 20 AMF propagules (i.e. isolated spores or root fragments containing spores/vesicles). Beads were air-dried to 35 % of their initial weight.

The mycorrhizal infective potential (MIP) of the beads was tested using the method described in Declerck *et al.* (1996a) slightly modified. The method was based on a dose-response relationship and involved the cultivation of a population of mycorrhizal plantlets on a range of inoculum density under controlled conditions. Briefly, a logarithmic scale of beads numbers (1, 3, 10, 30 and 100), were thoroughly mixed in 200 cm^3 pots containing autoclaved Terragreen® (a calcinated attapulgite clay soil conditioner; Oil-Dri, Cambridgeshire, UK; see Hodge (2001)). Seeds of *M. truncatula* (10 per pot) were sown into the pots and grown for 20 days in a growth chamber set at 22/18 °C (day/night) with 70% relative humidity, a 16 h photoperiod and a photosynthetic photon flux (PPF) of 300 µmol m^{-2} s^{-1}. The plantlets were then harvested and roots cleared (10 % KOH at 50 °C for 90 min) and stained (5 % blue ink (Parker®, USA) diluted in vinegar (7 ° acidity) at 50 °C for 60 min). The presence/absence of AMF was evaluated in each plantlet under stereo-microscope (Olympus SZ40, Olympus Optical GmbH, Germany) at × 1-40 magnification. Three replicates were set up. Data were expressed as the percentage of mycorrhizal plants (% MP) per pot. The % MP was plotted against the logarithm of bead number. Regression curves were used to determine the inoculum density, i.e. the number of beads needed to infect 90% of the plants. ID_{90} corresponded to 13 beads.

Molecular characterization of the *R. irregularis* MUCL 41833

Fungal DNA was extracted from spores and mycelium of *R. irregularis* MUCL 41833 produced *in vitro* using innuPREP Plant DNA kit (Analytikjena, Germany) and following manufacturer recommendations with slight modifications (the homogenization step was adapted to fungal material and explained further).

Spores/mycelium of 2-month-old cultures were extracted from MSR medium by citrate-buffer solubilization (Doner and Bécard, 1991), isolated with a micropipette under a dissecting microscope, placed in a 1.5 ml plastic tube (Eppendorf, Germany), repeatedly centrifuged to eliminate water and air-dried. The tip of the tube was put in liquid nitrogen and spores/mycelium were crushed with a plastic pestle (Eppendorf, Germany) before lysis step with the buffer. Extracted DNA was then subjected to PCR amplification of mitochondrial large ribosomal subunit (mtLSU) RNA gene. The region was amplified by nested PCR using the primers RNL28a (Börstler *et al.*, 2008) and RNL5 (Raab *et al.*, 2005) in the first step and then RNL29 (Börstler *et al.*, 2008) and GImt4510R (Krak *et al.*, 2012) in the second step (see Table 15). The PCRs were conducted using a total volume of 20 µl and contained 0.2 mM of each dNTP (Applied Biosystems, USA), 0.5 U of AmpliTaq® DNA polymerase (Applied Biosystems, USA), 1 × Taq buffer with KCL (Applied Biosystems, USA), 1 ng of genomic DNA and reaction-specific concentrations of primers and $MgCl_2$ (see Table 15). An initial denaturation step at 94°C (4 min) was followed by 35 cycles of denaturation (94 °C, 30 s), annealing (59 °C in the first step and 53 °C in the second step, 30 s), and extension (72°C, 1.5 min), with a final extension step at 72 °C (10 min). The resulting PCR products were sequenced using the original PCR and additional internal primers (Table 15). One sequence type was obtained. To design specific primers, sequence obtained was aligned together with mtLSU sequences of *R. irregularis* sensu lato as well as other species that are representative of all published haplotypes (www.ncbi.nlm.nih.gov/) using Mega6 software (http://www.megasoftware.net/).

Table 15. PCR primers and conditions used for the characterization of the haplotype of *R. irregularis* MUCL 41833 in the large subunit mitochondrial DNA

Primer name(s)	Primer sequences (5' -> 3')	Application	Primer conc. (µM)	Annealing temp. (°C)	MgCl2 conc. (mM)
RNL28a/ RNL5	CCATGGCCAAGTGCTATTTA (Börstler et al. 2008) / GAGCTTCCTTTGCCATCCTA (Raab et al. 2005)	1st step of nested PCR	0.2 / 0.2	59	1.5
RNL29/ GImt4510R	TAATAAGACTGAACGGGTGT (Börstler et al. 2008) / CATCCACGCTAGTGTTAGC (Krak et al. 2012)	2nd step of nested PCR	0.1 / 0.1	53	2
RNL27	CCAACTATGCAACCGTAGG	Sequencing			
RNL24	GAGCATACTAAGGCGTAGAG	Sequencing			

To prepare standards for the qPCR experiments, DNA extract from *R. irregularis* MUCL 41833 spores and mycelium grown *in vitro* were used. The concentration of DNA was measured with Quant-iT™ PicoGreen® dsDNA Assay kit (Life technologies, USA) using Fluoroscan Ascent FL instrument (Labsystems, USA) and diluted to 100 pg genomic DNA.

Primer design and optimization of qPCR assay

Two primer pairs targeted to the mtLSU region (Table 16) were designed. One primer pair was designed to amplify species-specific isolates and another to amplify haplotype-specific isolates. The first primer pair was able to amplify only *R. irregularis* strains and the second primer was able to amplify the specific strains MUCL 41833, MUCL 43204 and MUCL 43196 but no other known *R. irregularis* strains such as MUCL 46241, MUCL 46239 or MUCL 43194. Isolate specific primers of MUCL 41833 could not be found because mtLSU region sequenced from MUCL 41833 was identical to that of MUCL 43204 and MUCL 43196. All primers were tested for dimer formation using AmplifX 1.7.0 (by Nicolas Jullien; CNRS, Aix-Marseille Université -http://crn2m.univ-mrs.fr/pub/amplifx-dist).

Table 16. Parameters of the Real-Time PCR used for the quantification of mtLSU of *R. irregularis* species-specific or MUCL 41833 haplotype-specific isolates

Target region, isolate	Primer names (forward/reverse)	Primer sequences (5' -> 3')	Primer conc. (µM)	Annealing temp. (°C)	Amplicon size (bp)	Amplification efficiency[a] (SD)
mtLSU, *R. irregularis*	BF7 / BR5	TGGTCTAAACATGGTTGAAAAAT / ACGCTATCGCTAAAAGGTGG	0.2 / 0.2	62	67	96.2 (5.3)
mtLSU, *R. irregularis* MUCL 41833 haplotype	BF8 / BR6	AAGTCCTCTAGGTCGTAGCA / ACAGGTATTTATCAAATCCTTCCC	0.2 / 0.2	62	121	93.2 (4.5)

[a] Amplification efficiency was calculated from ten independent 5-fold dilutions of DNA template

The qPCR using LightCycler® Fast Start DNA Master SYBR Green I kit (Roche, Switzerland) was performed in 10 µl reaction mixtures on the LightCycler® 96 Real Time PCR instrument (Roche, Switzerland). The following cycling conditions were used: 10 min at 95°C, followed by 45 cycles of denaturation (95°C, 10 s), annealing (62°C, 10 s), and extension (72°C, 15 s). The cycling was finalized by a standard melting curve analysis.

The efficiencies of the qPCR assays were estimated from standard calibration curves based on serial 5-fold dilutions of genomic DNA standards (10^{-1} to 10^{-5} ng μl^{-1}). The absolute quantification of the target sequences was performed based on the standard calibration curves using the LightCycler® 96 software, version SW1.1 (Roche, Switzerland). The resulting concentrations are expressed as ng of *R. irregularis* DNA or ng of *R. irregularis* MUCL 41833 haplotype DNA/ ng template.

To ensure specificity of qPCR assays, cross-amplification tests were performed with a range of templates (DNA extracted from potato roots, DNA extracted from fungal spores/mycelium and DNA extracted from potato roots colonized by AMF from *in vitro* or *in vivo* cultures).

Validation of molecular markers to trace and quantify *R. irregularis* MUCL 41833 on potato under greenhouse conditions

A greenhouse experiment was setup to validate molecular tracers and to quantify mtLSU gene at different colonization stages of *R. irregularis* on potato. Potato tubers of cultivar Bintje were inoculated with *R. irregularis* MUCL 41833 via an *in vivo* mycorrhiza donor plant (MDP) system for fast colonization of potato plantlets (adapted from Voets *et al.* (2009)) under greenhouse conditions.

Briefly, maize cv. ES Ballade plantlets were inoculated with *R. irregularis* MUCL 41833 and cultured for two months in pots of 8 dm^3 filled with sand and vermiculite (1V/1V) to set up a profuse and active extraradical mycelium network. Seedlings of *M. truncatula* were subsequently placed in the active network, for 5 weeks. The *M. truncatula* plants were then used as mycorrhizal donor plant. A pre-germinated tuber and 3 donor *M. truncatula* plants were placed in biodegradable pots (6 cm, Jiffy) filled with autoclaved sand/ vermiculite (1V/1V). Roots of potato were harvested 2, 3, 4 and 5 weeks after planting in order to have plants colonized at different colonization stages. They were washed with deionized water, dried at room temperature under laminar flow and stored at -20°C before DNA extraction and root staining.

Field experimental setup

The field trial was conducted in Libramont (Belgium) at the Walloon Agricultural Research Centre, Life Sciences Department, Breeding and Biodiversity Unit (49°55'N, 5°22'E). The trial was conducted from May 2014 to September 2014. The climate of the region is temperate. Maximal temperatures, minimal temperatures and cumulative rainfall are presented in Figure 26. The soil was silty with a pH of 6.3 and 5.3 in H_2O and KCl, respectively. Soil organic matter and C content were 47.8 g kg^{-1} and 27.8 g kg^{-1}, respectively.

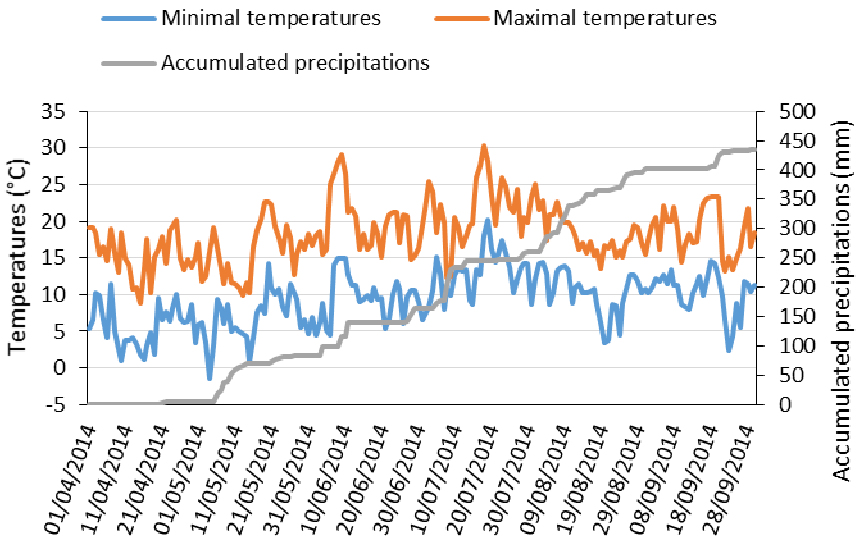

Figure 26. Climatic data (i.e. precipitations, minimal temperatures and maximal temperatures) during potato crop (source Pameseb asbl).

The soil was covered with a mowing grassland during three consecutive years before trial. Individual subplots were arranged in a randomized complete block design replicated four times. The trial was conducted under conventional agricultural itineraries but no fertilizers were added. The time line of the trial and the conventional agricultural factors applied are presented in Table 17.

Table 17. Time line of the trial and agricultural factors applied.

Time	Conventional agricultural factors
16th December 2013	Ploughing
9th April 2014	Herbicide Roundup (4 L ha^{-1})
16th May 2014	Tillage
19th May 2014	Tillage Plantation Mounding
26th May 2014	Herbicides Artist (1.8 kg ha^{-1}) + Challenge (2.5 L ha^{-1})
June, July and August	Fungicides Infinito (1.2 L ha^{-1}) × 6

	Revus (0.6 L ha^{-1}) × 3 Shirlan (0.4 L ha^{-1}) × 4 Cymopour (0.32 kg ha^{-1}) + Dithane (4.3 kg ha^{-1}) × 1 Cymbal 45 (0.25 kg ha^{-1}) + Dithane (2.5 kg ha^{-1}) × 1
28th August 2014	Defoliant Reglone (2.5 L ha^{-1})
2nd September 2014	Defoliant Reglone (2.5 L ha^{-1})
16th September 2014	Mechanical defoliation
24th September 2014	Harvest

The trial was conducted with three different potato cultivars (i.e. Bintje, Nicola and Charlotte). Two inoculation treatments were applied (i.e. beads containing *R. irregularis* MUCL 41833 (Ri41833) and beads without the AMF (Control)). Each potato tuber was inoculated with 120 beads. Inoculation was achieved in the planting hole close to the tuber. Each individual subplot had a surface of ± 21 m^2 (3 m × 7 m) with 60 plants and was separated from a neighboring subplot by a distance of 2 m. At the end of the experiment the subplots were chemically and mechanically defoliated. Harvest was done on the 24th of September 2014.

The roots of sixteen randomly-selected plants (i.e. four potato plants per plot over the 4 blocks) of each potato cultivar (i.e. Bintje, Nicola and Charlotte) and each inoculation treatment (Ri41833; Control) were harvested at the flowering stage (the 15th of July 2014), before defoliation (the 27th of August 2014) and at harvest (the 24th of September 2014). They were washed with deionized water, dried at room temperature under laminar flow and stored at -20°C until analysis.

Estimation of potato tuber weight, size and number

At harvest, tubers (four lines in each plot, i.e. 40 plants per subplot) were collected. Tubers were counted, weighed and their size distribution (< 60 mm or > 60 mm) determined.

Estimation of root colonization of potato by AMF

Roots were cleared (10 % KOH at 50 °C for 90 min) and stained (5 % blue ink (Parker®, USA) diluted in vinegar (7° acidity) at 50 °C for 60 min). Root colonization was subsequently assessed following the method of McGonigle et al. (1990). Two hundred intersections were observed under a compound microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 20-40 × magnifications. Total root colonization (% RC), abundance of arbuscules (% A) and intraradical spores/vesicles (% V) were determined.

Traceability and/or quantification of haplotype *R. irregularis* MUCL 41833 and species *R. irregularis* in potato with the mitochondrial marker

Frozen root samples of *S. tuberosum* were ground in liquid nitrogen, and DNA was extracted using the innuPREP Plant DNA kit (Analytikjena, Germany) according to the manufacturer recommendations with slight modifications (700 µl of SLS buffer and 25 µl of proteinase K were directly added to the grounded roots for the lysis). The concentration of DNA was measured with Quant-iT^{TM} PicoGreen® dsDNA Assay kit (Life technologies, USA) using Fluoroscan Ascent FL instrument (Labsystems, USA), and 10 ng of total genomic DNA was used as the template in qPCR.

qPCR was performed as described above. Standard curves constructed from 5-fold dilutions of genomic standard were included

in each run. The amplification of each dilution of genomic standard and experimental samples were performed in triplicate.

Data analysis and statistics

Data analysis was performed with the SAS statistical software version 9.3 (SAS Inc., Cary, NC). Data of the preliminary greenhouse experiment was analyzed by one-way ANOVA. Data of the field experiment was analyzed using a Linear Mixed Model (PROC MIXED) where “cultivar” and “inoculation treatment” were regarded as fixed factors, and block as a random factor. If necessary, data were either logarithmically or arcsine transformed prior to statistical analyses to meet the requirements of normal distribution and homogeneity of variance (as determined by Levene’s test). Differences between means were tested by Tuckey’s test ($P \leq 0.05$).

Results

Validation of molecular markers to trace and quantify *R. irregularis* MUCL 41833 on potato under greenhouse conditions

Three different mycorrhizal stages (early, intermediate and late stage) could be distinguished in the thirteen stained potato root samples (Table 18). Three samples were in the early stage of root colonization characterized by the presence of few hyphae and absence of arbuscules and vesicles/spores (Gallou *et al.*, 2010) (harvested at 2 weeks). Four samples were in the intermediate stage, with hyphae, a few arbuscules and no vesicles/spores (harvested at 2 weeks, 5 weeks and 6 weeks). Similarly, four samples (harvested at 3 weeks and at 4 weeks) were in the intermediate stage with the presence of hyphae, some arbuscules and the first vesicles/spores (the two intermediate stages were thus discriminated by the presence

of spores/vesicles). Finally, two samples (harvested at 5 weeks) were in the late stage of colonization with high amounts of hyphae, arbuscules and vesicles/spores (Gallou *et al.*, 2010). These results were compared with the amount of mitochondrial markers. The lowest concentration ($3.9\ 10^{-6} \pm 2.1\ 10^{-6}$ ng/ ng DNA) was found in the early stage of root colonization, while the highest concentration ($7.4\ 10^{-3} \pm 5.1\ 10^{-3}$ ng/ ng DNA) was found in the late stage. An intermediate concentration of mitochondrial markers ($5.7\ 10^{-5} \pm 1.7\ 10^{-5}$ ng/ ng DNA (A) and $3.0\ 10^{-4} \pm 1.2\ 10^{-4}$ ng/ ng DNA (B)) was found in the intermediate stage with absence and presence of spores/vesicles, respectively.

Table 18. Total (% RC), arbuscular (% A), spore/vesicles (% V) and hyphal only (% H only) root colonization and quantification of mtLSU of *R. irregularis* MUCL 41833 haplotype in potato roots grown under greenhouse conditions at different root colonization stages (i.e. Early, Intermediate (presence of arbuscules but no spore/vesicles (A) and presence of arbuscules and the first spore/vesicles (B)) and Late stage).

	Root colonization				mtLSU	
Mycorrhizal stage	**% RC**	**% A**	**% V/S**	**% H only**	***R. irregularis*/ Haplotype MUCL41833**	
					Cq	**Conc. (ng/ ng DNA)**
Early	18 ± 9^{a}	0 ± 0^{a}	0 ± 0^{a}	18 ± 9^{a}	35 ± 1	$3.9\ 10^{-6} \pm 2.1\ 10^{-6}$
Intermediate (A)	20 ± 5^{a}	2.2 ± 0.3^{b}	0 ± 0^{a}	18 ± 4^{a}	31 ± 1	$5.7\ 10^{-5} \pm 1.7\ 10^{-5}$
Intermediate (B)	33 ± 10^{a}	10 ± 3^{c}	1 ± 0^{b}	22 ± 7^{a}	28 ± 1	$3.0\ 10^{-4} \pm 1.2\ 10^{-4}$
Late	47 ± 16^{a}	15 ± 3^{c}	8 ± 0^{c}	25 ± 13^{a}	23 ± 2	$7.4\ 10^{-3} \pm 5.1\ 10^{-3}$

Data are means ± SE (n=2-4). The Tuckey's test compares means.

Potato tuber weight, size and number

The average tubers weight per plant, proportion of tubers > 60 mm and number of tubers per cultivar and inoculation treatment are presented in Table 19 and Figure 27. A significant effect of the factor “cultivar” was observed on potato tubers weight ($P < 0.01$). To the contrary, no significant effect on tubers weight was observed for the factor “inoculation treatment” and there was no interaction between both factors. As the factor “inoculation treatment” was not significant, average tubers weight per plant was calculated for the “Ri41833” and “Control” treatments combined. Potato tubers weight was significantly higher in Bintje (i.e. by 11 %) and in Nicola (i.e. by 10 %) than in Charlotte.

The % tubers > 60 mm ranged from 14 ± 2 % to 30 ± 2 % among the different treatments (Table 19 and Figure 27). A significant effect ($P < 0.0001$) of the factor “cultivar” was observed. To the contrary, no significant effect on the % tubers > 60 mm was observed for the factor “inoculation treatment” and there was no interaction between both factors. As the factor “inoculation treatment” was not significant, % tubers > 60 mm was calculated for the “Ri41833” and “Control” treatments combined. The % tubers > 60 mm was significantly higher for cultivar Bintje than for cultivar Nicola ($P < 0.001$) or Charlotte ($P < 0.0001$) and % tubers > 60 mm was significantly higher for cultivar Nicola than for cultivar Charlotte ($P < 0.05$) (Table 19).

The number of tubers per plant ranged from 14 ± 1 to 16 ± 1 among the different treatments (Table 19 and Figure 27). However, no significant effect on the number of tubers per plant was noticed for the factors “cultivar” or “inoculation treatment”.

Table 19. Tuber weight and characteristics (percentage tubers > 60mm, number of tubers/plant – N°/plant) with following "inoculation treatments" (inoculation with beads containing *R. irregularis* MUCL 41833 (Ri41833) or beads without the AMF (Control)) and of following "cultivars" (Bintje, Nicola and Charlotte).

Cropping scenarios	Weight (g/plant)	Tubers	
		Size > 60 mm (%)	N°/plant
Bintje/ Ri41833	2305 ± 37	30 ± 2	15 ± 1
Bintje/Control	2290 ± 61	28 ± 2	15 ± 0
Nicola/ Ri41833	2292 ± 66	21 ± 1	14 ± 1
Nicola/Control	2243 ± 37	18 ± 0	14 ± 0
Charlotte/ Ri41833	2195 ± 73	17 ± 2	16 ± 1
Charlotte/Control	1913 ± 119	14 ± 2	14 ± 1
Effects (p-values)			
Cultivar	**0.0056**	**< 0.0001**	0.0583
Inoculation treatment	0.0612	0.1152	0.2473
Cultivar × Inoculation treatment	0.1499	0.8581	0.2399
Tuckey's test			
Bintje	2298 ± 32	29 ± 1	
Nicola	2268 ± 37	20 ± 1	
Charlotte	2055 ± 83	15 ± 2	
Bintje vs Nicola	0.9065	**0.0006**	
Nicola vs Charlotte	**0.0195**	**0.0433**	
Charlotte vs Bintje	**0.0079**	**< 0.0001**	

Data are means ± SE of 4 blocks (40 plants per block). Main effects and interactions between the factors "cultivar" and "inoculation treatment" are presented. Tests that revealed significant differences at the 0.05 α level are indicated in bold. The Tuckey's test compares means.

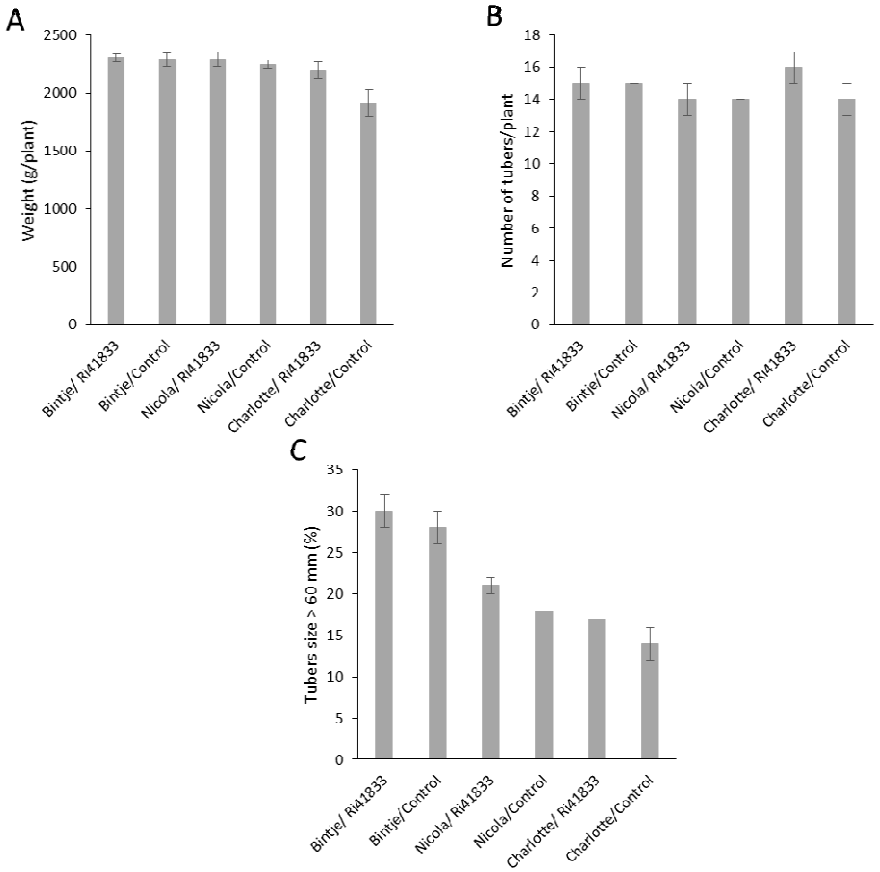

Figure 27. Tuber weight and characteristics (number of tubers/plant – N°/plant, percentage tubers > 60 mm,) with following "inoculation treatments" (inoculation with beads containing *R. irregularis* MUCL 41833 (Ri41833) or beads without the AMF (Control)) and of following "cultivars" (Bintje, Nicola and Charlotte).

Estimation of root colonization by AMF

Potato root colonization at flowering stage, before defoliation and at harvest is presented in Figure 28 and statistics are described in Table 20.

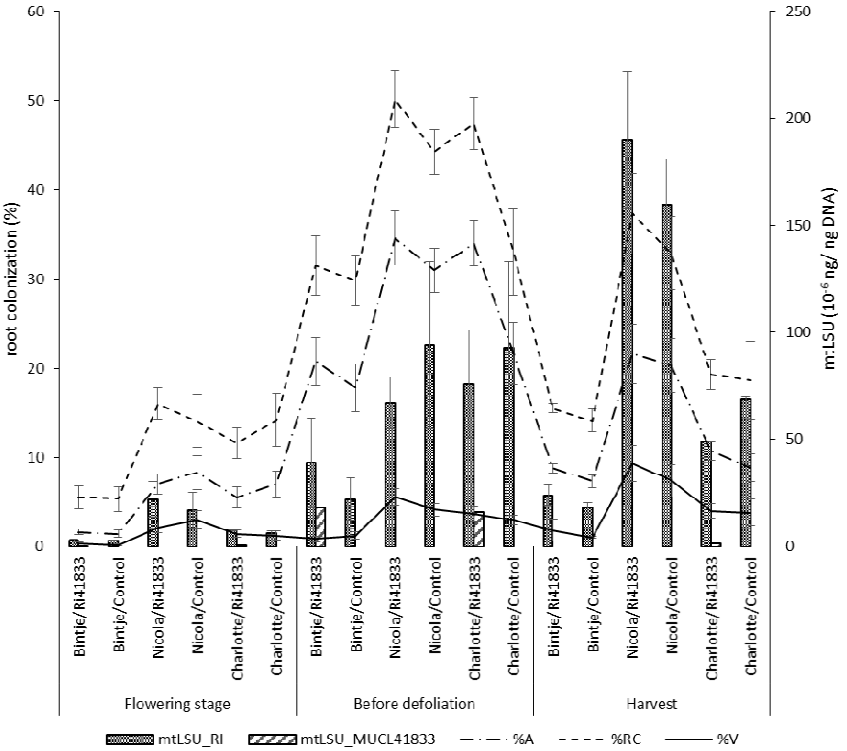

Figure 28. Root colonization of potato cultivars Bintje, Nicola and Charlotte by AMF (Total root colonization (%RC), Arbuscules (%A) and Vesicles/Spores (%V)) and mtLSU DNA concentration of *R. irregularis* (mtLSU-RI) or *R. irregularis* haplotype MUCL 41833 (mtLSU_MUCL41833).

At flowering stage, the % RC ranged from 5.3 ± 1.4 % to 16.0 ± 1.8 % and the % A from 1.4 ± 0.4 % to 8.3 ± 1.9 %. The % V remained low (from 0.2 ± 0.1 to 3.0 ± 1.0 %). A significant effect ($P < 0,0001$) of the factor "cultivar" was noticed on the % RC, the % A and the % V. In contrast, the factor "inoculation treatment" had no significant effect on % RC, % A or % V. No interaction was even noticed between the two factors for % RC, % A or % V. % RC, % A and % V were calculated for the "Ri41833" and "Control" treatment combined (Table 20). Significant ($P < 0.0001$) higher % RC and % A

were noticed for cultivars Nicola and Charlotte in comparison to cultivar Bintje, while no difference was observed between Nicola and Charlotte (Table 20). Moreover, significant higher % V was noticed for cultivars Nicola ($P < 0.0001$) and Charlotte ($P < 0.05$) in comparison to Bintje, while no difference was observed between Nicola and Charlotte. Before defoliation the % RC ranged from 29,9 ± 2,8 % to 50,2 ± 3,2 % and the % A from 17,8 ± 2,7 to 34,6 ± 3,0. The % V ranged from 0,8 ± 0,3 % to 5,6 ± 0,9 %. As at flowering stage, significant effect ($P < 0,0001$) of the factor “cultivar” was noticed on the % RC, the % A and the % V. The factor “inoculation treatment” had no significant effect on % RC, % A or % V. An interaction ($P < 0,05$) was noticed between the two factors for % RC but no interaction was noticed for % A or % V. Similarly as above, % RC, % A and % V were calculated for the “Ri41833” and “Control” treatment combined (Table 20). Significant ($P < 0.0001$) lower % RC, % A and % V were noticed for cultivars Bintje in comparison to cultivar Nicola (Table 20). Significant ($P < 0.05$) lower % RC, % A and % V were noticed for cultivars Charlotte in comparison to cultivar Nicola (Table 20) and significant ($P < 0,05$) lower %RC, % A and %V were noticed for cultivar Bintje in comparison to cultivar Charlotte (Table 20). At harvest the % RC ranged from 14.1 ± 1.3 % to 37.4 ± 4.5 % and the % A from 7.3 ± 0.7 % to 21.6 ± 3.3 %. The % V ranged from 1.0 ± 0.1 to 9.3 ± 2.0 %. A significant effect ($P < 0,0001$) of the factor “cultivar” was noticed on the % RC , the % A and the % V. The factor “inoculation treatment” had no significant effect on % RC, % A or % V. No interaction was noticed between the two factors for % RC, % A or % V. % RC, % A and % V were thus calculated for the “Ri41833” and “Control” treatment combined (Table 20). Significant ($P < 0.0001$) lower % RC and % A were noticed for cultivars Bintje and Charlotte in comparison to cultivar Nicola, while no difference was observed

between Bintje and Charlotte (Table 20). Moreover, significant different % V were noticed between the three cultivars (Table 20), with the highest % V for cultivar Nicola (8.3 ± 1.3 %), then for cultivar Charlotte (3.9 ± 0.8 %) and the lowest % V for cultivar Bintje (1.4 ± 0.2 %).

Table 20. Statistics of total (% RC), arbuscular (% A) and spore/vesicular (% V) colonization of the roots of potato at flowering stage and at harvest with following "inoculation treatments" (inoculation with beads containing *R. irregularis* MUCL 41833 (Ri41833) or beads without the AMF (Control)) and of following "cultivars" (Bintje, Nicola and Charlotte).

	Flowering stage			**Before defoliation**			**Harvest**		
	% RC	**% A**	**% V**	**% RC**	**% A**	**% V**	**% RC**	**% A**	**% V**
Effects (p-values)									
Cultivar	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Inoculation treatment	0.4996	0.9115	0.4248	0.3119	0.2662	0.5890	0.2059	0.1642	0.0839
Cultivar × Inoculation treatment	0.5813	0.2645	0.6764	**0.0466**	0.0833	0.3833	0.9557	0.9499	0.9397
Tuckey's test									
Bintje	5.4 ± 1.0	1.5 ± 0.3	0.3 ± 0.1	30.7 ± 2.2	19.3 ± 1.9	1.0 ± 0.2	14.8 ± 0.7	8.0 ± 0.5	1.4 ± 0.2
Nicola	15.0 ± 1.7	7.6 ± 1.1	2.6 ± 0.6	47.2 ± 2.1	32.8 ± 2.0	4.8 ± 0.6	35.2 ± 3.0	21.0 ± 2.2	8.3 ± 1.3
Charlotte	12.9 ± 1.7	6.3 ± 1.0	1.3 ± 0.3	40.3 ± 3.1	27.8 ± 2.4	3.3 ± 0.5	18.9 ± 2.3	9.8 ± 0.9	3.9 ± 0.8
Bintje vs Nicola	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Nicola vs Charlotte	0.4826	0.3568	0.1082	**0.0174**	**0.0327**	**0.0469**	**< 0.0001**	**< 0.0001**	**0.0003**
Charlotte vs Bintje	**< 0.0001**	**< 0.0001**	**0.0112**	**0.0105**	**0.0081**	**0.0001**	0.4397	0.6729	**0.0323**

Main effects and interactions between the factors "cultivar" and "inoculation treatment" are presented. Tests that revealed significant differences at the 0.05 α level are indicated in bold. The Tuckey's test compares means.

Traceability and/or quantification of native and inoculated *R. irregularis* in potato within field

MtLSU detection levels of haplotype-specific or species-specific *R. irregularis* at flowering stage, before defoliation and at harvest are presented in Figure 28 and statistics are described in Table 21.

MtLSU of haplotype MUCL 41833 (mtLSU_41833) was detected at a very low level at the flowering stage in only one plant of cultivar Bintje ($2.2\ 10^{-7} \pm 1.7\ 10^{-7}$ ng/ ng DNA) and Charlotte ($7.0\ 10^{-7} \pm 1.2\ 10^{-7}$ ng/ ng DNA) that were inoculated with this strain. Before defoliation, detection was again noticed in only one plant of the inoculated cultivar Bintje ($1.8\ 10^{-5} \pm 8.0\ 10^{-6}$ ng/ ng DNA) and inoculated cultivar Charlotte ($1.6\ 10^{-5} \pm 1.5\ 10^{-5}$ ng/ ng DNA). At harvest, detection was only noticed in one inoculated plant of cultivar Charlotte ($1.5\ 10^{-6} \pm 2.9\ 10^{-7}$ ng/ ng DNA). MtLSU of haplotype MUCL 41833 was never detected in cultivar Nicola, whatever the stage of development.

The species-specific mtLSU region of *R. irregularis* (mtLSU_RI) was detected in all the potato plants. Statistics are presented in Table 21. At flowering stage, before defoliation and at harvest, significant effects ($P < 0.001$) of the factor “cultivar” were observed. To the contrary, no significant effect for the mtLSU_RI was observed for the factor “inoculation treatment” and there was no interaction between both factors. As the factor “inoculation treatment” was not significant, detection of mtLSU_RI was calculated for the “Ri41833” and “Control” treatments combined. The detection of mtLSU_RI was significantly higher for cultivar Nicola ($P < 0.001$) and Charlotte ($P < 0.01$) than for cultivar Bintje at the flowering stage and

before defoliation (Table 21). At harvest, detection of mtLSU_RI of cultivar Nicola was significantly higher than for cultivar Charlotte ($P < 0.001$) and cultivar Bintje ($P < 0.001$). Detection of MtLSU_RI of cultivar Charlotte was significantly higher ($P < 0.0001$) than for cultivar Bintje.

Table 21. Quantification and statistics of the mtLSU specific to *Rhizophagus irregularis* detected in the roots of potato at flowering stage, before defoliation and at harvest for following "inoculation treatments" (inoculation with beads containing *R. irregularis* MUCL 41833 (Ri41833) or beads without the AMF (Control)) and following "cultivars" (Bintje, Nicola and Charlotte).

	mtLSU_*Rhizophagus irregularis* 10^{-6} ng/ ng DNA		
	Flowering stage	**Before defoliation**	**Harvest**
Bintje/Ri41833	2.9 ± 0.5	39 ± 21	24 ± 5
Bintje/Control	2.8 ± 0.2	22 ± 10	18 ± 2
Nicola/ Ri41833	22 ± 8	67 ± 12	190 ± 32
Nicola/Control	17 ± 9	94 ± 39	160 ± 21
Charlotte/ Ri41833	7.3 ± 0.2	76 ± 25	49 ± 8
Charlotte/Control	6.2 ± 0.8	93 ± 40	69 ± 1
Effects (p-values)			
Cultivar	**< 0.0001**	**0.0002**	**< 0.0001**
Inoculation treatment	0.3767	0.5212	0.7615
Cultivar × Inoculation treatment	0.0569	0.8484	0.6638
Tuckey's test			
Bintje	2.9 ± 0.3	31 ± 11	21 ± 2.7
Nicola	20 ± 5.9	81 ± 20	180 ± 19
Charlotte	6.7 ± 1.1	84 ± 23	59 ± 7.1
Bintje vs. Nicola	**< 0.0001**	**0.0002**	**0.0005**
Nicola vs. Charlotte	0.1814	0.4299	**0.0001**
Charlotte vs. Bintje	**0.0099**	**0.0095**	**< 0.0001**

Data are means ± SE of 4 blocks (4 plants per block). Main effects and interactions between the factors "cultivar" and "inoculation treatment" are presented. Tests that revealed significant differences at the 0.05 α level are indicated in bold. The Tuckey's test compares means.

Discussion

This study is the first, to our knowledge, that traces and quantifies native and inoculated *R. irregularis* by Real-Time quantitative PCR of mtLSU on three different potato cultivars grown under field conditions. MtLSU of the inoculant *R. irregularis* MUCL 41833 was characterized and two different markers were designed. The first marker was a haplotype-specific marker (to trace the field-inoculated haplotype) and the second marker a *R. irregularis* species-specific marker (to trace either the field-inoculated isolate or the native strains belonging to the *R. irregularis* species). The markers were validated on potato plants grown under controlled greenhouse conditions and paralleled with the root colonization stage as defined by Gallou *et al.* (2010) via microscopic observations. Our comparison suggested that the markers were good indicators of inoculation success of a specific isolate even as good indicators of the colonization stage. Krak *et al.* (2012) compared this mtDNA-based quantification to nrDNA-based quantification, which is explored in numerous studies (Alkan *et al.*, 2004, Filion *et al.*, 2003, Gamper *et al.*, 2008). They found that the ratio of mtLSU to nrLSU copy numbers was constant across the root colonization of various ages. But the higher-resolution power of the mtLSU compared to that of nrDNA (Börstler *et al.*, 2010) increased the chances of distinguishing an introduced isolate from a native background. This kind of marker was already used for the characterization of *R. irregularis* strains in pure cultures (Börstler *et al.*, 2008, Raab *et al.*, 2005) as well as for diversity studies (Börstler *et al.*, 2010) or tracing of introduced isolate (Sýkorová *et al.*, 2012) in the field. But the absolute quantification of the mtLSU gene of an introduced isolate was to our knowledge never performed on field samples.

In the field trial, haplotype-specific molecular markers revealed the virtual absence of the inoculated *R. irregularis* MUCL 41833 haplotype in the cultivars Charlotte and Bintje and its absence of detection in the cultivar Nicola. In contrast, the species-specific markers detected *R. irregularis* in all potato roots, indicating that native *R. irregularis* was naturally present in the soil since this AMF was never inoculated in this specific field. In the study of Farmer *et al.* (2007), inoculation success could be suggested by the increased frequency of the occurrence of inoculated species after inoculation. In our study, species-specific mtLSU markers indicated that there was no significant difference in the colonization by *R. irregularis* between inoculated and non-inoculated potato plants. With the haplotype-specific markers we could confirm that inoculation success after direct inoculation of potato plants was very low. The unsuccessful inoculation could be explained by different factors: viability/infectivity of inoculum (Declerck *et al.*, 1996), incompatibility with the soil environment or plant host (Oehl *et al.*, 2010, Öpik and Moora, 2012), competition with other microorganisms, lack of time for establishment (Dickie *et al.*, 2012) or distance from roots. In our study, viability/infectivity of inoculum was evaluated when developing the inoculum by estimating the number of beads necessary to infect 90% of the plants cultured under controlled conditions. Results showed that 13 beads were necessary to infect 90% of the plants. But, effectiveness of inoculum should also be evaluated under field conditions. Even if we over-inoculated with 120 beads per potato plant, field establishment seemed absent. Indeed, competition between isolates of the same species could occur, what was previously observed with a decrease in mtLSU levels for one of two coexisting isolates from the same species (Krak *et al.*, 2012). Finally, the distance of the inoculum from the host roots is crucial for

successful inoculation. In our study, beads were placed next to the potato tuber but the distance with the roots was probably not optimal for good establishment. We hypothesize that the first large roots, produced by reserves from the tuber, are less suitable to be colonized by AMF than secondary roots that are at a higher distance from the tuber. A pot experiment should be performed with potato to study which roots are colonized by AMF.

The three potato cultivars were colonized with *R. irregularis* at flowering stage, before defoliation and at harvest as determined by species-specific mtLSU detection. Interestingly, AMF root colonization and *R. irregularis* mtLSU detection of Nicola and Charlotte was significantly higher as compared to Bintje at flowering stage. Before defoliation, AMF root colonization of Nicola was significantly higher than Charlotte and root colonization of Charlotte significantly higher than Bintje. In parallel *R. irregularis* mtLSU detection of Nicola and Charlotte was significantly higher as compared to Bintje but no significant difference was observed between Charlotte and Nicola. At harvest, %RC and %A was significantly higher for cultivar Nicola than for cultivars Charlotte and Bintje and Nicola had the highest %V followed by Charlotte and then Bintje. In parallel, Nicola had the highest mtLSU detection followed by Charlotte and then Bintje. Even if the tendency of root colonization is in the same sense as the *R. irregularis* mtLSU detection, possible colonization of cultivars by other AMF species are also to be taken into acount when evaluating root colonization. These results are in accordance with Bhattarai and Mishra (1984) who observed a difference in root colonization between potato cultivars. They suggested that the highly disease resistant cultivars showed an earlier establishment and more rapid development of AMF than

susceptible ones. Previous studies showed also that crop breeding programs selecting for high yield varieties under fertilized conditions may have inadvertently select genotypes that are unresponsive to mycorrhiza (Johnson and Pfleger, 1992). In our study, mycorrhizal dependency could not be evaluated, because for each cultivar potatoes were all colonized at the same rate by AMF. Hence, greenhouse studies with these cultivars should be conducted in order to determine their mycorrhizal dependency, which is the degree to which a plant relies upon the mycorrhizal condition to produce its maximum growth or yield at a given level of soil fertility. We suppose that it will be the highest for Nicola and the lowest for Bintje. In the same way, studies (Goffart et al. 2002) have shown that cultivar Nicola makes better use of nitrogen fertilization than cultivar Bintje. A possible explanation to this could be the effect of AMF, probably in parallel with a cultivar genetic effect. Breeding for better symbiosis (Rengel, 2002) could thus be a strategy for a sustainable agriculture.

In conclusion, our study demonstrated, for the first time, that mtLSU-based qPCR assays could be used in the field for quantification of native and inoculated AMF strains. Traceability is important because using this tool we could confirm inoculation success and importance under complex environmental conditions. This study showed that efficiency of AMF inoculation under field conditions is complex and that colonization potential is cultivar dependent. Establishment potential of inoculated isolates and their persistance has to be further studied over longer periods with this molecular markers. Moreover, isolate specific markers should further be designed by using the whole mitochondrial genome.

Acknowlegments

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VII. GENERAL DISCUSSION

Potato (*Solanum tuberosum*) is the fourth largest food crop cultivated in the world (FAO, 2014). This crop is easy to grow, has high energy and protein contents per unit area and has beneficial effects on human health (Camire *et al.*, 2009). However, under conventional agricultural systems, heavy mechanization and application of high amounts of fertilizers and pesticides, with potential detrimental effects on the environment, are the rule. There is thus an increasing need of integrated potato cultivation systems combining different management strategies and itineraries with reduced use of mineral fertilizers and pesticides and complying with economic, health and environmental requirements. An alternative or complementary approach to fertilizers and pesticides is the use of beneficial microorganisms such as AMF or *Trichoderma* spp. Indeed, their beneficial effects on potato have been demonstrated repeatedly under controlled conditions (e.g. Duffy and Cassells (2000); Yao *et al.* (2002)). However, their application under conventional or organic potato agro-ecosystems is still in its infancy and the few results obtained so far remain inconclusive (e.g. Douds *et al.* (2007)). There is thus an urgent need to increase our knowledge on the effects of some agricultural factors on AMF and *Trichoderma* spp., on the modes and timing of application of these microorganisms and on their traceability within the field to demonstrate their potential in increasing health and yield of potato while decreasing mineral fertilizers and pesticides applications.

1. Understanding the impact of fungicides and herbicides on AMF

Pesticides can impact AMF directly or indirectly (Figure 29). *In vitro* cultivation systems associating potato plants with AMF were used to investigate the impacts of fungicides and herbicides on the

fungal symbiont (Voets *et al.*, 2005). These systems have several advantages, among which the possibility of non-destructive observations of the mycelium architecture (e.g. De La Providencia *et al.* (2005)) and sporulation dynamics (e.g. Declerck *et al.* (2001)). In addition, they are void of any unwanted contaminants, thus excluding the risk of confounding effects with undesirable microorganisms.

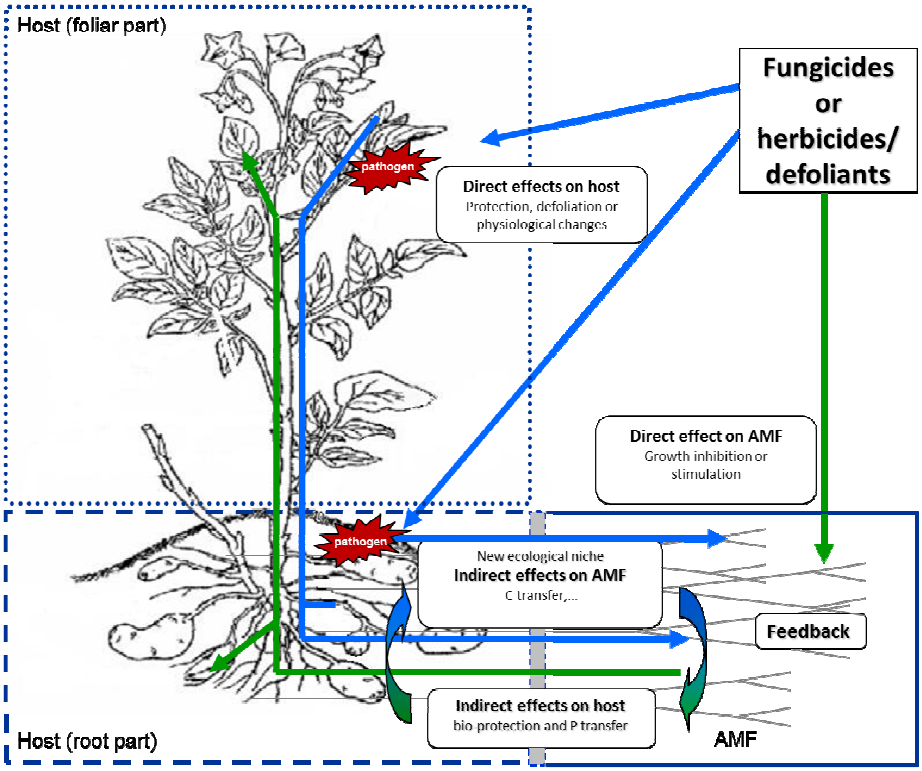

Figure 29. Direct and indirect effects of fungicides or herbicides/defoliants on AMF and plant host. Blue arrows: direct effect of fungicides or herbicides/defoliants on plants and subsequent indirect effect on AMF; green arrows: direct effect of fungicides or herbicides/defoliants on AMF and subsequent indirect effect on plants.

Rhizoctonia stem canker and black scurf are economically important diseases of potatoes worldwide. Currently, it is not possible to completely control these pathogens, but severity can be limited by a combination of cultural and crop protection factors. Seed or soil fungicide treatment is among the most widely used control strategy, but this may be harmful also to non-target beneficial soil fungi (e.g., AMF), another strategy increasingly considered to reduce belowground diseases. These fungi have recognized potentials to improve resistance of plants to diseases. For instance, Gallou *et al.* (2011) demonstrated under *in vitro* culture conditions, an increased resistance of mycorrhized potato plantlets against *R. solani*. Notwithstanding these promising results, it is doubtful that AMF may replace fungicides to control Rhizoctonia disease. They should rather be considered in an integrated management of the disease, favoring for instance a decrease in the level of fungicide application. In **Chapter I** we tested *in vitro* the impact of a broad-spectrum (azoxystrobin) or Basidiomycota-specific (flutolanil) systemic fungicides, blocking cell respiration, and a contact Rhizoctonia-specific fungicide (pencycuron), blocking cell division, and their respective formulations, Amistar, Monarch, and Monceren, on the AMF *R. irregularis* MUCL 41833. This approach was earlier tested with other compounds (e.g. Gong *et al.* (2014)) and thus represent a standardized approach to evaluate fungicide compounds and formulations. Several of these studies reported results closely-related to those obtained in field or in greenhouse conditions (e.g. Jin *et al.* (2013)) supporting the reliability of the *in vitro* experimental approach.

Spore germination was not impacted by the three tested fungicides at the IC_{50} established for *R. solani*, but was impacted at higher concentrations. Interestingly, the fungicides at concentrations

from 1 to 100 mg L^{-1} had a fungistatic effect on *R. irregularis* rather than a fungitoxic effect. Similar results were observed for fenhexamid and fenpropimorph at concentrations from 2 to 200 mg L^{-1} (Zocco *et al.*, 2008). Pencycuron, the contact and most specific fungicide did not impact *R. irregularis* MUCL 41833 at the IC_{50} of *R. solani*, whilst the systemic fungicide flutolanil had a pronounced effect on root colonization, possibly because of accumulation of the fungicide in the root zone. Azoxystrobin, known to have a lower systemic activity than flutolanil, did not affect intraradical AMF development but affected the extraradical mycelium development at doses above the IC_{50} of *R. solani.* This was earlier observed by Kjøller and Rosendahl (2000) in a greenhouse trial with propiconazole, another broad-spectrum fungicide, where the ALP activity of the extraradical mycelium was decreased suggesting a higher sensitivity to this fungicide as compared to the intraradical mycelium which was not affected. The effects of fungicides on AMF were thus dependent on the compound and dose of application.

This first experiment suggested that fungicides at adequate dosage to control *R. solani* or decrease its impact on potato may be combined with the beneficial fungus *R. irregularis* MUCL 41833. However we should take care that reduced fungicide doses do not result in the apparition of resistances in *R. solani*. Moreover, combined disease management (i.e. chemical and biological) should not increase costs for the farmers or complexify the management system. The advantage of AMF is that they will first reduce fertilizer input costs because they will permit a better exploitation and exploration of nutrients in soil. In the same context, a number of studies have reported on the combination of biocontrol fungi and fungicides. Van Den Boogert and Luttikholt (2004) demonstrated that

azoxystrobin (a broad spectrum fungicides) was toxic to the biocontrol agent *Verticillium biguttatum*, while the *Rhizoctonia* specific fungicides pencycuron and flutolanil, co-applied with *V. biguttatum*, showed additive control effects on black scurf. *Trichoderma harzianum*, another Rhizoctonia biocontrol fungus, was shown to tolerate over 100-fold higher doses of flutolanil than *R. solani* which supports this combined use (Wilson *et al*., 2008).

Herbicides or defoliants are commonly used in crops to control weeds, facilitate crop harvest or eventually suppress cover crops before cash crop. AMF colonize most crops and weeds indiscriminately and rely exclusively on photosynthetically fixed carbon for their life cycle (Yamato, 2004). Defoliation can thus affect the plant-to-fungus C pathway and impact indirectly AMF. In **Chapter II**, an *in vitro* culture system was used to evaluate the impact of two defoliation treatments (i.e. mechanical defoliation and chemical defoliation with a contact defoliant – diquat-formulation) and one systemic herbicide (glyphosate-formulation) on *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410. As observed in a recent study (IJdo *et al*., 2010), mechanical defoliation drastically impacted spore production of *R. intraradices.* IJdo *et al.* (2010) suggested that the production of novel spores was directly dependent on the C flow from the plant and that this AMF were able to modulate spore production according to C availability. *Rhizophagus* spp. sporulation dynamics follow generally a lag log and plateau phase (Declerck *et al*., 2001, Declerck *et al*., 1996). It is thus highly probable that defoliation of plants in the plateau phase of spore production is less detrimental to the AMF and spores population than defoliation in the lag or log phase. More interestingly, the systemic herbicide (e.g. glyphosate) had a faster and stronger effect than chemical (e.g.

diquat) or mechanical defoliation. However, the mechanism that inhibit so rapidly sporulation dynamic is not well understood. It is not excluded that the herbicide is impacting the AMF via its translocation in the roots in close contact with the IRM or release in the medium via root decay (Neumann *et al.*, 2006) and contact with the ERM. Indeed, *in vitro* (Wan *et al.*, 1998) and *in vivo* (Druille *et al.*, 2013) studies have shown that a direct contact of glyphosate with AMF and aminomethylphosphonic acid (AMPA), a glyphosate secondary metabolite, were relatively toxic to AMF (IC_{50} of 0.5 ± 0.3 ppm and 3.8 ± 2.8 ppm, respectively). However, Araújo *et al.* (2003) claimed that glyphosate is rapidely biodegraded by soil microorganisms. But, the persistence and transport of glyphosate in soil is dependent on soil composition, climatic conditions and microbial activity (Carlisle and Trevors, 1988), as wel as agricultural factors (Helander *et al.*, 2012). Mechanical and chemical defoliation and especially the systemic herbicide (glyphosate) reduced colonization by *R. irregularis* and *R. intraradices* and root dry weight. Druille *et al.* (2013) observed also a significant reduction of root colonization (in particular arbuscules) after glyphosate application. They explained that arbuscules were sensitive to the reduction in supply of carbohydrates caused by defoliation. Consequently, appropriate and timely defoliation or herbicide applications could possibly attenuate their impact on spore production or root colonization.

In the second part of our thesis, we focused on potato within the field. Interestingly, no significant difference was noted in AMF root colonization of potatoes grown in organic or conventional culture systems (**chapter III**). This suggested that the fungicides/herbicides used in both agricultural systems had similar effects (or absence of effects) on AMF. However, we could not exclude that there was a

shift in AMF species (not evaluated). We have also to note that preceding crop in this experiment was performed under conventional culture conditions which mean that we were not entirely under organic culture conditions. As observed in our *in vitro* studies (**chapter I**), the impact of pesticides on AMF varies with the type of compound, dosage or time of application. Within field, the effects of pesticides may be attenuated by microorganisms having the capacity to degrade pesticides, by adsorption on soil particles and by dilution and availability of pesticides. These factors should also be considered over the long term. Repeated or chronic exposure (due to soil build-up) could affect AMF.

2. Technical itineraries for optimal use of AMF

AMF are known to play a key role in agro-ecosystems by increasing plant growth and improving their resistance/tolerance to biotic and abiotic stresses (Gianinazzi *et al*., 2010). However, most studies were conducted under greenhouse or *in vitro* conditions and those conducted within the field were often inconclusive. AMF application or management in cropping systems necessitates the development of technical itineraries that stimulate their functions as plant growth promoters and plant resistance enhancers. Moreover, in most agricultural systems indigenous AMF are already present and could mask the effects of inoculated strains.

In **Chapter III**, different strategies were tested to increase the population density and efficacy of AMF. The first strategy consisted in the application of adequate agricultural itineraries favoring/stimulating the indigenous population of AMF, while the second strategy was to inoculate selected beneficial microorganisms with known benefits on plant growth and health. Here we combined both strategies. We used

cover crops before potato planting to increase the population of AMF. Indeed, studies have demonstrated that the use of mycotrophic cover crops before cash crop or as weed control strategy during cash crop, increased the natural population of AMF in soil and subsequent root colonization of cash crop (Boswell *et al.*, 1998, Kabir and Koide, 2000, Lehman *et al.*, 2012, Ramos-Zapata *et al.*, 2012). Nevertheless, in our study *Medicago sativa* did not increase significantly potato root colonization by indigenous AMF. A possible explanation was the very low population of native AMF. In consequence, under low indigenous population densities, inoculating the AMF is another option to increase AMF within the field (Douds *et al.*, 2007, Pellegrino *et al.*, 2011). The inoculation of a cover crop or directly the potato crop increased potato root colonization. This suggested that inoculated AMF was efficient in colonizing the potato plants as compared to indigenous AMF. But in both trials conducted in **Chapter III**, the most advantageous itinerary to increase potato yield was to inoculate a cover crop before potato planting. We suggested that the cover crop could have allowed a better establishment and increase of the population of the inoculated strain in the soil before potato crop than direct inoculation. Interestingly, for cultivar Bionta inoculating the cover crop increased potato yield and the size of the tubers. For cultivar Sarpo Mira inoculating the cover crop increased potato yield and the number of tubers per plant. This technical itinerary will not have only the adventage of increasing crop yield. The legume cover crop combined with beneficial fungal inoculants will also reduce soil erosion, weed control costs, disease control costs and fertilizer costs. However, in none of the experiments we could trace the inoculated AMF to differentiate its effects from the indigenous AMF population. In addition, it is unknown if the

introduced fungal inoculant have replaced the native strains or reduce their diversity.

In **Chapter III***, R. irregularis* MUCL 41833 was co-inoculated with *T. harzianum* MUCL 29707 because a number of studies have reported the synergism between AMF and *Trichoderma* spp. (Calvet *et al.*, 1993, Chandanie *et al.*, 2009). Moreover, preliminary experiments (not published) have shown that *R. irregularis* MUCL 41833 or *T. harzianum* MUCL 29707 inoculated separately via a cover crop did not increase significantly potato crop. Moreover, De Jaeger *et al.* (2011) suggested that the presence of *T. harzanium* in close contact with AMF (i.e. in the beads) could stimulate the latter.

The mycorrhizal dependency of the potato cultivars seems also to be an important point to considere. Yao *et al.* (2002) observed, for example, that potato cultivar Goldrush was more influenced (e.g. increase of shoot fresh weight, root dry weight and number of tubers produced per plant) by AMF than the potato cultivar LP89221. In **Chapter IV** three potato cultivars were compared. But mycorrhizal dependency could not strictly be evaluated because in the field all the plants (including the controls) were colonized. However, between cultivars, root colonization, mtLSU detection of *R. irregularis* and yield were different. Interestingly, root colonization and mtLSU detection of *R. irregularis* at different times of potato crop was the highest for Nicola, followed by Charlotte and then Bintje. Bhattarai and Mishra (1984) observed also differences in root colonization between cultivars.

3. Traceability of AMF in potato crop

If we want to link enhanced yield or disease control to an introduced AMF strain, we should be able to demonstrate, first, the presence of this strain in the roots. The design of molecular markers is thus mandatory and achieved in **Chapter IV,** to trace the inoculated strain *R. irregularis* MUCL 41833 and distinguish from native *R. irregularis*. The mtLSU of inoculated *R. irregularis* MUCL 41833 was first characterized. Indeed, the mtLSU seem to be a good region to characterize and distinguish different haplotypes of the same species of this fungus in the field (Börstler *et al.*, 2008, Börstler *et al.*, 2010) because of the higher resolution power of this region than nrLSU. Moreover, mtLSU was shown to be homogeneous within the same isolate in contrast to the nrLSU (Raab *et al.*, 2005). In this study, two different markers were thus designed: the first marker was a haplotype-specific marker (to trace inoculated strain) and the second marker was a species-specific marker (to trace inoculated strain but also native strains from the same species). The markers were validated on inoculated potato plants grown under greenhouse conditions and the molecular quantification was linked with the AMF root colonization stage as defined by Gallou *et al.* (2010), via microscopic observations. Our comparison suggested that the markers were good indicators of inoculation success of a specific strain and a good indicator of the AMF root colonization stage in potato roots.

In the field trial of **chapter IV**, haplotype-specific molecular markers revealed the virtual absence of the inoculated *R. irregularis* MUCL 41833 haplotype in the cultivars Charlotte and Bintje and its non-detection in the cultivar Nicola. In contrast, the species-specific markers could detect *R. irregularis* in all potato roots, indicating that

native *R. irregularis* was naturally present in the soil since this AMF was never inoculated in this field.

VIII. CONCLUSIONS & PERSPECTIVES

Potato is a high nutrient-demanding crop that requires the application of large quantities of fertilizers. This crop faces also numerous pathogens requesting several pesticides. Repeated application of these chemicals are harmful to the environment if not used properly. Nowadays, the application of beneficial fungal inoculants such as AMF or *Trichoderma* spp. represent a promising alternative or a complementary strategy to chemicals, for enhancing yield and health of crops. However, the use of beneficial microorganisms in agro-ecosystems is not yet optimal. Increased knowledge is necessary on the impact of agricultural factors on AMF in order to apply proper itineraries.

Here, we investigated, under *in vitro* controlled conditions, the effects of fungicides used to control an important belowground pathogen *R. solani*, on *R. irregularis* associated to potato plants. Moreover, the effects of mechanical or chemical (e.g. diquat) defoliation or the worldwide most used systemic herbicides glyphosate, used to defoliate crops or cover crops or control weeds, was evaluated on *R. irregularis* and *R. intraradices*. We further conducted field experiments to identify technical itineraries (e.g. cover crop co-inoculation) that improve AMF development and performance in potato crop. Finally, molecular tools to trace AMF in potato field were designed and assayed in greenhouse and under field conditions.

Four central questions were addressed:

Do fungicides used to control *Rhizoctonia solani* impact the non-target arbuscular mycorrhizal fungus *Rhizophagus irregularis* MUCL 41833?

With the easy-to-use, reproducible AMF *in vitro* culture system developed by Voets *et al.* (2009) we could evaluate the impact of fungicides conventionally used to control *R. solani* on *R. irregularis* associated to potato roots (**Chapter I**). Pencycuron, the contact and most *Rhizoctonia*-specific fungicide did not impact *R. irregularis* at the IC_{50} of *R. solani*, whilst the systemic fungicide flutolanil had an apparant effect on root colonization, possibly because of accumulation of the fungicide in the root zone. Azoxystrobin, known to have a lower systemic activity than flutolanil, did not affect intraradical AMF development but affected the extraradical development at doses above the IC_{50} of *R. solani.* Spore germination was not impacted by the three tested fungicides at the IC_{50} for *R. solani*, but only at higher concentrations. This results suggested that fungicides at adequate dosage to control *R. solani* or decrease its impact on potato may be combined with the beneficial fungus *R. irregularis*. However economical and environmental sustainability of this combination should be evaluated as well as proper technical itineraries for their application. The combined use should not increase input costs but rather reduce total fertilizer and pesticide costs and avoid the apparition of resistances in the pathogens.

Not only fungicides but also other beneficial microorganisms or bioactive molecules should be screened for compatibility with AMF. This was for example already investigated by De Jaeger *et al.* (2010) with *Trichoderma harzianum*.

However, the effects or compatibility could be different following the life history strategy of AMF strains (e.g. Jin *et al.* (2013)). Consequently, these compounds should also be evaluated in the same conditions with strains having different life history strategies. Moreover, semi-controlled greenhouse and microcosm field trials have shown that agricultural inputs could cause a shift in AMF diversity (e.g. Jin et al., 2013) supposing that some species are more sensitive than others to these compounds or microorganisms or that some strains have developed resistance mechanisms to these compounds to the detriment of their beneficial effect. In consequence, metagenomic studies should be conducted in field trials with distinct agricultural factors evaluated over the long-term, and health and yield characteristics of the crop should be assessed.

Is a systemic herbicide (i.e. glyphosate) more detrimental to the obligate root symbionts *R. irregularis* or *R. intraradices* than chemical (i.e. diquat) or mechanical defoliation?

The easy-to-use, reproducible AMF *in vitro* culture system developed by Voets *et al.* (2005) was used to study the impact of mechanical and chemical defoliation in comparison to systemic herbicide applications on spore production of *R. irregularis* and *R. intraradices* (**Chapter II**). This study suggested a clear and general decrease of spore production rate of *R. irregularis* and *R. intraradices* after chemical defoliation and systemic herbicide application as previously observed with mechanical defoliation. But, a difference of effect was revealed between defoliation and systemic herbicide application. The systemic herbicide (Glyphosate-formulation) had a faster and stronger effect than chemical (Diquat-formulation) or mechanical defoliation. It was suggested that glyphosate application impacted directly the root part of the plant because of its rapid

translocation to the roots (Shaner, 2006). The C flow from the root to the AMF was thus possibly directly interrupted, but studies measuring plant to fungus C-transport after systemic herbicide application in comparison to defoliation treatments should be performed to confirm this hypothesis. Moreover the release of glyphosate via the roots (Neumann *et al.*, 2006) could have impacted the ERM (i.e. spores) of AMF. But, studies measuring glyphosate concentration in the medium should further test this hypothesis. In contrast, defoliation inhibit only plant photosynthetic production (by eliminating or drying foliar part of the plant) but do not impact the roots. However, the chemical (Diquat-formulation) and mechanical defoliation affected spore production nearly as severely as the systemic herbicide (Glyphosate-formulation) six weeks after defoliation. We know that sporulation of *Rhizophagus* sp. follows a lag, log and plateau phase (Declerck *et al.*, 2001). The observations suggested that the time of defoliation may play an important role on AMF spores population. Defoliation or systemic herbicide applications of plants in the plateau phase of spore production should be less detrimental to AMF and spore population than defoliation in the lag and log phase.

The results obtained in **chapter II** indicate that crop defoliation or systemic herbicides applied on crops or weeds could be a threat to AMF establishment in agroecosystems if AMF are not in the plateau phase of sporulation. Not all AMF species respond in the same way to C-limitations. Therefore, further studies with species with other life history strategies should be performed as done by IJdo *et al.* (2010) after mechanical defoliation. Moreover, systemic herbicides that translocate through the roots and release in the soil seem to impact faster and stronger AMF growth. But the mechanism involved remains to be discovered. In addition other herbicides with different

absorption and translocation properties may be tested and in particular those that translocate through the roots.

In vitro studies give incomplete results and therefore the impact of defoliation and systemic herbicides on AMF should also be evaluated under greenhouse or field conditions because the mycorrhizosphere is an important part of plants coping with toxins.

Is the application of *R. irregularis* combined with *T. harzianum* beneficial to potato crops and what are the more pertinent agricultural itineraries to increase potato yield in presence of the microbial inoculants?

In the field experiments conducted in **Chapter III** inoculating a *Medicago sativa* cover crop with *R. irregularis* in combination with *T. harzianum* before potato plantation increased significantly potato yield in two different potato cultivars (i.e. Bionta and Sarpo Mira). This was mainly attributed to improved AMF colonization of the potato plants. But this strategy need to be further explored and other cover crops such as vetches or clover – more common in North-Europe – should be assessed. In contrast non-mycorrhizal cover crops such as mustard – cover crop common in North-Europe – should be avoided or intercropped with a mycorrhizal crop. Polyculture cover crops should also be assessed. Indeed, it has been shown that increasing diversity of plants, increase the diversity of AMF in soil. In most agroecosystems, AMF occurrence are to low or not efficient and an overwintering cover crop alone instead of bare soils to enhance native AMF does not have always a significant effect on yield of cash crop. Inoculation is thus necessary. In the same way Rhizobia are commonly inoculated on legumes. But persistence of these microorganisms should be evaluated.

Direct inoculation at potato plantation also increased potato tuber production for cultivar Bionta, but only significantly on the % of potatoes > 30 mm. The difference between inoculation via a cover crop or direct inoculation could be explained by a better establishment potential of the strain in agro-ecosystems via the cover crop. In our study, beads were placed on the potato tuber but the distance with the roots was probably not optimal for good establishment. The distance of the inoculum from the host roots is crucial for successful inoculation and need to be assessed. We hypothesize that the first large roots produced by reserves from the tuber, are less suitable to be colonized by AMF than secondary roots that are at a higher distance from the tuber. Pot experiments should thus be established to determine wich roots are colonized by AMF to determine the best place to put the inoculum. But, for crops with a short cycle such as potato, presence of well-established propagules (via cover crop inoculation) is supposed to be more effective. Potato pre-inoculation before planting is another strategy that need to be considered.

The advantages of entrapping fungal inoculants into alginate beads is their protection from external factors (e.g. attack of other microorganisms, soil environment (Oehl *et al.*, 2010)) which make them adapted to conservation and mechanical application into the field. However, inoculants (formulation and microorganism production) should not exeed costs of common fertilizers and pesticides. Combined products containing *R. irregularis* with *T. harzianum* has been shown to increase plant growth. Other microorganisms such as mycorrhiza helper bacteria (MHB) are also known to stimulate AMF (Frey-Klett et al., 2007) and need further to be explored to enhance microbial inoculants efficacy. But combined

inocula are not always easy to deposit for commercial use in particular when they harbor different legislations. For example, *T. harzianum* is more considered as a biopesticide in contrast to *R. irregularis* that is considered as a biofertilizer even if some studies show that *T. harzianum* also stimulate plant growth and *R. irregularis* also enhance plant resistance to pathogens. Deposit of combined products is thus complex because they have to complete with both legislation requirements. Therefore, the principal function of the product should be determined even if others can be performed. But a clear distinction shall be posed in the legal provision among the possible claims advertised by the product on the label.

The AMF inoculated in this study, *R. irregularis* is a worldwide distributed AMF, widely used for commercial applications owing to its easy mass-production (IJdo *et al.*, 2011) and life history strategy adapted to agricultural soils (Chagnon *et al.*, 2013). Strain MUCL 41833, originates from soil of Canary Islands with a subtropical climate. Even if this strain grow well under controlled temperate conditions (70% relative humidity – 22°/18°C (day-night)) (De Jaeger *et al.*, 2011). it is possibly not well adapted to our regions. In consequence, native strains should be sampled in our regions. Moreover, metagenomics studies should identify AMF diversity in regional potato crop areas.

In addition, future studies need to be conducted to screen the best AMF-potato associations and breeders should select cultivars with higher AMF dependencies. Hence, greenhouse studies with different cultivars should be conducted in order to determine their mycorrhizal dependency, which is the degree to which a plant relies upon the mycorrhizal condition to produce its maximum growth or yield at a given level of soil fertility (Plenchette *et al.*, 1983). Nutrient

uptake efficience should also be evaluated in parallel to mycorrhizal dependency. Studies with as objective to find strategies for a more integrated nitrogen fertilization in potato crop observed that in most cases cultivar Nicola makes better use of nitrogen fertilization than Bintje (Goffart, 2002). These observations could maybe be explained by the effect of AMF because our results show that Nicola is more colonized by AMF than Bintje, probably in parallel with a cultivar genetic effect. Breeding for better symbiosis (Rengel, 2002) could thus also be a good technical itinerary to enhance population densities and efficacy of AMF.

How can we trace and quantify inoculated *R. irregularis* MUCL 41833 in potato crop and distinguish from native AMF?

MtLSU of *R. irregularis* MUCL 41833 was sequenced and haplotype- and species-specific primers were designed. The markers were validated on potato plants inoculated with *R. irregularis* MUCL 41833 grown under greenhouse conditions. The molecular quantification by Real-Time qPCR seemed to match with the AMF root colonization stages evaluated by microscope observations. As earlier observed (Börstler *et al.*, 2008, Raab *et al.*, 2005) the mtLSU appeared a good region to distinguish different haplotypes of the same species what was not possible with nrLSU or microscopic observations. However, two strains of the six *R. irregularis* strains of GINCO presented the same mtLSU fragment as *R. irregularis* MUCL 41833 sequenced. The mtLSU region sequenced was in terms of base pairs length very limited (± 3.000 bp) in comparison to the whole mitochondrial genome (± 80.000 bp). Sequencing the whole mitochondrial genome could give more possibilities to find intra– or interspecific regions. Formey *et al.* (2012), for example, used next generation sequencing (NGS) approaches (454 pyrosequencing and

Illumina technologies) to sequence whole mitochondrial genomes from total genomic DNA of strains from the same or different species. But also the use of probes in addition to the primers could give more possibilities to find specific primers without dimer formation. Moreover, designing primers for other AMF species is necessary and should be done in parallel to *in vitro* culture development that is the only source of contaminant-free DNA.

But now further studies should be performed with designed primers to evaluate persistence and dynamics of field introduced species. How is the fitness of inoculated strains in the long-term? Is inoculation each year necessary? In contrast a possible risk of inoculation is that introduced strains take the place of native strains and thus reduce diversity.

In conclusion, the application of AMF within potato fields is desirable and offers many opportunities to decrease/adapt the application of pesticides and fertilizers for a more sustainable potato production. Some agricultural factors such as the use of microbial inoculants at cover crop sowing before potato crop seems a realistic and promising option to increase yield. Similarly, some pesticides frequently used to control root pathogens in potato seems not detrimental at reduced field dosages on AMF and may potentially been used in combination with AMF. Moreover, defoliation or herbicide applications are suggested to be less detrimental to *Rhizophagus sp*. in the plateau phase of sporulation than in the lag or log phase. However, systemic herbicide applications that could have a close contact with AMF via the plant should be avoided. Finally, to link enhanced yield and disease control to introduced AMF strains, Real-Time qPCR of mtLSU is a good tool to trace and quantify

introduced or native strains in the field. However, further studies are necessary to find the best combinations of Potato - AMF and it seems important to validate them under different cropping itineraries.

IX. OVERVIEW OF THE SCIENTIFIC ACHIEVEMENTS

Scientific publications

Published

1. Buysens C., Dupré de Boulois H., Declerck S. (2014) Do fungicides used to control *Rhizoctonia solani* impact the non-target arbuscular mycorrhizal fungus *Rhizophagus irregularis*? Mycorrhiza DOI 10.1007/s00572-014-0610-7

Submitted

1. Buysens C., Schtickzelle N., Declerck S. (2015) Glyphosate impacts sporulation dynamics of *Rhizophagus irregularis* and *Rhizophagus intraradices* more severely than chemical or mechanical defoliation. Fungal biology

2. Buysens C., César V., Ferrais F., Dupré de Boulois H., Declerck S. (2015) Cover crop inoculation with *Rhizophagus irregularis* and *Trichoderma harzianum* increases potato yield. Applied soil ecology

In preparation

1. Buysens C., Alaux PL., César V., Huret S., Cranenbrouck S., Declerck S. (2015) Tracing native and inoculated *Rhizophagus irregularis* in three potato cultivars (Charlotte, Nicola and Bintje) grown under field conditions. Mycorrhiza

Conference participation

1. Buysens C., Dupré de Boulois H., Schtickzelle N., Declerck S. (6 – 11 January 2013) Impact of chemical and mechanical defoliation strategies on arbuscular mycorrhizal fungi associated to potato plants. 7th International Conference on Mycorrhiza – poster presentation, ICOM 7. New Delhi (India).

2. Buysens C., Vincent César, François Ferrais, Hervé Dupré de Boulois, Stéphane Declerck (6 – 11 July 2014) Inoculation of a cover crop with *Rhizophagus irregularis* and *Trichoderma harzianum* prior to potato plantation increases potato yield – oral presentation, EAPR. Brussels (Belgium).

Teaching

1. International Training on *in vitro* Culture of Arbuscular Mycorrhizal Fungi (3 – 8 June 2012): Training organizer + *Host preparation* session. Louvain-la-Neuve, Belgium

2. International Training on *in vitro* Culture of Arbuscular Mycorrhizal Fungi (26 – 31 May 2013): Training organizer + *HAM-P system preparation* session. Louvain-la-Neuve, Belgium.

3. International Training on *in vitro* Culture of Arbuscular Mycorrhizal Fungi (18 – 23 May 2014): Training organizer + *spore disinfection* session. Louvain-la-Neuve, Belgium.

Student supervision

1. Nyssens Thiago (September 2011 - Septembre 2012). Détermination des densités de populations de CMA dans les systèmes bananiers de Martinique.

2. Tankoano Yentema Priscile (May 2012 - August 2012). Etude de l'impact d'une defoliation chimique et mécanique et d'un désherbant chimique sur le développement d'un champignon mycorhizien à arbuscules (CMA).

3. Demortier Marion (September 2013 - Septembre 2014). Effets d'une bactérie promotrice de croissance et d'un champignon

mycorhizien à arbuscules sur la croissance et le rendement de la pomme de terre au champ.

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