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**Does the arbuscular mycorrhizal fungus
Rhizophagus irregularis mitigate late blight in potato
plants?**

Thèse de doctorat présentée par **Pierre-Louis ALAUX** en vue de
l'obtention du grade de Docteur en Sciences agronomiques et
ingénierie biologique

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List of abbreviation

ACP	Acyl Carrier Protein
AFLP	Amplified Fragment Length Polymorphism
AM	Arbuscular Mycorrhiza
AMF	Arbuscular Mycorrhiza Fungi
ANOVA	Analysis of variance
As	Arsenic
ATP	Adenosine TriPhosphate
AUDPC	Area Under Disease Progress Curve
Avr-R	Plant Avirulent R gene
AzA	Azelaic Acid
BAS	Branched Absorbing Structure
BCAs	Biological Control Agents
BCCM	Belgian Coordinated Collections of Microorganisms
BTH	Benzo (1, 2, 3) thiadiazole-7-carbothioic acid
C	Carbon
Cd	Cadmium
CMN	Common Mycorrhizal Network
CO4	Chitotetraose
CO5	Chitopentaose
Cq	Quantification Cycle
CRA-W	Centre de Recherche Agronomique- Wallon
Cu	Copper
DA	Diterpenoid
DAOM	Canadian National Mycological Herbarium formerly Department of Agriculture, Ottawa, Mycology
DAPG	2, 4-DiAcetylPhloroGlucinol
DGA	Direction générale opérationnelle de l'Agriculture, des ressources naturelles et de l'environnement
DNA	Deoxyribose Nucleic Acid
dpi	Days post inoculation
ECM	Ecto-Mycorrhiza fungi
ELIM	Earth and Life Institute Applied Microbiology
EPA	Environmental Protection Agency
ER	Endoplasmic Reticulum
ERM	Extra-Radical Mycelium
ES	Ecosystem Services
ET	Ethylene
ETI	Effector Triggered Immunity
ETS	Effector Triggered Susceptibility

FA	Fatty Acids
FAO	Food and Agriculture Organization
G3P	Glycerol-3-Phosphate
GAs	Gibberellic Acids
GINCO-BEL	Glomeromycota In vitro Collection Belgium
GINCO-CAN	Glomeromycota In vitro Collection Canada
GlcNAc	N-acetylglucosamine
GPAT	Glycerol-3-Phosphate Acyl Transferase
Gpi	Glucose-phosphate isomerase
HAM-P	Half-closed Arbuscular Mycorrhizal-Plant
HC	Hyphal Compartment
HR	Hypersensitive Response
INVAM	International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi
IPM	Integrated Pest Management
IRM	Intra-Radical Mycelium
ISR	Induced Systemic Resistance
ISR	Induced Systemic Resistance
ITS	Internal Transcribed Spacers
JA	Jasmonic Acid
LPS	Lipopolysaccharides
LSU	Large Sub-Unit of RNA polymerase
LysMs	Lysin Motifs
MAMP	Microbe Associated Molecular Patterns
MDP	Mycelium Donor Plant
MeSA	Methyl ester of Salicylic Acid
MIR	Mycorrhiza Induce Resistance
MPK	Mitogen-activated Protein Kinase
MPN	Most Probable Number
mRNAs	messenger Ribo-Nucleic Acid
MS	Murashige and Shoog
MSR	Modified Strullu-Romand
MST	Mono-Saccharide Transporter
MT	Mating Type
MtLSU	Mitochondrial Large Sub-Unit
MUCL	Mycothèque de l'Université catholique de Louvain
Myc-LCOs	Mycorrhizal-LipoChitoOligosaccharides
N	Nitrogen
NADH	Nicotinamide Adenine Dinucleotide
NBCs	Nature-Based Compounds
NB-LRR	Nucleotide Binding and Leucine Rich Repeat

NGS	Next Generation Sequencing
NH ₄	Ammonia
nr	nuclear
P	Phosphorus
PAHs	Polycyclic Aromatic Hydrocarbon
PAI	Peri-Arbuscular Interface
PAM	Peri-Arbuscular Membrane
PAMP	Pathogen Associated Molecular Patterns
Pb	Plumb
PDA	Potato Dextrose Agar
Pep	Peptidase
PGPF	Plant Growth-Promoting Fungi
PGPR	Plant Growth-Promoting Rhizobacteria
Pi	Phosphorus
Pip	Pipecolic acid
PPA	Pre-Penetration Apparatus
PPF	Photosynthetic Photon Flux
PR	Pathogen Related
PRRs	Pattern Recognition Receptors
PTI	Pattern Triggered Immunity
qPCR	Real Time quantitative Polymerase Chain Reaction
QTLs	Quantitative Trait Loci
R	Gene-based Resistance
rAUDPC	relative Area Under Disease Progress Curve
RC	Root Compartment
rDNA	nuclear DeoxyriboNucleic Acid
RLKs	Receptor-Like Kinases
ROC	Root Organ Culture
ROI	Reactive Oxygen Intermediates
ROS	Reactive Oxygen Species
rRNA	ribosomal Ribonucleic Acid
RT	Reverse Transcription
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SBI	Sterol Biosynthesis Inhibitor
SE	Standard Error
SIS1	SL-Induced putative Secreted protein
SLs	Strigolactones
SMRT	Single Molecule Real Time
SNA	Single Nucleotide Analysis
SSRs	Sequence analysis and Single sequence Repeats

SSU	Small SubUnit
SyM	Symbiosis-related genes
TEs	Trace Elements
TF	Transcription Factor
UV	UltraViolet
VA	Vesicular Arbuscular
VOCs	Volatile Organic Compounds

Glossary

Arbuscular mycorrhiza (AM): *is the most ancient symbiosis and common mycorrhizal type. They are formed in an enormously wide variety of terrestrial host plants by obligatory symbiotic fungi which have recently been reclassified on the basis of DNA sequences into a separate fungal phylum, the Glomeromycota (Smith and Read, 2008).*

Biocontrol: *The control of one organism by another. Biocontrol agents used in plant productions are living organisms protecting plants against their enemies, i.e. reducing the population of pests or diseases to acceptable levels. Modes of action may include competition, antibiosis, parasitism and also induced systemic resistance which is mediated by the plant (du Jardin, 2015).*

Biofertiliser: *A biofertiliser is any bacterial and/or fungal inoculant applied to plants with the aim to increase the availability of nutrients and their utilization by plants, regardless of the nutrient content of the inoculant itself. Biofertilisers may also be defined as microbial biostimulants improving plant nutrition efficiency (du Jardin, 2015).*

Biostimulant: *A plant biostimulant shall be an EU fertilising product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: nutrient use efficiency, tolerance to abiotic stress, quality traits or availability of confined nutrients in the soil or rhizosphere. Regulation (EU) 2019/1009.*

Fertilising product: *means a substance, mixture, micro- organism or any other material, applied or intended to be applied on plants or their rhizosphere or on mushrooms or their mycosphere, or intended to constitute the rhizosphere or mycosphere, either on its own or mixed with another material, for the purpose of providing the plants or mushrooms with nutrient or improving their nutrition efficiency. Regulation (EU) 2019/1009.*

Common mycorrhizal networks (CMN): *The development of extra radical mycelium means that mycorrhizal fungi can form hyphal connections between the root systems of two or more host plants, forming 'common mycorrhizal networks' (CMNs) below ground between multiple plants (Gilbert and Johnson, 2017).*

Effector: “weapon” of a pathogen to manipulate its host; frequently a suppressor of PAMP- or MAMP-triggered immunity that, when recognized, induces hypersensitive cell death in the plant (Conrath, 2011).

Haplotype: a group of genes in an organism that are inherited together from a single parent (Cox et al., 2016).

Induced systemic resistance (ISR): type of systemic, broad-spectrum immunity in plants. Induced systemic resistance is elicited by colonization with beneficial microorganisms and depends on the plant hormones ethylene and jasmonic acid (Conrath, 2011).

Isolates: The first spore or pure isolation of a fungus from any place (Ainsworth, 2008).

Microbe-associated molecular pattern (MAMP): molecular signatures typical of whole classes of microbes. Their recognition plays a key role in innate immunity in plants and animals (Conrath, 2011).

Mycorrhiza induced resistance (MIR): During mycorrhiza formation, modulation of plant defense responses occurs, potentially through cross-talk between salicylic acid and jasmonate dependent signaling pathways. This modulation may impact plant responses to potential enemies by priming the tissues for a more efficient activation of defense mechanisms (Pozo and Azcón-Aguilar, 2007).

Obligate biotroph: An organism that is unable to complete a reproductive cycle in the absence of a living host (Parniske, 2008).

Pathogen-associated molecular pattern (PAMP): molecular signatures typical for potential microbial pathogens of a given host organism (Conrath, 2011).

Pathosystem: the combination of a specific host species and pathogen species (Poland et al., 2009).

Priming: sensitization of the whole plant for enhanced defense; characterized by a faster and stronger activation of cellular defenses upon invasion (Pieterse et al., 2014a).

Systemic acquired resistance (SAR): type of systemic, broad-spectrum immunity in plants. Systemic acquired resistance is induced by local contact with a MAMP, PAMP, or effector and depends on the plant hormone salicylic acid (Conrath, 2011).

Summary

Potato (*Solanum tuberosum*) is one of the most important food commodities worldwide. This crop is threatened by numerous pests and diseases, amongst which the most damaging is *Phytophthora infestans*, the causal agent of late blight. In organic farming, the most effective treatment to control this disease is copper. However, copper has a long remanence and may accumulate in soil over cropping seasons, leading to harmful effects on the environment and potentially affecting human health. Another approach, complementary or as a substitute for copper, is the use of biological control agents (BCAs) such as bacteria (e.g. *Bacillus* sp.) or fungi (e.g. *Trichoderma* sp., arbuscular mycorrhizal fungi (AMF)). Despite the undeniable effects observed with AMF on plant resistance against pathogens under controlled greenhouse conditions, few convincing results were obtained within the field and nearly none could attribute the observations to the fungal inoculant. In the present study:

- A tracing tool was first developed and field-tested with the standard AMF strain *Rhizophagus irregularis* MUCL 41833. Root colonization was high, and the inoculant detected by the partial mitochondrial genome (MtLSU).
- The impact of the AMF on late blight development was investigated with Bintje and Nicola over two cropping seasons. A delay and decrease of late blight symptoms were noticed during warm and dry (i.e. under low pathogen pressure) cropping season, while under wet conditions (i.e. strong pathogen pressure) no marked effects were noticed.
- Transfer defense signals from diseased to healthy plants was investigated *in vitro* in a system free from any unwanted microorganisms. The activation of defense related genes was noticed in healthy plants connected via a common mycelium network of *R. irregularis* MUCL 41833 to plants infected by *P. infestans* within 24 h after infection. Activated genes (*PR-1b*, *PAL*, *ERF3* and *LOX*) were from or involved in jasmonic acid (JA) or JA/ethylene (JA/ET) pathways.

These results provide a glimpse of the link between root colonization by a specific AMF inoculant and its effects on potato resistance to *P. infestans* under field conditions. They also raise

questions about the ecology of AMF and roles of CMN during late blight outbreak.

Keywords

Phytophthora infestans, late blight, *Rhizophagus irregularis* MUCL 41833, Arbuscular mycorrhiza fungus, *Solanum tuberosum*, potato, traceability, mycorrhiza induced resistance, common mycorrhiza network, interplant signaling, Real-Time quantitative PCR.

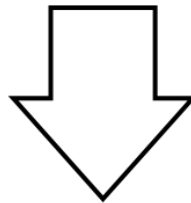
Outline of the thesis

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

1. Are molecular markers based on MtLSU specific to an inoculated AMF strain and can we link rate of roots colonization and molecular quantification made in greenhouse to field experiments?
2. Is *Rhizophagus irregularis* MUCL 41833 able to reduce late blight under field condition, following late blight outbreak and disease development?
3. Do hyphae connecting plants via a common mycelial network play a key role in the transmission of signals from diseased to healthy plants? What are the transcriptional changes in the healthy plants (i.e. the receiver) connected to a diseased plant (i.e. the donor) infested by *Phytophthora infestans* MUCL 54981

The successive steps to answer these questions are schematized:

Chapter I:	Chapter II:	Chapter III:
Tracing native and inoculated <i>Rhizophagus irregularis</i> in three potato cultivars (Charlotte, Nicola and Bintje) grown under field conditions.	Impact of <i>Rhizophagus irregularis</i> MUCL 41833 on disease symptoms caused by <i>Phytophthora infestans</i> in potato grown under field conditions.	Interplant signaling via common mycorrhizal networks might warn uninfected potato plants from late blight outbreak.



**General Discussion, Conclusion,
Perspectives,**

Annex I:
Selection of BCAs against *Phytophthora infestans* on cultivar Bintje

In the **introduction** section, we presented the subject and the general objective of the thesis.

In the **context of the Study** section, the state of the art was reviewed. In the first part, the state of knowledge on the AMF; from their life cycle to their beneficial effects for crops and impacts of agricultural practices on their development was addressed. In the second part, the pathosystem *Solanum tuberosum* / *Phytophthora infestans* was described as well as the methods currently used for late blight management. A third part was devoted to the plant defense mechanisms with presentation of the biotic stress recognition steps and plant defense dynamics. The involvement of genes or hormones in defense mechanism was reviewed. In a fourth part, the mechanism of mycorrhizal induced resistance was described. Finally, in a fifth part, the role of common mycelium networks for nutrients and defense signals transfer between plants was addressed.

In the **Materials and Methods** section, the biological materials were presented, and the most important techniques used in the thesis briefly described.

The objective of **Chapter I** was to characterize the MtLSU of the AMF strain *R. irregularis* MUCL 41833 used as inoculant in the field. It was the aim, in a first step, to validate the markers on inoculated potato plants grown under greenhouse conditions, linking the rate of root colonization with molecular detection. The markers allowed, in a second step, to evaluate field inoculation success by quantifying in potato roots the inoculant strain *R. irregularis* MUCL 41833 within the local AMF community (also hosting *R. irregularis* strains) colonizing the roots.

The results of Chapter I were published in Applied soil ecology (2017).

The objective of **Chapter II** was to determine whether *R. irregularis* MUCL 41833 was able to decrease late blight development in potato plants grown under field condition. Two experiments were conducted over two cropping seasons with Bintje and Nicola and results analyzed taking into consideration climatic conditions prevailing during the two trials.

The results of Chapter II were published in Crop protection (2018).

The objective of **Chapter III** was to study the role of AMF in the interplant transfer of defense signals from potato plants infected by late blight to disease-free potato plants. The experiment was conducted under strict *in vitro* culture conditions and gene expression and defense pathways analyzed in the disease-free plants.

The results of Chapter III were submitted to *Frontiers in plant science* (2019).

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

Finally, in the **Conclusion** and **Perspectives** section, the major outcomes of the thesis were reviewed and commented. Further studies were proposed to improve our knowledge on the mechanism involved in the late blight management by AMF and more precisely on the nature of CMN signals.

An **Annex** section was added to the thesis document on the role of fungal biological control agents (BCA), other than AMF, on *Phytophthora infestans*. Selection of the BCA started with an *in vitro* confrontation test, followed by two steps on plants, one *in vitro* and one in greenhouses.

Author's contribution

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

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

Chapter I is a research paper published in *Applied Soil Ecology* (2017). My contribution to this paper was estimated to 15%. Data analysis and writing of the manuscript have been done by Catherine Buysens 75% (PhD student at UCL Earth and Life Institute - Applied Microbiology - Laboratory of Mycology). The field trial was performed with the help of Vincent César (Engineer at the Walloon Agricultural Research Center – CRA-W) and Hugues Seutin (Technician at the Walloon Agricultural Research Center – CRA-W). The preliminary greenhouse experiment, part of the AMF-root colonization evaluation from field samples and the molecular marker validation on the field sample was performed by myself.

Chapter II is a research paper published in *Crop protection* (2018). My contribution to this chapter was approximately 90%. The experimental design, field trialing, data analysis and writing of the paper were conducted by myself. The field trial was performed with the help (monitoring late blight development) of Vincent César (Engineer at the Walloon Agricultural Research Center – CRA-W). Françoise Naveau (Technician at UCL Earth and Life Institute - Applied Microbiology - Laboratory of Mycology) helped for sample preparation. The data analysis with JmP software was performed with the help of Mailis Amico (“Support en Méthodologie et Calcul Statistique”, UCL).

Chapter III is a research paper submitted to *Frontiers in plant science* (2019). My contribution to this chapter was approximately 90%. The experimental design, data analysis and writing of the paper were conducted by myself. Françoise Naveau (Technician at UCL, Earth and Life Institute - Applied Microbiology - Laboratory of Mycology) helped for harvest, preparation of sample and qPCR run. The data analysis with JmP software was performed with the help of Rebecca Marion (“Support en Méthodologie et Calcul Statistique”, UCL).

Annex 1 is a research paper in preparation. My contribution to this chapter was approximately 90%. The experimental design, the planification and data collection (i.e. measure of mycelium growth using ImageJ, infection development) were performed by myself. The data analysis with JmP software was performed with the help of Catherine Rasse (“Support en Méthodologie et Calcul Statistique”, UCL). Writing of the paper was conducted by myself. Françoise Naveau (Technician at UCL Earth and Life Institute - Applied Microbiology - Laboratory of Mycology) helped for BCA strains maintenance

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

INTRODUCTION

Potato (*Solanum tuberosum*) is one of the most important crops worldwide. In Belgium, the cultivated area is estimated at 95k ha of arable land with a production in 2017 of 5.1 million tons (Fiwap, 2018). Unfortunately, this crop is threatened by numerous pests and diseases amongst which the most damaging is *Phytophthora infestans*, the causal agent of late blight (Haas et al., 2009). Late blight control measures are mostly based on agrochemicals, increasing cost for farmers to more than 1 billion €/year in Europe and 55 million € in Belgium (Haverkort et al., 2008; Vos and Kazan, 2016). These control measures are markedly impacting the environment and human health (Wilson and Tisdell, 2001; Beketov et al., 2013).

In organic farming, the primary treatment to control late blight is copper. However, copper has a long remanence, may accumulate in soil over cropping seasons and has a detrimental impact on below-ground organisms (Graham et al., 1986; Brun et al., 1998). In Belgium for instance, up to 16 treatments can be applied per season under conditions favorable to late blight outbreak (Haverkort et al., 2009).

In 1991, the European commission (Regulation No.2092/91 – (Regulation, 1991) limited the use of copper in organic farming to 6 kg/ha per year and in 2009, the EU directive 2009/128/CE – (directive, 2009) promoted the use of integrated pest management practices (IPM). IPM includes alternative approaches or techniques to control pests and diseases such as the application of nature-based compounds (NBCs) or biological control agents (BCAs). For instance, plant extracts, microorganisms such as *Trichoderma* spp., arbuscular mycorrhizal fungi (AMF) or *Bacillus* spp. have been shown effective against *P. infestans* or other *Phytophthora* species under laboratory or greenhouse conditions (Dorn et al., 2007).

Arbuscular mycorrhizal fungi are obligate biotrophs forming associations with an estimate of 72% of terrestrial plant species (Brundrett, 2018) among which potato. These organisms have been repeatedly reported to improve mineral (e.g. phosphorus) and water nutrition of plants (Smith and Read, 2008) as well as improving their resistance/tolerance to abiotic (e.g. drought, heavy metal) (Plouznikoff et al., 2016) and biotic (Whipps, 2004) stresses. Enhanced resistance/tolerance of AMF-colonized plants is not only due to a better

plant nutrition (Trotta et al., 1996; Hayek et al., 2014), but also involve the potentiation of plant defense mechanisms, called priming (Pozo and Azcón-Aguilar, 2007).

Priming induced by AMF may be local with notably the immunization of arbuscules-containing cells (Cordier et al., 1998) or systemic (Song et al., 2010; Ismail and Hijri, 2012) with the elicitation of defence genes in other plant parts such as shoots and leaves (Gallou et al., 2011). This mycorrhiza induced resistance (MIR) has been widely reported against root pathogens but to a lesser extent against leaf pathogens (Pozo and Azcón-Aguilar, 2007). For instance, Gallou et al. (2011) reported, under *in vitro* culture conditions, a decrease in disease symptoms caused by *P. infestans* on leaves of potato plants (var. Bintje) associated to the AMF *Rhizophagus irregularis*. Results under field conditions are far less numerous and more controversial, due among others, to the competition with the indigenous microorganisms (Berruti et al., 2015) and to harsh agricultural practices (e.g. ploughing, fungicides applications...) (Oehl et al., 2004; Avio et al., 2013) that could impact the establishment and development of the AMF inoculants.

The soil harbours an indigenous population of AMF and under field conditions most, if not all, plants are colonized by AMF. Therefore, to assess whether a fungal inoculant is adequately established in roots, potentially providing beneficial effects to plants, it is necessary to develop proper tracing tools. Unfortunately, the widely used genomic marker (i.e. ITS) are not efficient to discriminate AMF to the strain level. To identify strains of the same AMF species, the mitochondrial genome is a promising target (Badri et al., 2016) and the development of specific molecular markers for field application is particularly interesting with strains that have already shown promising result on *P. infestans* in controlled conditions (Gallou et al., 2011).

Interestingly, AMF have recently been reported to transfer signals from diseased to healthy plants via a network of hyphae connecting individuals, the so-named common mycorrhizal network (CMN). This mechanism of interplant signaling has been shown in pots, under conditions that could not discriminate the contribution of AMF from those of other microorganisms. The development of the mycelium donor plant donor (MDP) *in vitro* culture system (Voets et al., 2009) offers wide perspective to investigate the direct involvement of AMF hyphae in signal transmission.

In this thesis, our objective was to investigate the role of AMF on the disease caused by *P. infestans* in potato. Studies were conducted both *in vitro*, to investigate the molecular mechanisms in the hyphal transmission of signals between diseased and healthy plants, and within the field to assess the disease development in AMF-inoculated potato plants versus controls under organic agricultural practices. Molecular markers were developed to trace the AMF inoculants and disease evaluated over two contrasting growing seasons. Conclusions were drawn on the potential roles of AMF in the control of late blight and on the possible mechanisms involved.

STATE OF THE ART

I. Arbuscular mycorrhizal fungi

1. Introduction

Arbuscular mycorrhiza fungi (AMF) are worldwide distributed soil fungi, forming symbiosis with an approximate of 72% of terrestrial vascular plants including the vast majority of agricultural and horticultural crops (Brundrett and Tedersoo, 2018). These fungi have been found in plant fossils dating back 460 million years ago, suggesting their role in the colonization of terrestrial ecosystem by aquatic plants (Redecker et al., 2000). They are nowadays classified in a monophyletic phylum, the Glomeromycota (Schüßler and Walker, 2010; Redecker et al., 2013).

In the arbuscular mycorrhiza (AM) symbiosis (Fig. 1), the fungus develops an intraradical mycelium (IRM) within the roots and an extra radical mycelium (ERM) into the soil. Both are interconnected to form a wide mycelial network composed of countless coenocytic (i.e. without septa) hyphae, containing hundreds of nuclei sharing the same cytoplasm (Parniske, 2008). In its intimate association with plants, the fungus receive photosynthates (i.e. lipids and carbon) (Smith and Read, 2008; Keymer et al., 2017) in exchange for minerals (mainly phosphorus) and water (Smith and Read, 2008). This association often result in improved plant biomass/yield and increased resistance/tolerance to a/biotic stresses.

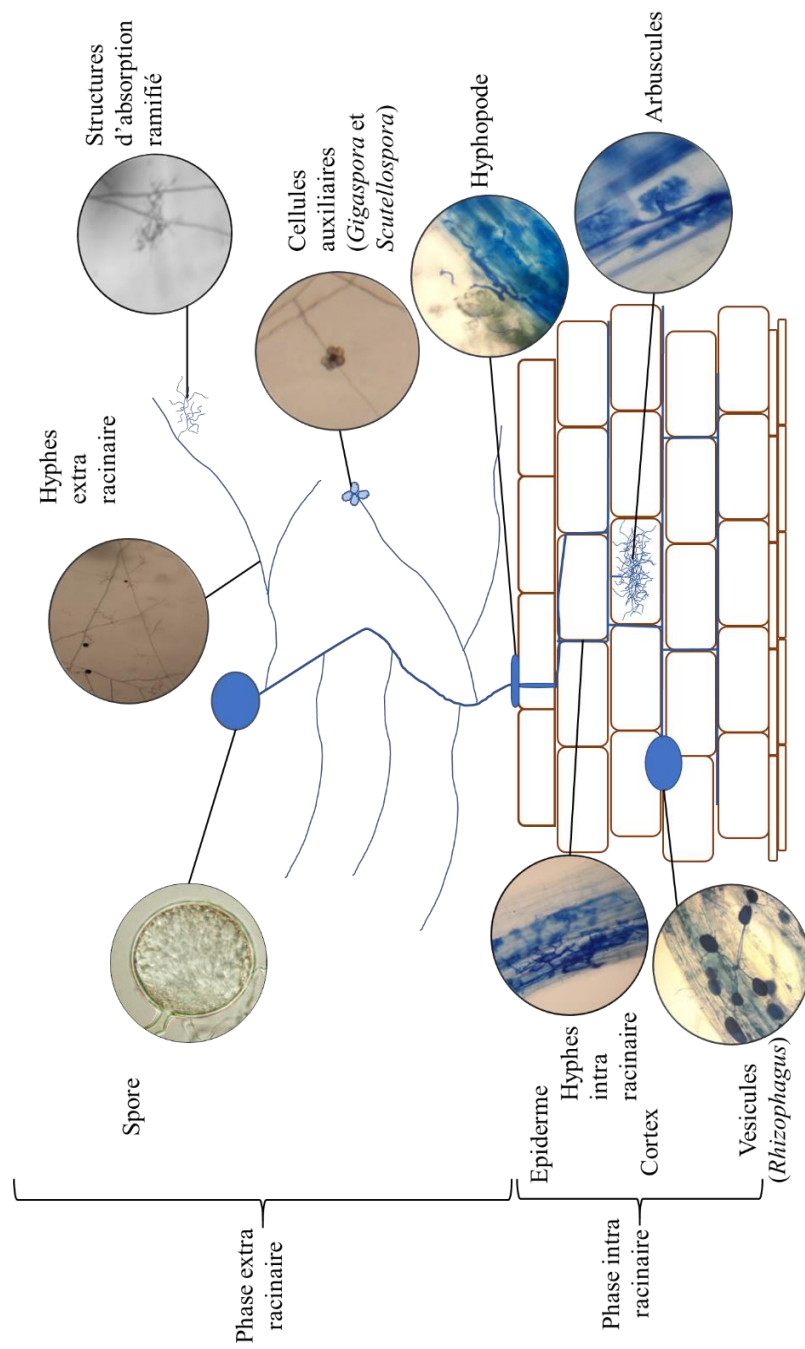


Figure 1. Arbuscular mycorrhizal fungus structures

2. The AMF life cycle

The primary sources of inoculum to initiate colonization are AMF-colonized root fragments, spores or vesicles, termed propagules. AMF hyphae extending from colonized living roots represents another important source of inoculum.

Starting from a spore (arbitrarily chosen as the propagule in the life cycle description of Fig. 2), several steps can be distinguished from pre-symbiotic communication, to late stage development with production of *de novo* spores.

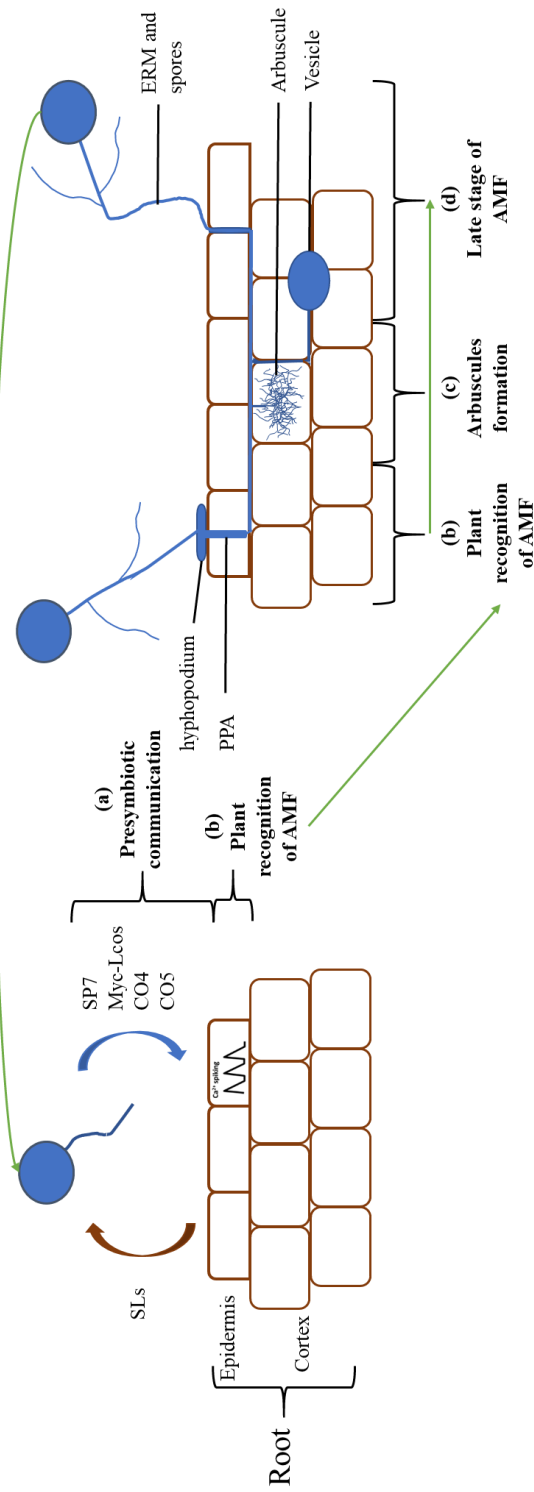


Figure 2. Schematic representation of life cycle of an arbuscular mycorrhizal fungus in presence of a suitable host plant starting from a spore. Several steps can be distinguished: (a) Presymbiotic communication, (b) Plant recognition of AMF, (c) Arbuscules formation, (d) Late stage. strigolactones (SLs), Secrete the protein 7 (SP7), chitotetraose (CO4) and chitopentaose (CO5), he mycorrhizal-lipochoitoligosaccharides (Myc-LCOs), pre-penetration apparatus (PPA), extra radical mycelium (ERM).

a. Pre-symbiotic communication: the role of root and fungal signaling molecules

Spore germination is stimulated by plant signaling molecules (Fig. 2), in particular by secondary metabolites such as carlactones and strigolactones (SLs) (Akiyama et al., 2005; Besserer et al., 2006; Mori et al., 2016). Strigolactones released by roots in the rhizosphere form a steep concentration gradient and therefore indicate the presence and position of suitable host roots (Parniske, 2008). Therefore, AMF possesses a highly sensitive perception system for SLs. For instance, concentrations as low as 10 nM of the synthetic model-SL GR24 has been shown to induce nuclear division, increase size of mitochondria and to stimulate the increase of NADH content, NADH dehydrogenase activity and ATP content in spore and germinating hyphae of *Gigaspora rosea* (Besserer et al., 2008). These modifications have been observed during spore germination, hyphal growth and extension prior to root colonization (Besserer et al., 2006; Gutjahr and Parniske, 2013). Upon perception of SLs (Fig. 2), the AMF release compounds that trigger the signaling transduction pathways that is required for symbiotic establishment (i.e. plant recognition of AMF as symbiotic partner) (Bonfante and Requena, 2011). For instance, *Rhizophagus irregularis* was reported to exudate SL-induced putative secreted protein (SIS1) into the rhizosphere upon SL treatment (Tsuzuki et al., 2016). Similarly, *Glomus intraradices* was shown to secrete the protein 7 (SP7) reducing the level of host defense responses (Kloppholz et al., 2011). Apart from these secreted proteins, AMF release oligosaccharide signals, known as Myc factors, which are somehow similar to the Nod-factors in the Rhizobium symbiosis (Denarie et al., 1996). Two Myc factors have been identified so far; the mycorrhizal-lipo-chitoooligosaccharides (Myc-LCOs) (Maillet et al., 2011) and short-chain chitoooligosaccharides (Bernaola et al., 2018), namely chitotetraose (CO4) and chitopentaose (CO5) (Genre et al., 2013). Perception of the Myc factor (Fig. 2) by the plant is mediated at the root cell surface by receptor-like kinases (RLKs) containing lysin motifs (LysMs) in their extracellular domain. RLKs are capable of binding with *N*-acetylglucosamine (GlcNAc) the basic component of chitin (Limpens et al., 2015; Zipfel and Oldroyd, 2017). Specific Myc Factor receptors have yet to be identified but calcium spiking has been measured in response to Myc factor (Choi et al., 2018).

b. Plant contact and colonization

After reciprocal recognition between the two partners, AMF hyphae form a “foot-like” attachment structures on the surface of the root epidermis (Fig. 2), named hyphopodium (Bonfante and Genre, 2008). Hyphopodium formation appears to be supported by host-derived cutin or cutin precursors (Wang et al., 2012). Hyphal contact with epidermis induces nuclear movement in root cells toward the fungus (Genre et al., 2005). To reach the inner cortex, the AMF first needs to cross epidermal and outer cortical cell layers. AMF penetration across epidermal cells (Fig. 3) is immediately followed by the transient formation of a novel intracellular organization comprising cytoskeletal and endoplasmic reticulum (ER) components (Genre et al., 2005). This assembly is named the pre-penetration apparatus (PPA). It includes a broad cytoplasmic bridge linking the site of fungal adhesion on the outer cell surface to the cell nucleus and defines the future intracellular path of hyphae penetration. This process also involves complex nuclear movements, including initial repositioning of the nucleus at the site of fungal adhesion and subsequent transcellular migration leading to the assembly of the PPA (Genre et al., 2008). Fungal hyphae enter the plant host cell, accompanied by high-frequency calcium spiking (Sieberer et al., 2012). The common Symbiosis (*SyM*) genes “does not make infection 2” (*dMi2* and *dMi3* are required for PPA induction and *dMi3* for a subset of genes induced during PPA formation (Genre et al., 2005).

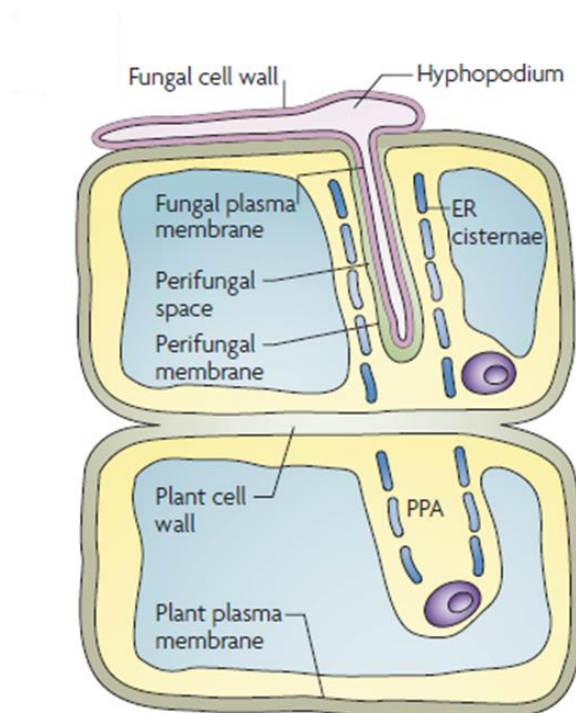


Figure 3. The prepenetration apparatus (PPA) is a cytoplasmic bridge across the vacuole of plant cell that are formed in anticipation of fungal infection (lower cell). The plant cell nucleus migrates ahead of the growing PPA and determines its orientation within the cell. The PPA contains a hollow tube that is formed by microtubules and is lined with endoplasmic reticulum cisternae. Only after completion of the PPA does a fungal hypha enter the PPA (upper cell) from (Parniske, 2008).

c. Arbuscules formation

Upon reaching the inner cortex, AMF develop highly branched, tree-shaped structures (Fig. 1 and Fig. 2), the arbuscules (Choi et al., 2018). Extensive cytological modifications are induced in cells containing arbuscules. The vacuole decreases in size as the relative volume of cytosol increases and cell organelles (mitochondria, plastids and endoplasmic reticulum) proliferate. Concomitant with arbuscules development and reactivation of the cytoplasmic compartment, the plant nucleus moves to the center of the cell and undergoes hypertrophy (Balestrini et al., 1992). There is evidence that such reorganization of host cell content requires synthesis of cytoskeletal components because it is accompanied by the transcriptional activation of a plant α -tubulin

gene in the colonized cells (Bonfante et al., 1996). The plant cell nucleus is characterized by enhanced fluorochrome accessibility, increased nuclease sensitivity and chromatin dispersion. All of these modifications reflect an increase in chromatin decondensation, which is a sign of greater transcriptional activity of the plant genome in the cells containing arbuscules (Berta et al., 1990). Within the inner cortex, the hypha develops and extends dichotomously. Concomitant with the development of intracellular fungal structures, the hosting plant cell envelops the growing fungus with the peri-arbuscular membrane (PAM), which is continuous with the cell membrane (Choi et al., 2018). Root colonization follows two distinct patterns: the Arum and Paris types (Fig. 3) (Smith and Read, 2008).

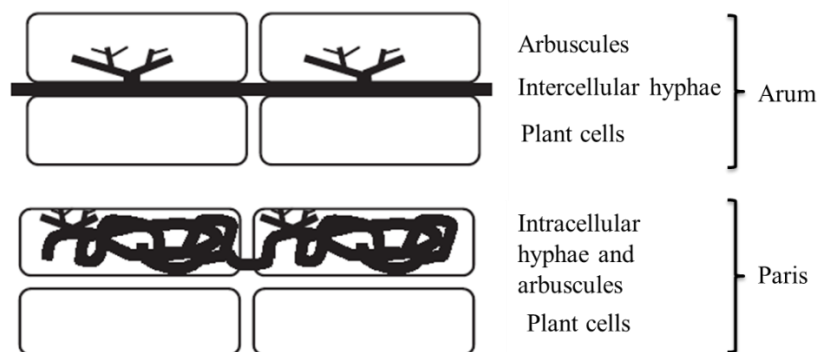


Figure 4. Stylized diagrams of colonization types: *Arum* and *Paris* from (Dickson, 2004)

In the *Arum* type (Fig. 4), intercellular hyphae grow longitudinally along the cells and penetrate each cell to differentiate into arbuscules. In the *Paris* type (Fig. 4), hyphae grow directly from cell to cell and the intercellular phase is restricted. Upon direct hyphal contact, the cortical cells respond with nuclear repositioning and localized cytoplasmic aggregations, as observed for the PPA formation. Inner cortical cell penetration is preceded by the assembly of large PPAs. Pre-branching cytoplasmic aggregations organize at sites along the intracellular hyphal trunk, preceding the development of the arbuscular fine branches (Genre et al., 2008). The size of the plant vacuole is reduced to accommodate the growing arbuscule, and the endoplasmic reticulum (ER), peroxisome, Golgi apparatus, nucleus, and plastids are densely compacted within the cytoplasm (Pumplin and Harrison, 2009). The PAM envelops the entire hyphae structure, thereby creating a large membrane surface area and peri-arbuscular

interface (PAI) for the exchange of nutrients and signals between the two organisms (Choi et al., 2018). Arbuscules have a short life span (i.e. a few days) and the collapse of branches seem to be synchronized (Alexander et al., 1988).

d. Late stage of AMF

In the late stage of a successful AMF colonization, AMF produce thick-walled lipid storage vesicles (Fig. 1 and Fig. 2), formed either within or between host cells, except for the Gigasporaceae that do not produce vesicles but auxiliary cells on the ERM. Similarly, asexual spores containing numerous nuclei and lipids are produced (Marleau et al., 2011). Extra-radical mycelium (Fig. 1) and branched absorbing structure (BAS) which are supposedly involved in mineral uptake as BAS and arbuscules seem to share several cytological characteristics: numerous nuclei, mitochondria, glycogen deposits, lipid globules, abundant polyvesicular bodies and electron-dense granules inside small vacuoles (Bago et al., 1998).

3. Plant-AMF exchanges and symbiosis control

Arbuscules are the main market place for exchange between plants (carbon) and AMF (nutrients) (Parniske, 2008). Via its ERM, the AMF acquire nutrients (principally inorganic phosphorus and nitrogen) from the soil, which are converted into polyphosphate granules for phosphorus (P) and in amino acid (mostly glutamate and arginine) for nitrate or ammonium. Both nutrients are hypothesized to be translocated along the hyphae and released in the PAI after hydrolyzation of the P and conversion of amino acids into ammonia (NH_4). From here, specific transporters ensure the transfer of nutrients across the PAM. Specific phosphate transporters from the MtPT4/OsPT11 type and possibly the MtAMT2;4 type take up the phosphate and ammonia, respectively, across the PAM (Choi et al., 2018). In exchange, the plant provides photosynthates to the AMF in the form of sugars and lipids. Sucrose coming from the plant are converted into hexose (principally glucose) by an invertase and then potentially transported by SUT or SWEET (direct sucrose transport) type transporter and released in the PAI (Casieri et al., 2013). From there, hexose is acquired by the AMF using monosaccharide transporters (MST). Regarding fatty acids (FA), AMF lack genes for long chain biosynthesis (encoding fatty acid synthase I subunits) and therefore are dependent on the plant for their supply

(Luginbuehl et al., 2017; Keymer and Gutjahr, 2018). The FA biosynthesis in PAM start by elongating C2:0-acyl carrier protein (ACP) to C4:0-ACP by FA synthase. The C4:0-ACP is then extended into C16:0-ACP. Acyl transferase releases C16:0 FA from acyl carrier protein (ACP) for subsequent export to the cytosol, where C16:0 FA is conjugated to Coenzyme-A for transport to the endoplasmic reticulum (ER). *RAM2* encodes a glycerol-3-phosphate acyl transferase (GPAT) and attaches C16:0-FA to the second carbon of glycerol-3-phosphate to make C16:0 monoacylglycerol (C16:0 β -MAG). PAM-localized lipid transporter candidates, *STR/STR2* release FA in the PAI. The FA provided by the plant are converted into triacylglycerol and used by the fungal metabolism (Choi et al., 2018).

The control of the symbiosis seems to be shared by both partners. On one hand, more “cooperative” fungi receive more carbon from the host and, on the other hand, fungi can allocate more phosphorus to host roots providing more carbon (Kiers et al., 2011). The control of the AM symbiosis involves a fine-tuned process, with pivotal role of several phytohormones, such as SLs, gibberellic acids (GAs), and auxin, involved in the modulation of AM symbiosis, through the early recognition of events up to the final stage (Liao et al., 2018).

4. Taxonomy, phylogeny and traceability of AMF

a. First step of classification

Historically, AMF identification relied primarily on the examination of spore characteristics. However, spores offer only a few discriminating characters (e.g. size, color) that are often influenced by the environment (Krüger et al., 2009). Moreover, some species in *Glomus* or *Acaulospora* have dimorphic spores (i.e. that shows two types of morphologies) making their identification even more complex (Rosendahl, 2008). Furthermore, if spores wall structure may appear simple, this is not always the case. For instance, *Scutellospora* species often have several inner membranous walls and a complex germination shield. Identification bias may also occur depending on how the preparation is made or on the age of the spore. Indeed, observation of the number of walls depends more on how hard you press the cover slip for microscopic observations than on the actual taxonomic identity (Rosendahl, 2008).

The first key to classification was based on descriptive characters and color of spores (Mosse and Bowen, 1968). Because of the superficial similarities of the spores with those of *Endogone*, the species belonging to the genus *Glomus* were first classified within the *Endogonaceae* (*Zygomycota*) (Trappe and Gerdemann, 1974).

b. Molecular classification

Since the last two decades, molecular techniques have been used in addition to the morphological methods for species identification and classification. In 2001, Schüßler et al. (2001) proposed the phylum *Glomeromycota* for these fungi, based on the small subunit (SSU) rRNA analyses, and in 2010, a revision of the *Glomerales* was published by Schüßler and Walker (2010), resulting in name modifications. For instance, one of the more widely used AMF species (*Glomus intraradices*) was renamed *Rhizophagus irregularis* (Stockinger et al., 2009; Tisserant et al., 2013). Recently, the classification of AMF was revisited by Spatafora et al. (2016). These authors proposed to place the AMF in a sub-phylum (i.e. *Glomeromycotina*) in the *Mucoromycota* phylum. However, this classification has been challenged by a more inclusive study of Tedersoo et al. (2018) and the *Glomeromycota* phylum was resurrected. Indeed, Spatafora et al. (2016) analyzed 25 genome including one AMF, whereas Tedersoo et al. (2018) analyzed 111 genome with at least two taxa from each phylum.

The current classification (Fig. 5) of the AMF comprises 1 phylum, 4 orders, 12 families, 34 genera for a total of 333 species (Schüßler and Walker, 2010; Redecker et al., 2013; Wijayawardene et al., 2018). Despite their worldwide distribution, only 333 species have been described so far, January 2020 (http://www.amf-phylogeny.com/amphylo_taxonomy.html). This low number of taxa is most probably largely underestimated (Senés-Guerrero and Schüßler, 2016) in regard to the % of plants hosting AMF. The democratization of the sequencing technics will surely help to identify new species.

order (4 in total)	family (12 in total)	genus (36 in total)	generic type	generic type basionym
Archaeosporales	Ambisporaceae	Ambispora	A. fennica	Ambispora fennica
	Archaeosporaceae	Archaeospora	A. trappei	Acaulospora trappei
	Geosiphonaceae	Geosiphon	G. pyriformis	Geosiphon pyriformis
Glomerales	Claroideoglomeraceae	Claroideoglomus	C. claroideum	Glomus claroideum
	Glomeraceae	Dominikia	D. minuta	Glomus minutum
		Funneliformis	F. mosseae	Endogone mosseae
		Funneliglomus	F. sanmartinense	Funneliglomus sanmartinense
		Glomus	G. macrocarpum	Glomus macrocarpum
		Halonatospora	H. pansihalos	Halonatospora pansihalos
		Kamienskia	K. bistrata	Glomus bistratum
		Microdominikia	M. litorea	Dominikia litorea
		Microkamienskia	M. peruviana	Microkamienskia peruviana
		Nanoglomus	N. plukenetiae	Nanoglomus plukenetiae
		Oehlia	O. diaphana	Glomus diaphanum
		Orientoglomus	O. emiratium	Dominikia emiratia
		Rhizophagus	R. populinus	Rhizophagus populinus
		Sclerocarpum	S. amazonicum	Sclerocarpum amazonicum
		Sclerocystis	S. coremioides	Sclerocystis coremioides
		Septoglomus	S. constrictum	Glomus constrictum
Diversisporales	Acaulosporaceae	Acaulospora	A. laevis	Acaulospora laevis
	Diversisporaceae	Corymbiglomus	C. corymbiforme	Corymbiglomus corymbiforme
		Diversispora	D. spurca	Glomus spurcum
		Desertispora	D. omaniana	Diversispora omaniana
		Otospora	O. bareai	Otospora bareai
		Redeckera	R. megalocarpa	Glomus megalocarpum
		Sieverdingia	S. tortuosa	Glomus tortuosum
		Tricispora	T. nevadensis	Entrophospora nevadensis
	Gigasporaceae	Bulbospora	B. minima	Bulbospora minima
		Cetraspora	C. gilmorei	Gigaspora gilmorei
		Dentiscutata	D. nigra	Gigaspora nigra
		Intraornatospora	I. intraornata	Racocetra intraornata
		Gigaspora	G. gigantea	Endogone gigantea
		Paradentiscutata	P. bahiana	Paradentiscutata bahiana
		Racocetra	R. coralloidea	Gigaspora coralloidea
		Scutellospora	S. calospora	Endogone calospora
	Pacisporaceae	Pacispora	P. scintillans	Glomus scintillans
	Sacculosporaceae	Sacculospora	S. baltica	Entrophospora baltica
Paraglomerales	Paraglomeraceae	Innospora	I. majewskii	Paraglomus majewskii
		Paraglomus	P. occultum	Paraglomus occultum
	Pervetustaceae	Pervetustus	P. simplex	Pervetustus simplex
unclear	insertae sedis	Entrophospora	E. infrequens	Glomus infrequens

Figure 5. Classification of Glomeromycota in October 2019 from http://www.amf-hylogeny.com/amphylo_taxonomy.html

c. International culture collection

One of the major flaws of the early classification was the conservation of material. Specimens in herbarium were often unavailable or with taxonomic traits unusable, making these type materials inadequate for species comparison (Rosendahl, 2008). Well characterized and managed cultures are indeed required for accurate identification. In 1985 a first international culture collection was erected in the US (i.e. the International Culture Collection of VA

Mycorrhizal Fungi – INVAM, <https://invam.wvu.edu/>), followed in 1994 by its European counterpart (i.e. the Banque Européenne des Glomales BEG, renamed the International Bank for the Glomeromycota, <http://www.i-beg.eu/>) and in 2001 by the Glomeromycota *In vitro* Collection hosted jointly in Belgium (GINCO-BEL, <http://www.mycorrhiza.be/ginco-bel/>) and in Canada (GINCO-CAN, http://res2.agr.gc.ca/ecorc/ginco-can/index_e.htm), the first and unique collection of AMF cultured *in vitro*. These collections allowed the long-term conservation and establishment of reference culture and are nowadays used by the international community.

d. Markers for phylogenetic analyses

Different molecular markers have been used to identify and classify AMF species. The selection of these markers is critical as the divergence in one locus may give an evolution history only of the locus, not of the species. Indeed, different genes may be subject to different mutation rates. The phylogenetic trees are also sensitive to sampling bias and therefore requires different samples from a wide range of areas (Rosendahl, 2008). Classically, the regions of ribosomal DNA sequence, as the internal transcribed spacers (ITS) are used for the identification of fungi but are more problematic for AMF. Indeed in AMF, ITS sequence exhibit within-spores variability and therefore was considered polymorphic (Sanders et al., 1995). For instance, up to 23 different sequences of rDNA have been found in single-spore isolates of *Glomus coronatum* (Clapp et al., 2001). Furthermore, ITS sequences within a single spore of one isolate of *Glomus intraradices* were as divergent as sequences from different isolates (Jansa et al., 2002b). This region is thus unsuitable as species specific markers (Croll et al., 2008) but can be used to measure impact on family distribution induced by ecological shifts and interactions across the kingdom fungi (Lekberg et al., 2018). Other parts of the nuclear ribosomal genes (rDNA) have been used such as the partial Small Subunit (SSU). However, this marker (e.g. NS31/AM1) does not cover the full AMF diversity (Redecker et al., 2003). The short partial SSU sequences does not resolve species of AMF, and even full-length SSU sequences fail to resolve closely related species (Redecker et al., 2013). Markers from the Large subunit of RNA polymerase (LSU) showed very low intra-isolate variation between two clones of *G. mosseae*. The LSU marker was used and allowed separation of almost all morpho-species tested, with the exception of *Gigaspora* sp. (Stockinger et al., 2014). Amplified fragment length

polymorphism (AFLP) was used to identify *G. intraradices* isolates from the field (Koch et al., 2004). Krüger et al. (2012) developed a phylogenetic reference data for systematics and taxonomy of AMF. The phylogeny was based on the nuclear ribosomal genes (rDNA) and more precisely on the partial small subunit rRNA gene (SSU), the partial large subunit rRNA gene (LSU), 5.8S rRNA gene (5.8S) and ITS. Those markers allowed both robust phylogenetic analyses and species resolution. AMF isolates characterization or field monitoring are possible using those markers (Senés-Guerrero and Schüßler, 2016) using the Single Molecule Real Time (SMRT) method. This technique allows long sequence read and therefore AMF specific 1.5kb sequence could be covered (Schlaeppli et al., 2016). To increase resolution of the phylogeny up to strain level, mitochondrial marker have been proposed (Badri et al., 2016).

e. Traceability of AMF

Phylogenetic analyses require discrimination of taxa at the species level, but traceability may require discrimination at the strain level. Therefore, other standardized molecular diagnosis tools are needed (Badri et al., 2016), even if the SMRT method may allow traceability of fungal inoculant species (Schlaeppli et al., 2016) and under field condition primer, targeting a fragment of the RNA polymerase II gene (*RPB1*) was successfully used recently (Thioye, van Tuinen, et al., 2019). The use of mitochondrial genes avoids potential complications attached to the heterogeneous sequences with nuclear genes (i.e. ITS). Typical mitochondrial genomes comprise a single molecule of DNA, which is usually circular and contains all mitochondrial genes (Nadimi et al., 2016) and unique within each strain (Formey et al., 2012). The mitochondrial Large Subunit (MtLSU) is homogenous within a same isolate (clones of the same strain) and can separate isolates from different geographic origin (different strains) (Raab et al., 2005). MtLSU haplotypes markers have been used to differentiate MtLSU haplotypes directly from colonized roots (Börstler et al., 2008). “Molecular tool kits” developed by Badri et al. (2016) based on mitochondrial genome made it possible to link the rate of colonization with the quantitative detection of a specific strain of AMF by real time quantitative polymerase chain reaction (RT-qPCR). This method was developed on *in vitro* produced spores (Badri et al., 2016). It allow traceability of AMF strains (Badri et al., 2016). Moreover, Krak et al. (2012) was able to quantify coexistence and dynamics of two closely

related *G. intraradices* strains in roots, showing competition between them. Development of this approach is still in progress. Nowadays, 16 Mt genomes are fully sequenced covering 11 taxa from AMF species belonging to *Rhizophagus*, *Glomus* and *Gigaspora* (Nadimi et al., 2016). Exploration of mitochondrial genome lead to the identification of genes (i.e. *rnl*, *nad5*, *cox1*, and *nad2*) that are potentially suited for an accurate phylogeny of AMF (Nadimi et al., 2014; Nadimi et al., 2016). Strain level of traceability may use recent observation of putative mating type homologues in AMF genome. Mating type loci are multi allelic and offer a great variability opening the perspective of discrimination between closely related strains (Halary et al., 2013; Ropars et al., 2016).

II. Benefits and agronomic applications of AMF

1. Introduction

Conventional agriculture, resulting from the “first green revolution”, largely depends on pesticides and mineral fertilizers. However, inadequate applications of these chemicals may seriously affect the environment by polluting soils and water, decreasing biodiversity and in the worst cases affecting human health (Knapp and van der Heijden, 2018). The impact on soil biodiversity may affect major ecosystem services (ES - i.e. multiple resources and processes from natural and management ecosystems that benefits human society (Costanza et al., 1997)) offered by flora and fauna. These ES include among others, soil formation, nutrient cycling, biological control and food production (Costanza et al., 1997). Arbuscular mycorrhizal fungi also offer certain ES. They increase soil stability and water retention, improve plant mineral nutrition, buffer the detrimental effects of biotic and abiotic stresses, and increase food production (bioregulation of plant development and increase in plant quality for human health) (Smith and Read, 2008 ; Gianinazzi et al., 2010).

2. Ecosystem services provided by AMF to agriculture

a. Impact on plant nutrition

Arbuscular mycorrhizal fungi have been shown to increase the mineral content of plant hosts (Gianinazzi et al., 2010), increasing

notably phosphorus and zinc content in shoot and fruits in experiments conducted in pots (Cavagnaro et al., 2006) and within the field (Cavagnaro, 2008). Nutrients uptake by AMF-colonized plants follows two pathways (Fig. 6): a direct uptake via the root and an indirect uptake via the AMF (Bucher, 2007).

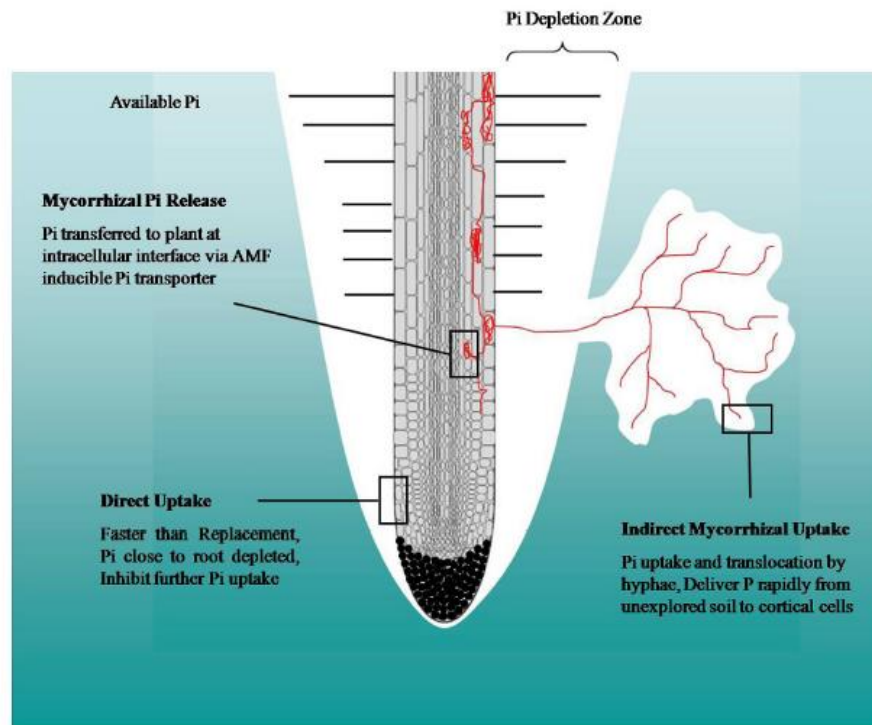


Figure 6. *Phosphorus (Pi) uptake by AMF-colonized plants following two pathways: the direct uptake via the plant roots and the indirect uptake via the AMF extra radical mycelium. This second pathway allows the plant to acquire Pi far beyond the rhizosphere (Johri et al., 2015).*

The ERM is able to take up various nutrients, in particular those with low mobility or sparingly soluble such as phosphorus (Marschner and Dell, 1994; Clark and Zeto, 2000). Arbuscular mycorrhizal fungi can decrease the nutrient depletion zone around the roots by extending the volume of soil explored far beyond the rhizosphere (up to 40 times) (Giovannetti et al., 2001). In addition, small diameter fungal hyphae (average diameter 3-4 μm) can penetrate soil pores inaccessible to root hairs (diameter $>10 \mu\text{m}$) (Clark and Zeto, 2000). Phosphorus direct acquisition by plants create a depletion zone immediately around the

roots (Li et al., 1991a). With white clover, this depletion zone was 10 mm for non-mycorrhized plants and more than 20 mm in plants colonized with *Glomus mosseae* (Li et al., 1991b). White clover associated with *G. mosseae* or *Acaulospora leavis* the soil phosphorus depletion zone was up to 110 mm and 80 mm, respectively (Li et al., 1991a).

Arbuscular mycorrhizal fungi possess a limited number of high affinity transporters expressed in intra and extra radical mycelium, (Garcia et al., 2016). Indeed, most fungal transporter transcripts were predominantly detected in ERM but also in intraradical mycelium and in cells containing arbuscules, suggesting a complex regulation (Casieri et al., 2013). Nutrients transporters of AMF have been identified for phosphorus, nitrogen, water, zinc, iron, sugars (di Fossalunga and Novero, 2019) and potassium (Courty et al., 2016). AMF induced transporters are notably essential for phosphorus uptake via the mycorrhizal pathway (Casieri et al., 2013).

Mycorrhizal symbiosis also plays a critical role for plant nutrients use efficiency in natural ecosystems and cultivated areas that are often characterized by nutrient limitations, especially with regard to nitrogen and phosphorus (Smith and Read, 2008; Verzeaux et al., 2017). Indeed, the C cost per unit absorptive area of AMF hyphae is approximately 10 times less than that of root hairs and about 100 times less than that of roots (Leake et al., 2004).

b. Impact on soil formation and water retention

Arbuscular mycorrhizal fungi develop within roots and extend into the soil as a complex and dense network of hyphae (Miller et al., 1995). This ERM represent between 20 and 30% of the total soil microbial biomass (Leake et al., 2004) and can account for up to 100 m of hyphae per cm³ of soil (Miller et al., 1995). Plants involved in AM symbiosis induce an increase of total soil carbon by 5 to 8% (Daynes et al., 2013). The ERM has been shown to fix and aggregate soil particles, increasing soil stability and thus reducing erosion (Tisdall and Oades, 1982; Mardhiah et al., 2016). The efficacy of AMF in soil aggregation is species-dependent. For instance, in a comparative study of three AMF species, i.e. *Septoglomus deserticola*, *Gigaspora gigantea* and *Rhizophagus irregularis*, the later was demonstrated to be the most effective to increase the percentage of stable aggregates in semiarid soils (Kohler et al., 2017). Interestingly, AMF produce and release a

hydrophobic “sticky” glycoprotein named Glomalin (Rillig et al., 2002). The direct effect of glomalin was much stronger than the direct effect of AMF hyphae themselves on the stabilization of soil aggregates, at least for the 1–2-mm size class of aggregates (Rillig et al., 2002). This glycoprotein accumulates within the soil and can represent as much as 5% of soil C and N (Treseder and Turner, 2007).

Arbuscular mycorrhizal fungi have been shown to increase soil water retention, notably thanks to Glomalin (Gianinazzi et al., 2010) and to increase water accessibility for the plants (Daynes et al., 2013). Indeed, AMF increase roots hydraulic conductivity and hyphae are able to access water that roots cannot reach (Ruiz-Lozano and Azcón, 1995). Furthermore, AMF have been reported to increase the water use efficiency in plants. For instance, in *Phaseolus aureus* a 50% decrease of water was needed to produce 1 g of dry matter (Honggang et al., 1989). This was recently corroborated by Le Pioufle et al. (2019). These authors demonstrated that the AMF *R. irregularis* MUCL 41833 can play a role in drought resistance/tolerance of maize plants by notably increasing their water use efficiency and phosphorus uptake (Le Pioufle et al., 2019).

c. Bioregulation of plant development and modification in plant compounds

Arbuscular mycorrhizal fungi induce modification of plant metabolism and physiology, often leading to an increase in plant quality for human health, notably by increasing antioxidant and secondary metabolites production (Gianinazzi et al., 2010). AMF can stimulate production of phenolic compounds (phenolic acids, flavonoids) and activate carotenoid pathway in roots (Harrison, 1993; Strack and Fester, 2006). Furthermore, increased yield and quality of essential oils have been shown in AMF-colonized fennel plants (Kapoor et al., 2004), as well as increased production of health-promoting phytochemicals like rosmarinic/caffeic acids (Toussaint et al., 2007; Sbrana et al., 2014) and sugar or carotene content (Farmer et al., 2007). Plant medicinal compounds can be increased by AMF colonization. For example, *G. fasciculatum* and *G. macrocarpum* induced a 95% increase of artemisin concentration in leaves of *Artemisia annua* (Chaudhary et al., 2008). Review of the effect of AMF on secondary metabolism in leaves, roots or fruits/tubers of different crops used as food or for medicinal purposes have been made by Gianinazzi et al. (2010). Interestingly, increase of

mineral plant nutrition and mineral content in AMF plant used for human consumption can prevent unbalanced dietary (Gianinazzi et al., 2010).

d. Abiotic stress alleviation by AMF

Arbuscular mycorrhizal fungi have been shown to increase plant tolerance/resistance to abiotic stresses such as drought, salinity, high temperatures, heavy metals, trace elements (TEs), petroleum, crude oil, and PAHs (see review by Plouznikoff et al. (2016)). Most results were obtained under controlled conditions and only a few within the field. A number of examples are reported below:

- Drought stress: In presence of AMF, this effect was decreased in plants such as sugarcane, citrus, mung bean, apple, tomato, maize, wheat (Wu, 2017). Trifoliolate orange colonized with *F. mosseae* had significantly higher root total length, surface area, volume, and number of first-, second-, and third-order lateral roots under drought stress conditions as compared to non-colonized plants (Liu et al., 2016). A number of mechanisms have been associated with AMF mitigation of drought stress: improved cell turgor via osmotic adjustment in shoots (Wu and Xia, 2006), neutralization of reactive oxygen species in tissues via the synthesis of enzymes (e.g. superoxide dismutase) (Ruiz-Lozano et al., 1996), increased transport of water via the extra radical mycelium to the root cells (Sanchez-Diaz and Honrubia, 1994; Khan et al., 2003) and increased root hydraulic conductivity through modulation of root aquaporin expression (Quiroga et al., 2018).
- Salinization: Agricultural practice, such as irrigation increases the salinity of soils. Arbuscular mycorrhizal fungi have been shown to maintain growth (i.e. increase of yield or number of fruit) of onions, lettuce, tomato, maize and pearl millet under salt stress as compared to non-colonized plants (Plouznikoff et al., 2016).
- Heavy metals: Cadmium is often found as a trace element in rock phosphates used as fertilizer (Lugon-Moulin et al., 2006) and often accumulates in soil. However, the ERM of AMF has been shown to buffer the effects on cadmium uptake reducing its toxic effects on plant (Gianinazzi et al., 2010). Furthermore, glomalin has been shown to induce sequestration and inactivation of pollutants like Pb, Cu, As, Cd (Gonzalez-Chavez et al., 2004).

- Anthropogenic activities (e.g. combustion of petroleum hydrocarbon, release of polyaromatic hydrocarbons (PAHs) that accumulate in soil and are susceptible to contaminate the food chain: The AMF *R. irregularis* MUCL41833 was shown to maintain phosphorus uptake of *M. truncatula* in presence of PAHs (Calonne-Salmon et al., 2018).

e. Biotic stress mitigation by AMF

Several studies (see Table 1) have reported an increasing resistance/tolerance of AMF-colonized plants against above and below-ground pathogens (Whipps, 2004). The effects were either local (at the site of colonization of the AMF) (Trotta et al., 1996; Hayek et al., 2014) or systemic (away from the site of colonization by the AMF) (Cordier et al., 1998; Khaosaad et al., 2007) and mostly concerned root pests and diseases. The effects on above-ground pathogens are less numerous and conclusive (Pozo and Azcón-Aguilar, 2007), even if systemic effects have been reported in tomato against *Fusarium sambucinum* or *Alternaria solani* (Song et al., 2010; Ismail and Hijri, 2012) and in potato against *P. infestans* (Gallou et al., 2011). Against insects on aerial part, experiments with AMF lead sometimes to increase of pest fitness. However, pathosystems involving combinaison of insect parasite wasp and AMF revealed synergistical beneficial effects (Bennett et al., 2016). Song et al. (2010) have shown the importance of the common mycorrhizal network (CMN) formed by AMF in the transmission of defence signals. These signals could potentially have a long-distance effect on neighbouring plants, allowing potentiation of plant defence.

As reported for the abiotic factors, most studies with pests and diseases were conducted under controlled conditions (in the greenhouse or *in vitro*). Results in the field are less abundant and more controversial due among others to the competition with the indigenous microorganisms that could impact the establishment and development of the AMF inoculants (Berruti et al., 2015). Indeed, amongst the papers published over the past 4 years, only 10 were conducted in the field (see Table 1). Diversity of AMF is also underrepresented in the studies aiming to mitigate biotic stresses. Indeed, amongst the 333 known species only 23 were used to investigate potential bio-protection effect against biotic stress and almost half of the studies were conducted with

two species, *F. mosseae* and *R. irregularis*. For both species only six and seven strains have been used, respectively.

Table 1. *Studies conducted between 2015 and 2018 on the effects of AMF on plant protection against below- and above-ground pests and diseases.*

AMF	<i>Rhizophagus irregularis</i> MUCL 41833				
Plant or crop	Soybean: <i>Glycine max</i> cv. Don Mario 4800				
Pathogen (Below ground)	Sudden Death Syndrome: <i>Fusarium virguliforme</i> MUCL 53605				
Context	Growth chamber (<i>In vitro</i>)				
References	Marquez et al., 2018b				
Biocontrol effect of AMF and proposed mechanisms	Decreased root infection by 33% as compared to the control (44%). Upregulated genes related to defense: resistance proteins, WRKY transcription factors, auxins, receptors kinases and proteases.	<i>Rhizophagus irregularis</i>	Tomato: <i>Lycopersicon esculentum</i> cv. Roma Fusarium wilt: <i>Fusarium oxysporum</i> f. sp., <i>Lycopersici</i> race 2, isolate RAF 70	Growth chamber Bidellaoui et al., 2018	Decreased disease incidence by 57%. Plant growth (height and dry weight) was similar to the control free from pathogen and AMF.

AMF	Natural inoculum Species not mentioned	<i>Funneliformis mosseae</i> <i>Glomus fasciculatum</i> <i>Rhizophagus irregularis</i>	Combination of <i>Glomus mosseae</i> , <i>Glomus fasciculatum</i> , <i>Glomus hoi</i>
Plant or crop	<i>Cryptocarya concinna</i> , <i>Ormosia pachycarpa</i> , <i>Engelhardia fenzelii</i> , <i>Schima superba</i> , <i>Ormosia glaberrima</i> , <i>Canarium</i>	Chickpea; <i>Cicer arietinum</i> cv. JG 62	Tomato: <i>Lycopersicon esculentum</i>
Pathogen (Below ground)	Natural pathogen	Fusarium wilt: <i>Fusarium oxysporum</i> f. sp. <i>ciceris</i>	Fusarium wilt: <i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i>
Context	Field and shade house	Net house	Glasshouse
References	Liang et al., 2015	Shukla et al., 2015	Singh, 2015
Biocontrol effect of AMF and proposed mechanisms	Neutralized the inhibition effect of pathogens on seedling. The beneficial effect on seedling survival and growth plant is host specific.	Pre-inoculation with AMF decreased severity by 89.5% and increased growth and P uptake that makes plant more tolerant against pathogen infection.	Reduced damage by 40%.

AMF	<i>Glomus mosseae</i>	<i>Glomus intraradices</i> , <i>Glomus hoi</i> , <i>Gigaspora margarita</i> , <i>Scutellospora gigantea</i>	<i>Funneliformis mosseae</i> , <i>Glomus dussii</i>
Plant or crop	Tobacco: <i>Nicotiana tabacum</i> seeds (Yunyan 85)	Bean: <i>Phaseolus vulgaris</i>	Yam: <i>Dioscorea alata</i> TDa98-01183, TDa98-165 <i>Dioscorea rotundata</i> TDr97-00551, TDr 745
Pathogen (Below ground)	Bacterial wilt: <i>Ralstonia solanacearum</i>	Root rot: <i>Fusarium solani</i> f. sp. <i>phaseoli</i>	Root Knot Nematode: <i>Meloidogyne</i> spp.
Context	Greenhouse	Greenhouse	Greenhouse
References	Yuan et al., 2016	Eke et al., 2016	Tchabi et al., 2016
Biocontrol effect of AMF and proposed mechanisms	Decreased incidence by 14.7% and population of the pathogen in the soil by 10%. Increased activity of various defense-related enzymes: polyphenol oxidase, phenylalanine ammonia-lyase and peroxidase.	Decreased disease incidence by 30 to 40% and severity by 25 to 39%, maintained root biomass of infected plant. Activity of phenylalanine ammonia-lyase was increased by two-fold in the AMF-pathogen treatment as compared to the control.	Reduced galling symptoms by 25-50% as compared to 76-100% in control and increased the number of <i>F. mosseae</i> , decreased the number of nematodes by a third compared to the control.

AMF	<i>Funneliformis mosseae</i> <i>Glomus cerebriforme</i> <i>Rhizophagus irregularis</i>	<i>Rhizophagus intraradices</i>	<i>Glomus mosseae</i>
Plant or crop	Pigeon pea: <i>Cajanus cajan</i>	Soybean: <i>Glycine max</i>	Eggplant: <i>Solanum melongena</i>
Pathogen (Below ground)	Wilt: <i>Fusarium udum</i>	Charcoal rot: <i>Macrophomina phaseolina</i>	Root knot nematode: <i>Meloidogyne incognita</i>
Context	Greenhouse	Glasshouse	Greenhouse
References	Deharia et al., 2018	Spagnoletti et al., 2017	Khan et al., 2017
Biocontrol effect of AMF and proposed mechanisms	Disease reduction by 78% when AMF was inoculated prior to the pathogen and with well-established colonization.	Reduced number of pathogens by half. Root biomass of infected plants was significantly higher in presence of AMF as compared to the infected controls without AMF and was similar to the plants free from AMF and pathogen.	Decreased invasion and development rate of life stages of the pathogen in the roots of eggplant treated with AMF resulting in almost ten times less eggs mass after three weeks and tree time less after five weeks. Developmental stages of <i>M. incognita</i> appeared progressively lower in the roots treated with AMF.

AMF	Species not mentioned	<i>Rhizophagus intraradices</i> BEG141	<i>Rhizophagus irregularis</i> MUCL 41833
Plant or crop	Jerusalem artichoke: <i>Helianthus tuberosus</i>	Grapevine: rootstock SO4 <i>Vitis berlandieri</i> <i>Vitis riparia</i>	Soybean: <i>Glycine max</i> . cv. Don Mario 4800
Pathogen (Below ground)	Stalk rots: <i>Sclerotinia sclerotiorum</i> Brown rot: <i>Rhizoctonia solani</i>	Nepovirus: Grapevine fanleaf virus nematode vector: <i>Xiphinema index</i>	<i>Macrophomina phaseolina</i> MUCL 53604
Context	Field and Greenhouse	Glasshouse	Growth chamber (<i>In vitro</i>)
References	Ezzat et al., 2015	Hao et al., 2018	Marquez et al., 2018a
Biocontrol effect of AMF and proposed mechanisms	Reduced disease incidence, improved yield and enhanced tuber quality.	Decreased proliferation of nematodes by 1.6-fold, 90 days post infection by the pathogen. Under low dose of nematodes, AMF prevent virus development in the plant (No detection by molecular tools).	Size and color of lesions were more intense in infected plant without AMF, 72 hours post infection compared to the AMF plants. Only a small set of genes showed higher expression in AMF-colonized plants as those coding for DIR-like protein, glutathione S transferase (GST) and PR-1.

AMF	Commercial inoculum : <i>Claroideoglomus entunicatum</i> , <i>Funneliformis coronatum</i> and <i>mossea</i> ; <i>Rhizophagus irregularis</i>	<i>Glomus intraradices</i> <i>Glomus mosseae</i> <i>Glomus etunicatum</i>	<i>Glomus intraradices</i>
Plant or crop	Wheat: <i>Triticum aestivum</i> Ventura and Yitpi	Potato: <i>Solanum tuberosum</i> cv. Tigoní and Clone	Eggplant: <i>Solanum melongena</i>
Pathogen (Below ground)	Nematode: <i>Pratylenchus neglectus</i>	Bacterial wilt: <i>Ralstonia solanacearum</i> race 3 biovar 2A	Verticillium wilt: <i>Verticillium dahliae</i>
Context	Growth chamber	Field	Field
References	Frew et al., 2018	Aguk et al., 2018	Pirinc et al., 2017
Biocontrol effect of AMF and proposed mechanisms	Negative effects of AMF on defense compounds (benzoxazinoid glucoside defense). Nematode populations were 47-117% higher in AMF inoculated plants, depending on wheat variety.	Decreased area under disease progress curve by 50% in organic farming.	Reduced symptoms by 17%.

Organism	<i>Rhizophagus irregularis</i>	<i>Rhizophagus irregularis</i> MUCL 41833
Culture	Common bean: <i>Phaseolus vulgaris</i> , Genotypes A-55 is a common bean breeding line. Azufrado Regional 87 (Az Reg87) and Azufrado Higuera (Az Hig) Tomato: <i>Solanum lycopersicum</i> cv. Micro-Tom	Bananas: <i>Musa acuminata</i> cv. Grande Naine, AAA genome, Cavendish group
Pathogen/herbivory (Above ground)	White mold: <i>Sclerotinia sclerotiorum</i> Bacterial spot: <i>Xanthomonas campestris</i> pv. vesicatoria	Black Sigatoka: <i>Mycosphaerella fijiensis</i> MUCL 47740
Activity ab/field	Growth chamber	Growth chamber in vitro
References	Mora-Romero et al., 2015	Anda et al., 2015
AMF effect on plant health against pathogen	Decreased disease symptoms (fewer lesions and lesions diameter reduced by 40%) via grafting of shoots onto mycorrhizal rootstocks. This suggested that a signal moved from colonized roots to the upper parts of plants, enabling the induction of pathogen defense in shoots grafted to mycorrhizal rootstocks.	Decreased severity index by more than 80% and 60%, respectively, 21 and 35 days after infection by the pathogen. Area under disease progress curve reduced by 59% in the leaves of the AMF plants, 35 days following infection by the pathogen.

Organism	<i>Funneliformis mosseae</i> BEG 167	Commercial inoculum : <i>Funneliformis mosseae</i> BEG 198
Culture	Tomato: <i>Solanum lycopersicum</i> cv. Jin Bao and Castlemart wild type, defective spr2 or overexpressing 35S::PS	Ribwort Plantain: <i>Plantago lanceolata</i>
Pathogen/herbivory (Above ground)	Early blight: <i>Alternaria solani</i> ACCC36110	Southern beet Armyworm: <i>Spodoptera exigua</i>
Activity ab/field	Growth chamber	Growth chamber
References	Song et al., 2015	Wang et al., 2015
AMF effect on plant health against pathogen	Reduced disease incidences and indices by 54.3% and 72.8%, respectively. Pre-inoculation by AMF led to a significant increase in activities of β -1,3-glucanase, chitinase, phenylalanine ammonia- lyase and lipoxxygenase in tomato leaves upon pathogen inoculation.	Reduced shoot biomass by 7.1% of plant colonized by AMF. Increase by 64% of catalpol (a defense metabolite) in shoots. Altered time course of induction of this defense metabolite in response to foliar insect herbivory.

Organism	<i>Entrophospora infrequens</i> , <i>Funneliformis mosseae</i> , <i>Claroideoglomus claroideum</i> , <i>Racocetra fulgida</i>	<i>Rhizophagus irregularis</i> DAOM 197198, commercial inoculum: <i>Funneliformis mosseae</i> FR140, mix of <i>Glomus</i> sp.
Culture	Soybean: <i>Glycine max</i>	Wheat: <i>Triticum aestivum</i> cv, Orvantis and Lord
Pathogen/herbivory (Above ground)	Bacterial blight: <i>Pseudomonas syringae</i> pv. <i>glycinea</i>	Wheat powdery mildew: <i>Blumeria graminis</i> f. sp. <i>tritici</i>
Activity ab/field	Growth chamber	Growth chamber
References	Malik et al., 2016	Mustafa et al., 2016
AMF effect on plant health against pathogen	Pathogens colonization was lowest in plants associated with <i>E. infrequens</i> and pathogen increased between 40 and 120-hour post infection by less than 2% in <i>E. infrequens</i> treated soybean, which is about seven times less than in the control.	Decreased symptoms on leaves with <i>F. mosseae</i> (74 %), then mix of <i>Glomus</i> sp. (58 %) and <i>R. irregularis</i> (34 %), suggesting that inoculum type rather than mycorrhizal levels determined the protection level of wheat against <i>B. graminis</i> .

Organism	Commercial inoculum <i>Rhizophagus irregularis</i> (DAOM197198)	Commercial inoculum <i>Funneliformis mosseae</i> FR140
Culture	Barrel medic: <i>Medicago truncatula</i> A17	Wheat: <i>Triticum aestivum</i> cv. Orvantis
Pathogen/herbivory (Above ground)	Pea aphids: <i>Acyrtosiphon pisum</i> lineage LSR1	Powdery mildew: <i>Blumeria graminis</i> f. sp. <i>Tritici</i> MPEbgt1
Activity ab/field	Growth chamber	Growth chamber
References	Garzo et al., 2018	Mustafa et al., 2017
AMF effect on plant health against pathogen	High level of AMF colonization hindered the aphid's ability to find and reach the phloem (twice the time needed in control treatment) with thicker leaves and poor food quality (reduced nitrogen).	Reduced infection of leaves by 78%. No upregulation in the expression of the targeted genes (phenylalanine ammonia-lyase, lipoxxygenase, peroxidase, chitinase) was observed in leaves of mycorrhizal wheat plants challenged with <i>B. graminis</i> .

Organism	<i>Rhizophagus intraradices</i>	<i>Glomus mosseae</i>
Culture	Tomato: <i>Solanum lycopersicum</i> cv. Castlemart	Tomato: <i>Lycopersicon esculentum</i> cv. Castlemart
Pathogen/herbivory (Above ground)	Beet armyworm: <i>Spodoptera exigua</i>	Leaf Mould: <i>Cladosporium fulvum</i>
Activity ab/field	Greenhouse	Growth chamber
References	Shrivastava et al., 2015	Wang et al., 2018
AMF effect on plant health against pathogen	Reduced weight of armyworm larvae, from 0.084g to 0.070g in the control and AMF treatment, respectively. Enhanced transcription of genes encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS), an enzyme that catalyzes the initial step of the MEP pathway in AMF treatment.	Induced systemic resistance against air-borne pathogen. Disease index was 36.4% in AMF treatment and 59.6% in the control. AMF colonization played an important role in plant resistance against air-borne pathogen infection by maintaining redox balance (increase of superoxide dismutase along with decreases in levels of H ₂ O ₂) and photosynthetic activity.

Organism	<i>Glomus intraradices</i> UT126	<i>Glomus fasciculatum</i>
Culture	Oil palm: <i>Elaeis guineensis</i>	Tomato: <i>Solanum lycopersicum</i> cv Pusa Ruby
Pathogen/herbivory (Above ground)	Basal stem rot: <i>Ganoderma boninense</i>	Early blight: <i>Alternaria alternata</i>
Activity ab/field	Greenhouse	Greenhouse
References	Sundram et al., 2015	Nair et al., 2015
AMF effect on plant health against pathogen	Reduced disease severity at 58% compared to the 91% in control treatment with <i>G. intraradices</i> UT126 applied single.	Smaller and fewer lesions 7 days after application of spores of <i>A. alternata</i> . Percentage disease index in AMF-colonized plants was half of the control plants. JA levels increased twofold in AMF-colonized plants when subjected to <i>A. alternata</i> . Systemic increase in JA in response to AMF colonization plays a key role in expression of MIR against <i>A. alternata</i> .

Organism	<i>Rhizophagus irregularis</i>	Commercial: <i>Rhizophagus intraradices</i> <i>Funneliformis mosseae</i> , <i>Glomus species</i>
Culture	Bittersweet nightshade: <i>Solanum dulcamara</i> Eastern black nightshade: <i>Solanum ptycanthum</i>	Strawberry: <i>Fragaria x ananassa</i> genotypes: cv. Elsanta, cv. Senga Sengana and K40 somaclone
Pathogen/herbivory (Above ground)	Specialist herbivore: <i>Manduca sexta</i>	Verticillium wilt: <i>Verticillium dahliae</i>
Activity ab/field	Greenhouse	Greenhouse
References	Minton et al., 2016	Sowik et al., 2016
AMF effect on plant health against pathogen	Caterpillar growth was unaffected by AMF. For <i>S. dulcamara</i> AMF increased the defenses measured (peroxidase 30% and protease inhibitor two-fold) but did not influence herbivore growth.	Decreased disease development in highly susceptible Elsanta genotype (reduce loss by half compared to infected treatment without AMF). Increased stomatal conductance, transpiration rate and leaf water potential in Elsanta genotype (around 30%, 25% and 30% respectively). Photochemical activity decreased with disease development only in 'Elsanta' plants and AMF significantly counteracted this effect: effective quantum yield was five times higher than in control.

Organism	Indigenous AMF <i>Funneliformis mosseae</i> <i>Rhizophagus irregularis</i>	<i>Rhizophagus irregularis</i>
Culture	Potato: <i>Solanum tuberosum</i> STN4709 and tetraploid Chilean From diploid wild potato species: <i>Solanum berthaultii</i> <i>Solanum polyadenum</i>	Grapevine : <i>Vitis vinifera</i> cv Chasselas, Pinot noir and the interspecific hybrid Divico: on the rootstock 41B
Pathogen/herbivory (Above ground)	Aphid: <i>Macrosiphum euphorbiae</i> Wasp: <i>Aphidius ervi</i>	Downy mildew: <i>Plasmopara viticola</i>
Activity ab/field	Glasshouse	Greenhouse
References	Bennett et al., 2016	Bruisson et al., 2016
AMF effect on plant health against pathogen	Increased proportion of aphids from which a wasp emerged in aphids that had fed on AMF-colonized plants. Parasitism was highest for aphids that had fed on plants in the AMF treatment as compared to the control, resulting in six times more total parasitism and attack on the aphids by the wasp.	Increased amounts of stilbenoid in leaves of AMF- colonized plants 48 h after pathogen inoculation. AMF potentiated plant genes expression against pathogens: up-regulation of phenylalanine ammonia- lyase, stilbene synthase and gene encoding a resveratrol O-methyltransferase, involved in the stilbenoid biosynthesis pathway. Pterostilbene increased by 8 to 10-time in AMF-colonized plants.

Organism	AMF inoculum Species not mentioned	AMF consortium Species not mentioned	<i>Rhizophagus irregularis</i>
Culture	Potato: <i>Solanum tuberosum</i> TBR5642 <i>Solanum berthaultii</i> BER7348	Poblano pepper: <i>Capsicum annuum</i> cv. San Luis	Bittersweet nightshade: <i>Solanum dulcamara</i> Eastern black nightshade: <i>Solanum ptycanthum</i>
Pathogen/herbivory (Above ground)	Aphid: <i>Macrosiphum euphorbiae</i>	Pepper Wilt: <i>Phytophthora capsici</i> CH11 stain	Specialist herbivore: <i>Manduca sexta</i>
Activity ab/field	Pots	Greenhouse	Greenhouse
References	Karley et al., 2017	Reyes-Tena et al., 2017	Minton et al., 2016
AMF effect on plant health against pathogen	No significant impact of AMF on number of nymphs produced per plant.	Reduced of severity by half.	Caterpillar growth was unaffected by AMF. For <i>S. dulcamara</i> AMF increased the defenses measured (peroxidase 30% and protease inhibitor two- fold) but did not influence herbivore growth.

Organism	<i>Rhizophagus irregularis</i> <i>Funneliformis mosseae</i> , <i>R. clarum</i> , <i>Gigaspora margarita</i> , <i>G. gigantea</i>	<i>Glomus fasciculatum</i> , <i>Acaulospora</i> <i>laevis</i>	<i>Glomus mosseae</i> strain 1404
Culture	Wheat: <i>Triticum aestivum</i>	Tomato: <i>Solanum lycopersicum</i>	Cucumber seeds: <i>Cucumis</i> <i>sativus</i> cv. Keyhan F1
Pathogen/herbivory (Above ground)	Stem rust: <i>Puccinia graminis</i> f.sp. <i>tritici</i>	Tomato wilt: <i>Fusarium oxysporum</i> f sp <i>lycopersici</i> .	Cucumber damping-off: <i>Phytophthora melonis</i> Zab101
Activity ab/field	Greenhouse	Pots greenhouse	Greenhouse
References	El-Sharkawy et al., 2018	Manila and Nelson, 2017	Sabbagh et al., 2017
AMF effect on plant health against pathogen	Reduced disease severity by 40% as compared to the control. Induced peroxidase and polyphenol oxidase enzymes production and increased total phenol content.	Increased resistance and shoot and root dry weights, height of shoots and stem thickness. <i>Acaulospora laevis</i> was the most effective, with disease severity scale limited to 26-49% infection compared to the 100% reached in the control, 20 days after infection.	Decreased disease severity by 40%. Genes over-expression (phenylalanine ammonia-lyase, pathogen-induced 4, lipoxygenase and galactinol synthase) during the first 72h post-infection in leaves of AMF-colonized plants.

Organism	<i>Funneliformis mosseae</i>	<i>Funneliformis mosseae</i> , <i>Gigaspora margarita</i> , <i>Claroideoglomus claroideum</i> , <i>Rhizophagus irregularis</i>	<i>Funneliformis mosseae</i> (BEG12)
Culture	Lucerne alfalfa: <i>Medicago sativa</i>	Tomato	Wheat: <i>Triticum aestivum</i> cv Chinese Spring
Pathogen/herbivory (Above ground)	Spring black stem and leaf spot: <i>Phoma medicaginis</i>	Bacterial wilt: <i>Clavibacter michiganensis</i> sub sp. <i>michiganensis</i>	Bacterial leaf streak: <i>Xanthomonas translucens</i>
Activity ab/field	Greenhouse	Greenhouse	Greenhouse
References	Gao et al., 2018	Duc and Posta, 2018	Fiorilli et al., 2018
AMF effect on plant health against pathogen	Decreased plant infection with the lowest disease incidence 18–41% for 59% in controls. AMF treatment had higher b-1,3-glucanase, chitinase and phenylalanine ammonia-lyase activities.	Decreased disease index by 20% with <i>R. irregularis</i> .	Reduced disease area on leaves by six times as compared to control. Induced local and systemic induction of defense genes and proteins in AMF plants. Transcriptomic and proteomic data sets revealed a strong activation of nutrient transporter genes and proteins in AMF colonized plants, leading to an increased mineral content in both roots and leaves.

Organism	<i>Rhizophagus irregularis</i> CD1	<i>Funneliformis mosseae Rhizophagus irregularis</i>
Culture	17 cotton cultivars: <i>Gossypium spp.</i>	Apple tree: <i>Malus pumila</i>
Pathogen/herbivory (Above ground)	Verticillium wilt: <i>Verticillium dahliae</i>	Apple canker: <i>Neonectria ditissima</i>
Activity ab/field	Field	Field (Pots placed)
References	Zhang et al., 2018	Berdeni et al., 2018
AMF effect on plant health against pathogen	Increased resistance to pathogen. The disease index in AMF-colonized plants was decreased by 28% as compared to the control. Induced PR, jasmonic related and lignin synthesis-related genes.	Decreased amount of plant material infected by the fungal pathogen by 18%.

Organism	Commercial AMF inoculum: <i>A. columbiana</i> , <i>R. clarus</i> , <i>C. etunicatum</i> , <i>R. intraradices</i> , Native species: <i>A. spinosa</i> , <i>E. infrequens</i> <i>C. claroideum</i> , <i>C. lamellosum</i> , <i>F. mosseae</i> <i>R.a fulgida</i> , <i>C.pelluci</i>
Culture	Little bluestem: <i>Schizyachrium scoparium</i> (Michx.) Nash Grey-headed coneflower: <i>Ratibida pinnata</i> Round-headed bush clover: <i>Lespedeza capitata</i> Nodding onion: <i>Ilum cernuum</i> Prairie dropseed: <i>Sporobolus heterolepis</i>
Pathogen/herbivory (Above ground)	Sod web-worms <i>Herpetogramma phaeopteralis</i>
Activity ab/field	Field experiment
References	Middleton et al., 2015
AMF effect on plant health against pathogen	Native, locally adapted AMF improved restoration of prairie plant species and these benefits were extended to neighbors up to two meters from the inoculation point. Plants grew larger with the native AMF over the commercial AMF, one month after the herbivory infestation. Leave number of AMF <i>Allium cernuum</i> was twice that measured in control treatment and shoot length of <i>Lespedeza capitata</i> was increased by a third compared to control.

Organism	<i>Rhizophagus irregularis</i> BEG 72	Local inoculum Species not mentioned
Culture	Tomato: <i>Solanum lycopersicum</i> variety San Marzano Nano	Dominant plants C4 grasses, Big bluestem: <i>Andropogon gerardii</i> . Little bluestem: <i>Schizachyrium scoparium</i> . Indian grass: <i>Sorghastrum nutans</i> . Switch grass: <i>Panicum virgatum</i> with subdominant C4 and C3 graminoids, composites, legumes, other forbs
Pathogen/herbivory (Above ground)	Mirid bug: <i>Macrolophus pygmaeus</i>	Herbivory
Activity ab/field	Glasshouse	Field grass land
References	Prieto et al., 2017	Kula and Hartnett, 2015
AMF effect on plant health against pathogen	Increased mirid bug adult emergence and progeny by two-fold. Mirid bug recaptured on AMF leaves were around 30% higher compared to non-mycorrhized treatment.	Higher herbivory on mycorrhizal C3 graminoids plants (40%). For C4 grasses AMF increased the plant biomass by around 30%.

Organism	Commercial inoculum: <i>Rhizophagus intraradices</i> , <i>Funnelformis mosseae</i> , <i>Glomus aggregatum</i> , <i>Claroideoglomus etunicatum</i>	Commercial AMF “Rootgrow” was provided by PlantWorks
Culture	Milkweed: <i>Asclepias curassavica</i> , <i>Asclepia latifolia</i> , <i>Asclepia syriaca</i> , <i>Asclepia incarnata</i>	Wheat: <i>Triticum monococcum</i> lines MDR037, MDR045 and MDR049 <i>Triticum aestivum</i> variety Solstice
Pathogen/herbivory (Above ground)	Phloem-feeding <i>Aphis nerii</i>	Aphid: <i>Sitobion avenae</i>
Activity ab/field	Greenhouse	Growth chamber
References	Meier and Hunter, 2018	Simon et al., 2017
AMF effect on plant health against pathogen	No effect on caterpillar. AMF-colonized plants generally produced tougher leaves with higher cardenolide concentrations than did plants without AMF, therefore aphids accumulate twice the amount of cardenolides.	<i>S. avenae</i> was attracted to and reproduced more on the mycorrhizal plants (between 20 and 30%). Increased reproductive success (decrease of the number of days needed for the first nymph from 2 to 5 depending of the host plant), but no effect on the aphid growth rate. Aphids on mycorrhizal plants fed on average for longer.

Organism	<i>Entrophospora infrequens</i> , <i>F. mosseae</i> , <i>Claroideoglomus claroideum</i> , <i>Racocetra fulgida</i>	<i>Claroideoglomus etunicatum</i> <i>Glomus versiforme</i> <i>Funneliformis mosseae</i>
Culture	Tomato: <i>Solanum lycopersicon</i>	Milkvetch: <i>Astragalus adsurgens</i> var. Shanxi Yulin
Pathogen/herbivory (Above ground)		Powdery mildew: <i>Erysiphe pis</i>
Activity ab/field	Glasshouse	Glasshouse
References	Malik et al., 2018	Liu et al., 2018
AMF effect on plant health against pathogen	Increased plant tissue consumption by the beetle (more than 10 time) and increased beetle survival at early stage but not in late stage.	<i>G. versiforme</i> increased (2.5 fold) powdery mildew disease significantly above the NM control. The peroxidase had consistently higher activity (15% to 72%) in plants with AMF than plants without AMF. Shoot and roots dry weight were 50% higher in AMF plants.

Red box indicates negative effect of AMF on plant protection.

3. Impact of agronomic practice on AMF

AMF are particularly sensitive to physical, chemical and biological disturbances caused by agricultural practices (Verzeaux et al., 2017), some may be beneficial and others detrimental. Adequate agricultural practices may favor AMF.

- Absence of tillage: this practice has been associated with an increase in the number of viable AMF propagules in soil after crop rotation of sunflower, spelt, grass-clover, maize, and at harvest for winter wheat (Säle et al., 2015; Verzeaux et al., 2017) and in root colonization of maize and wheat (Hu et al., 2015). Similarly, the spore density and species richness was higher in the top soils under minimal tillage as compared to ploughed soils with a crop rotation consisting of rapeseed (*Brassica napus* L.), winter wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), and winter wheat (Jansa et al., 2002a; Säle et al., 2015). In intensive cropping systems, the highest diversity of AMF has been found just below the sole of ploughing and may represent a “gene-bank” facilitating agroecological restoration (Oehl et al., 2005).
- Cover cropping: This practice has been reported to contribute to the restoration and maintenance of AMF in sunflower and maize in a typic calcixercept soil in the central Tajo River basin in Spain (Verbruggen et al., 2010). For instance, when weeds are not controlled (chemically or mechanically) mycorrhizal infectivity is maintained (Plenchette et al., 2005). Similarly, cover cropping during winter periods allow the maintenance of a potential AMF inoculum increasing colonization in the subsequent cultivated crops (Verzeaux et al., 2017). Both the choices of species in cover crop mixtures as well as their sowing density represent important practical issues. Indeed, mixtures of different plant species can supply broad spectrum of hosts that are able to enhance AMF diversity in soils and favor their complementary effects.
- Rotations: Diverse rotations encourage a diverse AMF community (Gosling et al., 2006). In organic farming, rotations generally consist of a period of fertility building ley followed by a period of cash cropping, before return to ley. In conventional farming, crop rotation is the practice of growing a series of dissimilar or different types of crops in sequenced seasons. Crop rotation has often been associated with an increase of AMF inoculum in the soil (Oehl et al., 2003; Gosling et al., 2006). Growing a crop that is strongly

dependent on AMF immediately after the ley, such as maize will make the best use of this potential (Gosling et al., 2006).

Inconclusive results on plant growth promotion or bio-protection in the field may be attributed partly to inadequate agronomic practices (e.g. monoculture, ploughing, fertilization or pesticides applications) impacting the AMF population density, diversity and efficacy (Oehl et al., 2004; Avio et al., 2013).

- Ploughing: This practice may physically disrupt the extra radical network of hyphae developing into the soil reducing uptake and transport of minerals from soil to plant (Helgason et al., 1998). Ploughing contribute to induce a strong decrease in AMF diversity in arable soil (Oehl et al., 2005).
- Monoculture: This practice that involves the cultivation of a single crop species or some crops such as sugar beet and rapeseed, which are non-hosts, prevents AMF development (Cosme et al., 2018) and potentially decreases the density of actives propagules. Even under maize (good AMF host) monoculture, the diversity of AMF may decrease (Oehl et al., 2003). Shifts in the population structure of AMF were observed in experiments in which undisturbed grasslands were converted to arable cropping fields: rotated crops became dominated by *Glomus* species (Daniell et al., 2001).
- Long fallow periods: This practice contributes to decreasing the AMF diversity in arable soil (Daniell et al., 2001).
- Fungicides: Even though AMF are not the primary target of fungicides, they may be affected either directly or indirectly. Fungicides have different effect on AMF, some like Monceren (pencycuron) or Amistar (azoxystrobin) seems harmful to AMF (*R. irregularis* MUCL 41833), while others like Monarch (flutolanil) have been reported to affect root colonization of potato under *in vitro* conditions (Buysens et al., 2015). *In vitro* experiments on transformed carrot roots have shown that two sterol biosynthesis inhibitor (SBI) fungicides (i.e. fenpropimorph and fenhexamid) affect mycelium architecture and strongly reduce spore production of *G. intraradices* (MUCL 43194) at high concentration (20 mg l⁻¹) (Zocco et al., 2008). Propiconazole (triazoles) was shown *in vitro* to alter the sterol metabolism of AMF (*G. irregularis*) resulting in the reduction of root colonization, sporulation and hyphal development (Calonne et al., 2012). In greenhouse, Benomyl (carbamate) has been reported to decrease AMF (*R. fasciculatus*)

rate of colonization on proso millet, while captan (trichloromethyl) seemed to increase AMF development and spores production (Channabasava et al., 2015).

- Herbicides: Some formulations have been reported to impact AMF via the destruction of host plants or by direct contact with fungal structures. Glyphosate has been shown to decrease spore viability and root colonization of *Lolium multiflorum* (Druille et al., 2013) and decrease soil AMF spore biomass and AMF propagules in white clover (Zaller et al., 2014).
- Fertilization: Over-utilization of phosphorus has a negative effect on AMF diversity and abundance. Some species were reported to be more sensitive to fertilization (unidentified *Glomus*) and other to be more tolerant (*G. mosseae*, *G. geosporum* and *Scutellospora fulgida*) (Bhadalung et al., 2005). High fertilization selects AMF that are inferior mutualists (i.e. providing less benefit) and disrupts the local co-adapted AMF communities that may exist (Johnson, 1993; Chen et al., 2018).
- Breeding programs: These practices are generally conducted on experimental stations where mineral nutrients are not limiting factors and may select genotypes that are unresponsive to AMF (Plenchette et al., 2005). In general, domestication reduced mycorrhizal benefits for crops under high phosphorus supply (Plouznikoff et al., 2019).

4. AMF diversity in arable soil

Arbuscular mycorrhizal fungi are present in most terrestrial ecosystems but may exhibit different distribution patterns, resulting in a high variability of taxon richness and composition between ecosystems (Öpik et al., 2006). The abundance and diversity of AMF in arable soils may drastically vary with agricultural practices. Indeed, even if diversity of AMF is not always low in arable soil (Hijri et al., 2006), most studies clearly demonstrated the negative impact of intensive agricultural practice on AMF. The number of species is limited with “Typical AMF of arable land” or “AMF generalists”: *G. aggregatum*, *G. geosporum*, *G. etunicatum*, *G. fasciculatum* and *G. mosseae* (Oehl et al., 2003; Oehl et al., 2005). *Rhizophagus irregularis* and *R. intraradices* are also dominant species in arable soils (Schlaeppi et al., 2016). Diversity of AMF was much lower in arable soils comparing to nearby woodland, with both soils at equal phosphorus content (Daniell et al., 2001). Indeed, meta-analysis made by Öpik et

al. (2006) comparing different ecosystems showed that plants in arable soil are hosting significantly less AMF taxa comparing to non-anthropogenic soils (e.g. tropical forest, grassland).

5. AMF functional diversity and plant breeding program

Even if molecular approaches considerably improved the identification and traceability of AMF in the field, their functional diversity is still lacking. Indeed, plant growth promotion and phosphorus uptake may vary between AMF species as well as between isolates of a same species (Munkvold et al., 2004). Functional diversity is important for the growth of individual plants and for the composition of plant communities (van Der Heijden et al., 2004). However, most available commercial inocula are composed of only few species (Brito et al., 2018). Some species like *Acaulospora* sp. find ecological niche in soil of organic farming systems. Even for the generalist *R. irregularis*, arable soils and grasslands contained completely different set of MtLSU haplotype (Börstler et al., 2010). Hypotheses is that these AMF species are functionally important for natural ecosystem (Oehl et al., 2004). Indeed, AMF contribute to the increase of nutrient use efficiencies (Verzeaux et al., 2017). AMF mediated agricultural practices may also benefit from a change in the breeding strategy and biocide usage. Indeed plants with increased capacities to exploit AMF attributes (i.e. alleviation of abiotic and biotic stress) may be selected (Gianinazzi et al., 2010; Verbruggen and Kiers, 2010). Therefore, it appears important to identify quantitative trait loci (QTLs) that may explain the establishment and functioning of the AMF plant symbiosis (Plouznikoff et al., 2019).

III. Pathosystem

1. *Solanum tuberosum*

a. History of potato domestication

Potato (*Solanum tuberosum* L.) was domesticated 8000 to 10000 years ago from wild species natives to the Andes of southern Peru (Hardigan et al., 2017). Although many crops were brought to Europe by Christopher Columbus soon after the discovery of the “new world” in 1492, potato arrived much later in Europe by Spanish

explorers (Hawkes and Francisco-Ortega, 1993). The first traces of potato in Europe dates back mid-16th century in the Canary Islands before expanding to the rest of the continent. In the 18th century, potato was common in gardens as a safeguard crop against grain crop failure or for military requisitions and started to be used as a field crop (McNeill, 1999). This is because potato production is high, and the crop is adapted to the climates found throughout Europe. The increase in population in Europe was partially supported by this crop. In some part of the continent, notably in Ireland, it became the main crop, making the population highly dependent with dramatic consequences in case of late blight outbreak (McNeill, 1999).

b. Potatoes genetic characteristics

Solanum tuberosum belong to the Solanaceae family. Outside its place of origin in South America, the cultivated potato varieties grown in Europe are considered to have a narrow genetic base resulting originally from a limited number of germplasms introduced in Europe. Most potato varieties are autotetraploid ($2n=4x=48$) and highly heterogenous (Consortium, 2011). Furthermore, most varieties have reduced their ability for sexual reproduction with the shift to asexual reproduction (Hardigan et al., 2017).

c. Potato production

Nowadays, potato is the third most important crop grown for direct human consumption (Hardigan et al., 2017). Its worldwide production was estimated above 370 million tons in 2016. China and India are the largest producers with an approximate of 30% of the total world production (Faostat, 2016).

The most widely grown variety in North America is Russet Burbank and in Belgium, Bintje. Both were released several dozen years ago but remains reference for tuber yield (e.g. in Europe, new varieties tuber yield are evaluated has a percentage of Bintje tuber yield) (Hardigan et al., 2017).

Belgium potato production was evaluated at 3.8 million tons in 2016, more than half of France, making this country one of the top 5 of EU producers (FAOSTAT, 2016). In the EU, 1.5% of arable soils are dedicated to potato but this surface rises to 9.1% in Belgium (Europotato, 2018). Indeed, in 2017 Belgium dedicated 95000 ha to

potato production with half of this surface grown only for Bintje (Fiwap, 2018). Yield is generally around 54t/ha, which is quite high compared to the 17t/ha in China, the first world producer (Faostat, 2016).

High yields are obtained by the application of high doses of fertilizers. One of the principal factors controlling the rate of leaf production is the supply of nutrients, i.e. nitrogen. When this element is present in large amount, much of the carbohydrate is diverted to leaf production. At first, tuber growth suffer from this carbohydrate allocation, but will benefit from the increased leaf production over the cropping season (Ivins and Bremner, 1965). In order to achieve a yield of around 50 tons/ha, fertilization recommendation per year for potassium are 30 to 200 Kg P_2O_5 /ha and for the nitrogen 60 to 300 Kg N/ha (Zebarth et al., 2004).

d. Market price and pest management cost

Transformed potatoes are the first food export product in Belgium and Belgium is one of the world leaders in the export of transformed potatoes (Faostat, 2016). The price of Bintje is comprised between 100 and 200 € per ton and profitability is generally achieved if price is superior to 100 € per ton (Fiwap, 2011). Price depends on the variety and caliber but is generally around 200 € per ton (Belgapom, 2017).

Profitability is markedly affected by pathogen management. For instance, in northern Europe and the USA, late blight management alone contributes to about 10 to 20% of total production cost of the crop (Haverkort et al., 2009). In the Netherlands, the value of potato production was estimated at 787 million € and costs, including chemical application, was evaluated at 115 million € (Haverkort et al., 2008). The number of pesticides treatments varies between 10 and 16 per season (Haverkort et al., 2008). In Belgium and more generally in EU, alert systems have been developed to help farmers to optimize the management of the disease.

e. Nutritional value of potatoes

Potato is primarily perceived as a carbohydrate source, but is also a good source of high quality proteins (Friedman, 1996). Potato also provides (Table 2) fibers, ascorbic acid, potassium and other

minerals or vitamins (Burlingame et al., 2009), which are of high nutritional value.

Table 2. *Macronutrient component of potatoes (data from aprifel.com, 2018)*

Macronutrient component	Quantity (g)	% Daily nutritional values
Water	78.3	NC
Proteins	1.8	0.5
Lipids	0.34	0.36
Saturated fatty acids	0.094	0.26
Carbohydrates	16.7	6.42
Sugar	0.86	0.96
Fiber	1.8	NC

2. *Phytophthora infestans*

a. Introduction

Phytophthora infestans (Mont.) de Bary, the causal agent of late blight, is the most devastating potato pathogen worldwide (Haas et al., 2009). The first record of late blight outbreak outside its natural area (Mexico), was made in 1843 in the USA, then in EU in 1845 (i.e. Ireland, Belgium) (Dyer et al., 1993). Thirty years was necessary to link the disease to a fungus-like organism (Ristaino, 2002). Indeed, *P. infestans* is an Oomycete (belonging to the Chromists) and unlike true fungi, it produces mobile zoospores and its cell wall contain mostly cellulose rather than chitin (Ristaino, 2002). *Phytophthora infestans* is a hemibiotroph that behaves as a biotroph (i.e. feeding on living host tissue) during the early stages of potato infection turning to a necrotroph (i.e. killing host tissue and feeding on the remains) in the later stage (Glazebrook, 2005; Gallou et al., 2011). This pathogen is known to produce a large set of effectors during its biotrophic phase (Lee and Rose, 2010). Genome sequencing and analyses showed rapid turnover (i.e. renewal and production of new effectors) and extensive expansion

of secreted disease effector proteins altering host physiology (Haas et al., 2009).

b. Life cycle of *Phytophthora infestans*

Phytophthora infestans principally reproduces asexually (Fig. 7), allowing a rapid population growth in susceptible host tissues (Fry, 2008). It forms sporangia on infested host tissues that either germinate directly to form infection hyphae or release zoospores. Sporangia can be dispersed in the air by wind and water to other plant tissues. The pathogen typically survives from season to season as mycelium in infected potato tubers and plant debris when the asexual life cycle is predominant (Ristaino, 2002). Tubers are generally infected by inoculum coming from the leaves, washed down by the rain or irrigation. Climate is a key parameter of disease development of *P. infestans*.

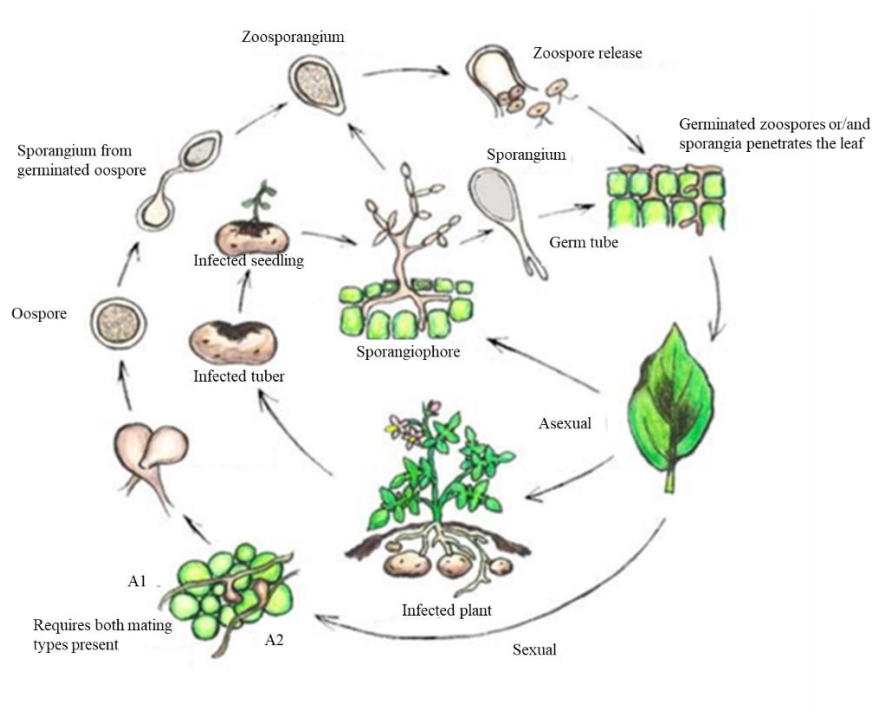


Figure 7. *Phytophthora infestans* life cycle on potatoes (from illustration 2013: H. P. Hovmalm).

c. Sexual reproduction

Phytophthora infestans is heterothallic and reproduce sexually (Fig. 7) by outcrossing of two mating types, termed A1 and A2 (Ko, 1988). Outcrossing of the two-mating types leads to the formation of oospores. Oospores are thick-walled structure which can survive extreme winter or summer conditions and may act as an additional source of primary inoculum (Arora et al., 2014). Mating type are compatibility types and do not correspond to dimorphic organism (Judelson, 1996). During sexual reproduction (outcrossing), genetic recombination occurs as the oospores are formed and leads to genetic diversity in subsequent generations of the pathogen (Judelson, 1996). *Phytophthora infestans* of the A2 mating type and oospores in infected plant material were first discovered in central Mexico in 1956 (Gallegly, 1958). First observations of the A2 mating type in Europe were made in the 1980s (Hohl and Iselin, 1984). Before population appeared only composed of A1 mating type. However, oospores were found in dried herbarium materials dating from 1876 through 1946 (Ristaino, 2002). Nowadays, in Belgium the ratio of mating types changed from more than 80% of A1 in 2004 to below 30% in 2017 (Eucablight, 2018). However, no definite trend exists and both mating types are detected. For example a 50/50 ratio is still detected in Mexico (Arora et al., 2014). Polyploidy appears to be common in *P. infestans* population, with diploids, triploids and tetraploids observed in Indian populations (Singh et al., 1997). Studies on sexual compatibility amongst different polyploids revealed that isolates of the same ploidy status mated freely, whereas those with varying ploidy status did not (Arora et al., 2014). The polyploids differed in their aggressiveness; diploids of A1 mating type are more aggressive than triploids. This trend was, however, not observed in polyploids of the A2 type (Arora et al., 2014).

d. Infection models

The duration of a wet climate combined with mild (~around 15°C) temperatures has been used by Magarey et al. (2005) for developing infection models. In these late blight models, infection by *P. infestans* is optimal with a minimum of 6 h of wetness (relative humidity of 95%) at 15°C. Furthermore, pressure of the pathogen based on disease severity and frequency of infection was classified in groups, notably low or high, by Bangemann et al. (2014) also depending on the

relative humidity and temperature. The pathogen pressure is also strain-dependent (Fig. 8) as shown by (Maziero et al., 2009)

e. *Phytophthora infestans* molecular marker

Different phenotypic and genotypic markers have been used for studying the diversity of *P. infestans*: mating types, virulence, metalaxyl resistance, isozyme and aggressiveness, restriction fragment length polymorphism (RFLP, mtDNA analysis, single nucleotide analysis (SNA), sequence analysis and single sequence repeats (SSRs) (Chmielarz et al., 2014). Isozymes (i.e. Glucosephosphate isomerase (Gpi) and peptidase (Pep) are useful markers to estimate the level of genetic variability in populations, to study species dispersion and to conduct phylogenetic analyses (Arora et al., 2014). Significant variations of aggressiveness have been noticed among isolates of the same geographic area (Carlisle et al., 2002).

f. Potato defense response against *P. infestans*

Potato defense response to *P. infestans* can be divided in three different stages corresponding to the biotrophic, transition and necrotrophic phase of the pathogen development (Wang et al., 2005). Whatever the stage, a large set of plant genes are up or down regulated, particularly those involved in transduction or transcription (Wang et al., 2005). Transcription factors like WRKY family are up-regulated starting 2h post infection and are involved in the activation of the pathogen related (PR) protein genes, in the late stage, by binding to the promoter W-box of those genes (Wang et al., 2005). Late stage involves notably, hypersensitive response (HR), phenylpropanoid pathway or thaumatococin like gene activation (Tian et al., 2006). On cv. Bintje, detection of *P. infestans* has been shown to be greatly increased 23h post infection (Orłowska et al., 2012) and priming of plant defense by AMF on cv. Bintje was shown to increase gene expression starting 24h post infection (Gallou et al., 2011). In fully resistant *Solanum* species and non-host plants, the HR occurred within 22h and was localized to cells surrounding the hyphae of the pathogen. Furthermore, depositions of callose and extracellular globules containing phenolic compounds have been observed in cells in addition to the HR. These compounds may be involved in cell wall strengthening (Vleeshouwers et al., 2000).

3. Late blight management

a. Introduction

Late blight caused by *P. infestans* was first observed in Europe in the 1840s and led to the devastating Great Irish Potato Famine. Nearly 160 years later, the disease continues to rage posing a major threat to potato production, particularly in the cooler, wetter northern European countries. During the first half of the 20th century, breeding programs focused on the introduction of major genes for late blight resistance. However, this approach was largely abandoned after the 1960s when it was found that even multiple resistance genes were rapidly countered by the pathogen (Cooke et al., 2011). Subsequent breeding efforts were conducted to produce cultivars with substantial levels of quantitative resistance, but most of the European varieties remain late blight-sensitive. Production is thus mostly reliant on the application of fungicides (Cooke et al., 2011).

The damages caused by late blight and the measures to control the disease accounts for more than 1 billion € per year in Europe (Vos and Kazan, 2016). In 2009, a EU directive (2009/128/CE – (directive, 2009)) established a framework to achieve a sustainable use of pesticides by reducing the risks associated with their application, particularly on human health and environment. This directive promotes the use of integrated pest management (IPM) and of alternative approaches or techniques such as the application of nature-based compounds (NBCs) or biological control agents (BCAs). *Biological control agents used in plant productions are living organisms protecting plants against their enemies, i.e. reducing the population of pests or diseases to acceptable levels* (du Jardin, 2015).

The EU also aimed to decrease copper use in organic farming (Regulation No.2092/91 – (Regulation, 1991). In 2013, the European potato production under organic farming represented only around 1.5% of the total area dedicated to this crop (<https://europatat.eu/activities/the-eu-potato-sector/>). Under organic potato production only 50 to 80% of conventional yields are reached. The differences in yields show up in the farm gate prices that can be obtained for organic potatoes: farm gate prices of organic potatoes are consistently higher (260 to 440 Euro/t) whereas conventional potatoes are sold at 60 to 300 Euro/t. Additionally, strong limitation for potato

organic farming remains also the management of pests and more specifically of late blight.

b. Late blight management via copper

In potato organic farming, management of late blight is mainly depending on copper treatment. Copper has been used intensively in pest management, as Bordeaux mixture, since the discovery of its effects on diseases by the end of the 19th century. Soluble copper ions have a direct action on enzymatic activity and cell metabolism preventing notably the germination of spores. However, the repeated applications of copper at doses 50% to 100% higher than the admitted norm have resulted in its accumulation in soils with significant impacts on below-ground organisms (Graham et al., 1986; Brun et al., 1998). In 1991, the EU issued a directive (Regulation No.2092/91 – (Regulation, 1991) limiting the use of copper in organic farming to 6 kg/ha per year. Even with this limitation, copper remains a threat to the environment. Indeed, only 200 to 300 g per ha/year are taken up by plants as micronutrients (Bouws and Finckh, 2008). Some European countries have already banned copper from organic management, like Denmark and the Netherlands or have reduced drastically its use like Germany.

c. Integrated late blight management

Natural and biological treatments tested in controlled or field environments are not always conclusive for the control of late blight. However, when used in combination with other control measures, they may contribute to late blight management under more environmental-friendly strategies (Olanya and Larkin, 2006). Furthermore, under low disease pressure, some non-chemical treatments were reported to reduce the impact of the disease (Bouws and Finckh, 2008).

Integrated pest management (IPM) has been defined as ‘*careful consideration of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of populations of harmful organisms and keep the use of plant protection products and other forms of intervention to levels that are economically and ecologically justified and reduce or minimize risks to human health and the environment*’ (directive, 2009). Using IPM, it is possible to reduce disease pressure with preventive strategies such as field hygiene (e.g. the removal of infected seed tubers before planting,

the crop rotation with non-host plants). Also if microclimate conditions in the field could be improved, then alternative treatments could be more reliable (Bouws and Finckh, 2008). Indeed, Bouws and Finckh (2008) noticed that growing potato in narrow strips perpendicular to the wind and separated by non-potato plants resulted in an overall decrease of epidemic pressure under organic farming conditions. The use of resistant cultivars is also an option. Some of them (e.g. Sarpomira, Nicola) are especially developed for the organic market (Pacilly et al., 2018). Potato varieties that can be harvested early in the season could decrease the risk of resistance breakdown, since the pathogen often appears at the end of the growing season (Pacilly et al., 2018).

d. Beneficial microorganism and late blight management

To replace copper-based fungicides, NBCs including plant extracts or BCAs such as *Trichoderma spp.*, AMF (See Table 1 and 3) or *Bacillus spp.* have been assayed with some success against *P. infestans* or other *Phytophthora* species under laboratory or greenhouse conditions (Dorn et al., 2007).

The selection of efficient BCAs is a long process that include several steps. The target crop and pathogen must first be identified. If the potential BCA come from the microbiome associated with the crop, it must be isolated and identified (Velivelli et al., 2014). The first step starts with the *in vitro* screening of the potential efficient BCAs against the target pathogen (e.g. antagonisms test) but this set up only represent a measurement of the metabolite potential of the strains grown on rather rich laboratory media and do not guarantee that the strains that are active *in vitro* will also be efficient *in planta* (Guyer et al., 2015). Additional experiments must be conducted before transfer to the field. Greenhouse and controlled trials under microplots are often used to evaluate consistency of performance. The microplot assays must be conducted under different geographic areas, climatic conditions and under different crops against different pathogens to evaluate a broad spectrum of action (Velivelli et al., 2014). For non-endophytic microbes, the challenge of a successful application as leaf spray is particularly high in view of the harsh conditions that prevail in the phyllosphere, such as UV irradiation, as well as variations in temperature and humidity (Guyer et al., 2015). de Vries et al. (2018) noticed that the diversity of microbes living inside plants is largely

underestimated. Endophytes may directly compete with pathogens of their host plants, induce plant defense and/or produce bioactive antimicrobial metabolites (de Vries et al., 2018). Different microorganisms or organic farming compatible treatments have been tested on late blight management. Most of them rely on bacteria but plant extracts or fungi have also been used (see Table 3). Selected BCAs are living organisms and should therefore be preserved in international collection to ensure long terms conservation. Then pilot industrial small-scale productions can be implemented, allowing evaluation of shelf life, compatibility with application practices, costs, ease of application. Before marketing BCA biosafety must be tested and danger to man (assessed notably, using different model organisms) must be evaluated (Velivelli et al., 2014).

Table 3. Overview of Biological control agents (BCAs) and nature-based compounds (NBCs) effective against *Phytophthora infestans*

BCAs and NBCs	<i>Bacillus</i> sp. JC12GB43	32 potato-associated bacterial strains	<i>Pseudomonas</i> strains
Experimental context	<i>In vitro</i>	<i>In vitro</i>	<i>In vitro</i> Controlled environment
References	Cray et al., 2016	Hunziker et al., 2015	Guyer et al., 2015
Biocontrol effects and mechanisms	Near-complete inhibition of <i>P. infestans</i> was observed.	Amongst the 32 strains tested, most belonged to the <i>Pseudomonas</i> genus and this genus had the highest proportion of active isolates. Some strain like R32 or R84 inhibited the growth of <i>P. infestans</i> by 100%. Volatile compounds produced by bacteria inhibited the growth of <i>P. infestans</i> . Analysis of the released volatile revealed the production of 1-undecene compound, an antifungal compound.	<i>In vitro</i> , <i>P. chlororaphis</i> R47 and R32 limited the growth of <i>P. infestans</i> to 8% and 10% as compared to the control. On leaf disk assay, <i>P. fluorescens</i> R76 strongly decreased the surface of necrosis (8% against 48% for the untreated control) but was unable to reduce the formation of sporangiophores. In contrast, <i>P. chlororaphis</i> R47 and <i>P. protegens</i> CHA0 significantly inhibited sporangiophore production.

BCAs and NBCs	<i>Bacillus</i> , <i>Pseudomonas</i> , <i>Rahnella</i> , and <i>Serratia</i>	<i>P fluorescens</i> S22:T:04 <i>P fluorescens</i> Sll:P:12. <i>P fluorescens</i> P22:Y:05 <i>Enterobacter cloacae</i> SI	<i>Streptomyces melanosporofaciens</i> EF-76
Experimental context	<i>In vitro</i> Growth chamber	Controlled environment (Storage bay)	<i>In vitro</i>
References	Daayf et al., 2003	Slininger et al., 2007	Clermont et al., 2011
Biocontrol effects and mechanisms	Eleven bacteria (P1, B2, Y1, B3, W1, M1, B1, C1, DF35, DF40, and W2) provided 50 to 89% inhibition of <i>P. infestans</i> growth. On detached leaves, DF1 was the most effective strain with inhibition of <i>P. infestans</i> growth above 75%. On plant experiment, B1 and W2 had the highest local inhibition of <i>P. infestans</i> growth (60%) and M1, P1 and B1 had the highest systemic effect (inhibition around 70%).	Disease suppression ranged from 35% up to 86% during the first year of assay and from 35 to 91% for the second year. The highest biocontrol effect was obtained with a mix of the four strains, then with <i>P fluorescens</i> S22:T:04, and <i>P fluorescens</i> Sll:P:12 alone.	The bacteria decreased soft rot symptoms caused by <i>P. infestans</i> by 40%. Activity of the strain was linked with the production of secondary metabolites such as eldanamycin, an antibiotic, as well as other secreted metabolites.

BCAs and NBCs	<i>Pseudomonas koreensis</i> 2.74	<i>Metschnikowia pulcherrima</i> <i>Curibasidium pallidicorallinum</i> <i>Candida saitoana</i>	<i>Bacillus subtilis</i> <i>Bacillus pumilus</i>
Experimental context	Greenhouse Leaf assays	Greenhouse Leaf assays	Storage trials
References	Hultberg et al., 2010	Hadwiger et al., 2015	Gachango et al., 2012
Biocontrol effects and mechanisms	Direct effect of the BCA on <i>P. infestans</i> was observed, with decrease of mycelial growth of 28, 26, 22 and 12%, 3, 6, 8 and 12 days after infection with <i>P. infestans</i> respectively. A significant reduction in the diameter of lesions was observed when the leaves were treated with <i>P. koreensis</i> 2.74 (more than 50% diameter of lesions decrease).	<i>M. pulcherrima</i> was the most effective in suppressing late blight symptoms. Maximal protection against <i>P. infestans</i> was found in the treatment using <i>M. pulcherrima</i> cells in the range of 4.3×10^6 through 3.5×10^7 cells/ml. The % of leaf infection ranged from 0 to 18% at those concentration compared to the 27% in control, 7 days after infection.	<i>Bacillus subtilis</i> induced a near complete inhibition of tuber late blight the first trial and reduced by half the disease severity the second trial.

BCAs and NBCs	<i>Bacillus subtilis</i> var. <i>amyloliquefaciens</i> FZB24	52 <i>Bacillus</i> spp. and eight <i>Pseudomonas</i> spp.
Experimental context	Glasshouse Field	<i>In vitro</i> , Greenhouse Assays Field
References	Keerthana et al., 2018	Caulier, Gillis, et al., 2018
Biocontrol effects and mechanisms	Significant reductions by 49.7% of late blight severity compared to the control. The potato plants inoculated with <i>B. subtilis</i> var. <i>amyloliquefaciens</i> showed higher activity of defense-related enzymes such as peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase, which resulted in higher accumulation of total phenols as compared to the untreated controls. Under field conditions, <i>B. subtilis</i> var. <i>amyloliquefaciens</i> reduced the severity of late blight disease on potato (45.9% reduction in disease severity) with higher tuber yield (15.5 t/ha) as compared to the controls (9.4 t/ha).	<i>In vitro</i>, the antagonism against late blight (<i>P. infestans</i>), 18 <i>Bacillus</i> spp. and 7 <i>Pseudomonas</i> spp. strains displayed a strong inhibitory activity Growth Inhibition Percentage (GIP) $\geq$ 70%. In greenhouse, three <i>Bacillus</i> spp. have shown significant protection index compared to control (PI > 60 %) (30B-B6, PI: 67%; 15A-B2, PI: 65%; 17A-B3, PI: 62%) and two <i>Pseudomonas</i> spp. (44R-P8, PI: 83%; 43R-P1, PI: 64%) gave significant protection against <i>P. infestans</i> <i>B. subtilis</i> 30B-B6 strain was able to decrease from 44% the late blight severity and showed significant protection until the end of the pilot field trial.

BCAs and NBCs	12 fungal endophytes	<i>Rhizophagus irregularis</i> MUCL 41833	<i>Chaetomium globosum</i> Isolate Cg-6
Experimental context	<i>In vitro</i> Growth chamber	<i>In vitro</i>	Field
References	de Vries et al., 2018	Gallou et al., 2011	Shanthiyaa et al., 2013
Biocontrol effects and mechanisms	<i>Phoma eupatorii</i> 8082 caused the greatest average relative growth inhibition of <i>P. infestans</i> with 50.6% growth inhibition. <i>Phoma eupatorii</i> isolate 8082 nearly inhibited completely infection by <i>P. infestans</i> in <i>planta</i> , with significant decrease of molecular detection of the pathogen (3.9 less in relative abundance).	Reduction by 25 % of the area under disease progress curve 1 and 2 days post infection by <i>P. infestans</i> . Induction of two pathogenesis related genes (PR1 and PR2) in the leaves of mycorrhizal plants 24h after infection with <i>P. infestans</i> .	<i>C. globosum</i> decreased late blight infection in potato plants and leaves infection index by 30%.

BCAs and NBCs	26 plant extracts	<i>Trichoderma</i> strains	<i>Pseudomonas</i> strains
Experimental context	<i>In vitro</i>	<i>In vitro</i> <i>In planta</i> Field	<i>In vitro</i>
References	Yanar et al., 2011	Yao et al., 2016	De Vrieze et al., 2018
Biocontrol effects and mechanisms	Plant extracts from <i>Xanthium strumarium</i> , <i>Lauris nobilis</i> , <i>Salvia officinalis</i> and <i>Styrax officinalis</i> were the most active and completely inhibited <i>P. infestans</i> radial growth.	<i>In vitro</i> , the <i>Trichoderma</i> isolate HNA11 showed the highest inhibition value of mycelium growth (60.4 %), followed by HNA14 with a percentage of reduction of 56.8 %. <i>In planta</i> , the lowest disease index (10.0) was obtained with <i>Trichoderma</i> isolate HNA12, followed by isolate HNA14 with a disease index of 11.5 compared to the control 42.5. Under field conditions, the <i>Trichoderma</i> isolate HNA14 was the most efficient against the pathogen out of all the <i>Trichoderma</i> strains tested and significantly reduced by almost half the disease severity compared to the control.	<i>Pseudomonas</i> S19 and S49 in combination were particularly efficient with complementary modes of action. S49 strain had much stronger inhibiting effect on mycelial growth than S19. S19 strain was a very efficient inhibitor of zoospore release. Three strains (R32, R47 and S49) were able to inhibit completely the mycelial growth of <i>P. infestans</i> .

BCAs and NBCs	24 Cu-free alternative products (chitosan, citrus product and liquorice)	<i>Chaetomium</i> -mycofungicide Strains of cellulose degrading fungi	<i>Trichoderma viride Pseudomonas fluorescens</i>
Experimental context	Field trials	<i>In vitro</i> Field	<i>In vitro</i> Greenhouse
References	Nechwatal and Zellner, 2015	Soytong et al., 2005	Zegeye et al., 2011
Biocontrol effects and mechanisms	In a plot trial, chitosan (4.5% infection rate) and citrus product 2 (3.1% infection rate) and liquorice products (2.4% infection rate) cause disease reduction compared to control (21% infection rate). In larger field test, one month after late blight outbreak, chitosan as well as copper applications were able to reduce late blight (between 10-20% of leaf area infection).	Chaetomium-mycofungicide inhibited <i>P. infestans</i> growth by more than 53% over 10 days in an <i>In vitro</i> dual test experiment. Under field, <i>Chaetomium</i>-mycofungicide reduced late blight incidence by 19% and pathogen colonization by 29% leading to an increased yield of 45.5% compared to the control.	<i>In vitro</i>, <i>Trichoderma viride</i> reduced by 36.7% radial growth of <i>P. infestans</i> after 3 days and a complete overgrowth of <i>T. viride</i> on <i>P. infestans</i> was observed after 9 days, whereas <i>P. fluorescens</i> inhibited the radial growth of the pathogen by 88%. <i>T. viride</i> and <i>P. fluorescens</i> reduced the disease severity by 75% and 25%, respectively as compared to the control. The combination of both microorganisms did not show any effect on late blight development.

BCAs and NBCs	53 copper-free preparations (CFPs) based on natural compounds, including plant extracts	chitosan oligomers (COS) and pectin-derived oligogalacturonides (OGA)	Essential oils (lavender, thyme, thyme borneal, and oregano) <i>Bacillus subtilis</i> QST 713
Experimental context	<i>In vitro</i> Growth chamber Field	Greenhouse	<i>In vitro</i> Growth chamber
References	Dorn et al., 2007	Clinckemallie, Decroës, et al., 2017	Olanya and Larkin, 2006
Biocontrol effects and mechanisms	<i>In vitro</i>, 19 CFPs inhibited germination of sporangia at 100% and 17 CFPs inhibited mycelial growth at 100%. In growth chamber, <i>Xenorhabdus bovienii</i> reduced foliar blight by 85%. In field experiment, maximal efficacy was 17% and no effect on tuber yield was observed with the CFPs.	Mean percentage of leaf area covered by symptoms increased to $73 \pm 24\%$ for control plants and $27 \pm 12\%$ for COS-OGA treated plant. The expression of <i>PR-1</i> and <i>PR-2</i>, encoding for basic PR-1 protein and acidic PR-2 protein, was significantly up-regulated after COS-OGA treatments, whether inoculated or uninoculated, from the first day after the last treatment (0 hpi) until 72 hpi. At 72 hpi, a significant up-regulation of the <i>PI-1</i> gene	In <i>vitro</i>, all treatments significantly reduced growth of <i>P. infestans</i>. <i>B. subtilis</i> strain QST 713 and oregano (essential oil) consistently inhibited growth of <i>P. infestans</i> over time. In growth chamber, oregano reduced late blight severity by 20-38%, and <i>B. subtilis</i> strain QST 713 by 30-40%.

e. Regulation framework

One of the major drawbacks for implementing the application of BCAs is the legislation. Indeed, biological control products based on microorganisms are considered as plant protection products in most countries. Modes of action may include competition, antibiosis, parasitism and also induced systemic resistance (ISR) which is mediated by the plant (du Jardin, 2015). Consequently, government regulation for registration and use are applied in the same way as for synthetic plant protection products.

Toxicology studies must be conducted and evaluation for producers, consumer, and user's exposure must be done. In the USA, the environmental protection agency (EPA) relies on the *a priori* assumption that biocontrol products are safer than chemical products. This considerably simplifies the registration process, which is completed within 12 to 24 months (compared with a mean of 84 months in Europe) (Velivelli et al., 2014). Furthermore, characterization of the active substance (e.g. identification, mode of activity, toxicity testing) must be conducted, and the microbial product requirements (e.g., formulation) have to be presented. An additional EU-specific requirement is the validation of the formulation efficacy. In every EU member state where a product is awaiting authorization, data certifying product efficacy and safety must be provided over a 24-month period. BCAs registration are twice as long and twice the price of a biofertilizer registration (Velivelli et al., 2014). Furthermore, ideally an understanding of the mode of action of the plant bioregulators on the molecular level requires the identification of the receptor site for each regulator, as well as the elucidation of the subsequent reactions. Unfortunately, identification of the molecular targets of all bioavailable (frequently uncharacterized) compounds within a given extract cannot be easily achieved. Therefore, most of the registration are made under the biostimulant legislation i.e. *any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits regardless of its nutrients content or biofertilizer* (du Jardin, 2015). A biofertilizer is *any bacterial or fungal inoculant applied to plants with the aim to increase the availability of nutrients and their utilization by plants, regardless of the nutrient content of the inoculant itself* (du Jardin, 2015). Biofertilizers may also be defined as microbial biostimulants improving plant nutrition efficiency (du Jardin, 2015).

IV. Plant defense

1. Introduction

Plants are susceptible to various pests and diseases caused by a wide diversity of organisms (e.g. bacteria, fungi, viruses, nematodes, insects). Plants, unlike mammals, lack mobile defender cells and a somatic adaptive immune system. Instead, they rely on the innate immunity of each cell and on systemic signals originating from infected sites (Jones and Dangl, 2006a). To survive and prosper, plants have developed various defense mechanisms, many of which being induced by pathogen attack (Glazebrook, 2005). Basically plant immunity is based on two defense mechanisms: (1) pattern triggered immunity (PTI), which is an incomplete (or quantitative) resistance mechanism based on microbes or pathogen associated molecular patterns (MAMP or PAMP), previously named basal disease resistance; (2) effector triggered immunity (ETI), which is a complete (or qualitative) resistance mechanism, also known as (R) gene-based resistance (Boller and Felix, 2009; French et al., 2016). PTI and ETI are aspects of a single overarching principle, the perception of danger signals (Boller and Felix, 2009). In quantitative disease resistance, phenotype follows a continuous distribution from susceptibility to resistance in host populations. Conversely, in qualitative resistance the plant is either susceptible or resistant (Roux et al., 2014). Loci controlling quantitative resistance contain some of the same causal genes that mediate qualitative resistance, but the predominant mechanisms of quantitative resistance extend beyond pathogen recognition. Indeed, most genes involved in quantitative resistance encode specific defense-related outputs such as strengthening of the cell wall or biosynthesis of defense compounds, but also a wide range of genes (e.g. kinases, metabolic enzymes, transporters) (French et al., 2016). Conversely, qualitative resistance loci are predominantly involved in detecting pathogens and/or transmitting signals resulting from pathogen detection (Corwin and Kliebenstein, 2017).

The perception of danger signals occurs around wounding and sites of pathogen attack. In addition to local defense responses, a pathogen attack can trigger defense responses far from the site of attack. This mechanism is called systemic acquired resistance (SAR). Systemic acquired resistance is a distinct signal transduction pathway involving

notably hormone modulation (i.e. salicylic acid accumulation) and pathogen related (*PR*) genes (Ryals et al., 1996).

Beneficial microbes of the plant microbiome can elicit a mechanism called induced systemic resistance (ISR). Those microorganisms prime plant defense allowing a faster and stronger response when disease outbreak occur (Pieterse et al., 2014a).

Amongst the beneficial microorganism, AMF have been shown to protect plant against a wide range of pathogens via the mycorrhiza induce resistance (MIR) (Pozo and Azcón-Aguilar, 2007).

a. Pathogen recognition

There are two pathways in the plant immune system recognition. One uses transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs), such as flagellin (Boller and Felix, 2009). The second acts largely inside the cell, using the polymorphic NB-LRR protein products encoded by most R genes (Dangl and Jones, 2001). They are named after their characteristic nucleotide binding (NB) and leucine rich repeat (LRR) domains. NB-LRR proteins are broadly related to animal Caterpillar/NOD/NLR proteins and Stand ATPases (Ting and Davis, 2005). Pathogen effectors from diverse kingdoms are recognized by NB-LRR proteins and activate similar defense responses. NB-LRR-mediated disease resistance is effective against pathogens that can grow only on living host tissue (i.e. obligate biotrophs) or hemibiotrophic pathogens, but not against pathogens that kill host tissue during colonization (i.e. necrotrophs) (Glazebrook, 2005).

b. The “Zig Zag” model

The Zig-Zag model of plant immunity incorporates PTI and ETI into a single model. Natural selection results in new ETI specificities so that the plant immune system can be represented as a five phase ‘zigzag’ model (Fig. 9).

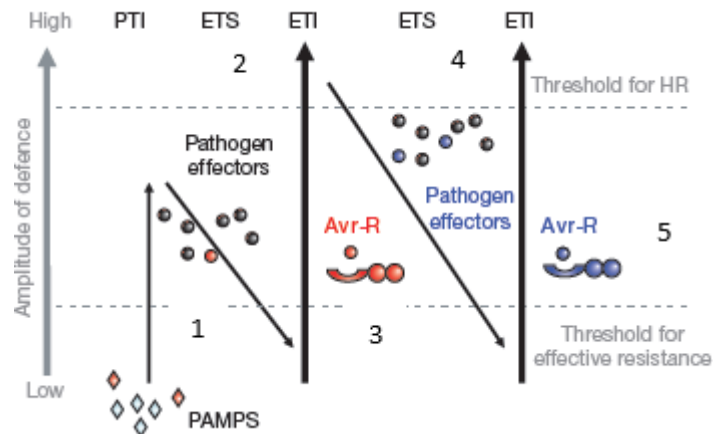


Figure 9. A zigzag model illustrates the quantitative output of the plant immune system. In phase 1, plants detect microbial/pathogen-associated molecular patterns (MAMPs/PAMPs, red diamonds) via PRRs. In phase 2, pathogen produce effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) induce hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the red effector or gain new one (blue). In phase 5, selection favors new plant NB-LRR alleles. Plant avirulent R gene (Avr-R) (Jones and Dangl, 2006a).

In phase 1 (Fig. 9), PAMPs (or MAMPs) are recognized by PRRs, resulting in PAMP-triggered immunity (PTI) that can halt further infection. In phase 2, successful pathogens deploy effectors that contribute to pathogen virulence. Effectors can interfere with PTI. This results in effector-triggered susceptibility (ETS). In phase 3, a given effector is ‘specifically recognized’ by one of the NB-LRR proteins, resulting in effector-triggered immunity. Recognition is either indirect or through direct NB-LRR recognition of an effector (Jones and Dangl, 2006b). Effector-triggered immunity (Jones and Dangl, 2006b) are formerly called R-gene-based or vertical resistance. Effector triggered immunity (ETI) is an accelerated and amplified PTI response, resulting in disease resistance and, usually, a hypersensitive cell death response (HR) at the infection site and rapid production of reactive oxygen intermediates (ROI) called the oxidative burst. This may have a direct antimicrobial effect, as well as serving as a signal for activation of other defense responses. Production of reactive oxygen species (ROS) is associated with the HR and loosening/stiffening of the plant cell wall. HR is a type of programmed cell death thought to limit the access of the

pathogen to water and nutrients (Glazebrook, 2005). In phase 4, natural selection drives pathogens to avoid ETI either by shedding or diversifying the recognized effector genes, or by acquiring additional effectors that suppress ETI (Jones and Dangl, 2006a). PTI is generally effective against non-adapted pathogens in a phenomenon called non-host resistance, whereas ETI is active against adapted pathogens. However these relationships are not exclusive and depend on the elicitor molecules present in each infection (Dodds and Rathjen, 2010).

c. Plant defense: hormones and genes

ETI is also associated with activation of salicylic acid (SA)-dependent signaling pathway that leads to the expression of certain pathogenesis-related (PR) proteins contributing to the host resistance. Some other plant defense responses are controlled by mechanisms dependent on ethylene (ET) and/or jasmonate (JA) (Glazebrook, 2005). Accumulation of PR is observed in plants in which resistance is locally or systemically induced. PRs have dual cellular localization – vacuolar and apoplastic – the apoplast being the main site of their accumulation. Apart from being present in cell walls of infected plants, PRs are also found in cell wall appositions (papillae) deposited at the inner side of the cell wall in response to fungal attack (Edreva, 2005). PRs are recognized as markers of the SAR, and PRs genes are involved in the list of the so-called SAR-genes. In some pathosystems, mRNAs for certain PR members accumulate to similar levels in compatible and incompatible interactions, but the maximum level of expression is reached much faster in the latter. In soybean, the activation of the phenyl ammonia-lyase (PAL) gene, a marker of the phenylpropanoid pathway, is linked with an increased accumulation of total phenols and lignin (Abdel-Monaim, 2011).

d. Systemic plant defense induction

The perception of danger signals occurs in the immediate surroundings of wounding and at the sites of pathogen invasion. Apart from local defense responses, a microbial attack can trigger systemic defense responses. In recent years, two forms of systemic resistance have been described (Fig. 10), termed systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Boller and Felix, 2009). The different forms of induced resistance are regulated by a complex network of interconnecting signaling pathways in which plant

hormones play an important regulatory role (Pieterse et al., 2012). Induced resistance signaling pathways that are triggered by pathogens, beneficial microbes, and insects partly overlap and share common signaling components (Pieterse et al., 2014a).

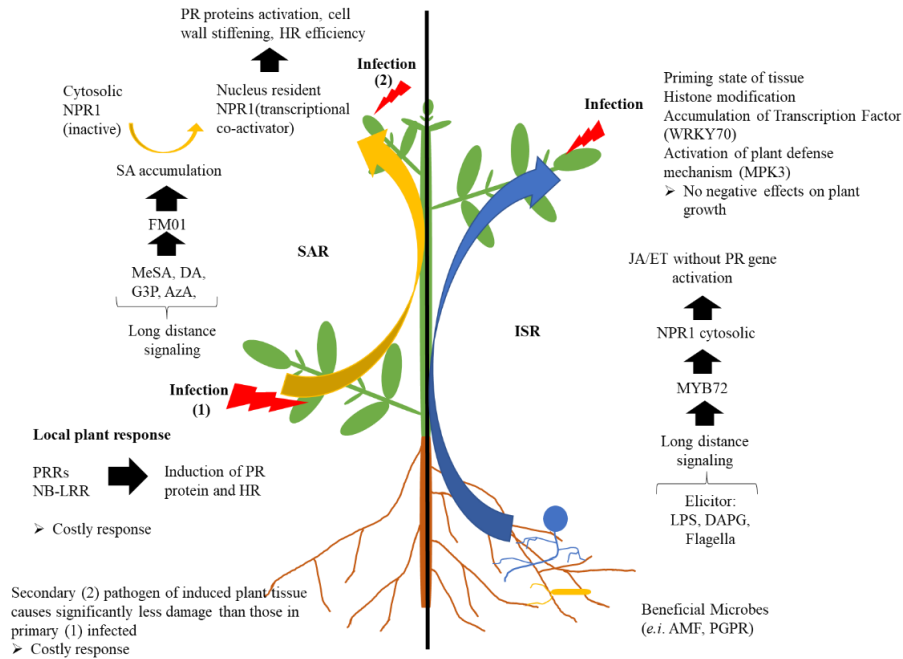


Figure 10. Schematic representation of systemic acquired resistance (SAR) and induced systemic resistance (ISR).

2. Pathogen-induced systemic acquired resistance (SAR)

The first formal characterization of SAR (Fig. 10) was made by Ross (1961). He observed that uninfected plant parts become more resistant in response to a prior infection elsewhere in the plant. Accumulating SA is enough to induce a subset of SA-responsive SAR genes such as the classical marker *PR-1* (Sticher et al., 1997). In tobacco, gene families are coordinately induced to high levels of expression during the onset of SAR. These gene families include the *PR-1* to 5 (Ward et al., 1991). *PR-1* is amongst the best characterized *PR* genes and in many plant species is used as a marker for SAR (van Loon et al., 2006). Even though, SA accumulates in the phloem sap of SAR-expressing plants, grafting experiments with tobacco showed that SA itself is not the mobile SAR signal (Vernooij et al., 1994). Genetic and biochemical studies uncovered several plant metabolites involved

in long-distance SAR signaling. These include the methyl ester of SA (MeSA), the diterpenoid (DA), a glycerol-3-phosphate (G3P)-dependent factor, azelaic acid (AzA), and pipecolic acid (Pip) (Pieterse and Van Wees, 2015). In systemic-triggered tissues, the onset of SAR requires the function of flavin-dependent monooxygenase 1 protein (FMO1) (Mishina and Zeier, 2006), possibly to transduce or amplify long-distance signals originating from primary leaves, which then results in enhanced SA biosynthesis in healthy tissues (Pieterse and Van Wees, 2015). The transcriptional co-activator *NPR1* is essential for SAR and is required for the predominant part of SA downstream responses, including activation of defense genes expression (Shah and Zeier, 2013). A model was suggested in which SA accumulation during SAR provokes redox changes driving the transition from the inactive, cytosolic NPR1 oligomer, to the active nucleus-resident NPR1 monomer (Mou et al., 2003). NPR1 has been identified as a direct SA receptor unraveling that SA perception and subsequent transcriptional activation of defense genes are contiguous events (Wu et al., 2012), leading to systemic defense.

3. Induced systemic resistance (ISR) by beneficial microbes

Besides pathogens, large communities of commensal and mutualistic microorganisms interact with plants (Fig. 10) providing protection or increasing resistance against pathogens (Pieterse and Van Wees, 2015). It was demonstrated that colonization of plant roots by selected strains of plant growth-promoting rhizobacteria (PGPR) can stimulate the plant immune system in above-ground plant parts, resulting in a broad-spectrum disease resistance called rhizobacteria-mediated ISR (Alström, 1991). Since the first publications on rhizobacteria-mediated ISR, hundreds of studies in dicots and monocots have reported on the ability of PGPR or plant growth-promoting fungi (PGPF) to promote plant health via ISR. These studies mainly concerned *Pseudomonas*, *Serratia*, and *Bacillus* PGPR strains and nonpathogenic *Fusarium oxysporum*, *Trichoderma*, and *Piriformospora indica* PGPF strains. Arbuscular mycorrhizal fungi were also shown to trigger ISR (Pieterse et al., 2014a). In order to stimulate ISR, beneficial microbes must produce elicitors lipopolysaccharides (LPS), other microbial ISR elicitors including antibiotics, like 2,4-diacetylphloroglucinol (DAPG) and pyocyanin, flagella, N-acyl homoserine lactones, siderophores, and biosurfactants that are responsible for the onset of systemic immunity (Pieterse et al.,

2014a). Because of its broad-spectrum effectiveness, ISR was initially thought to be mechanistically similar to pathogen-induced SAR. However, along with SA, the plant hormones JA and ET are also important regulators of the plant immune system (Thomma et al., 2001). By using *Arabidopsis* mutants impaired in JA or ET signaling, it was demonstrated that JA and ET are central players in the regulation of rhizobacteria-mediated ISR (Pieterse et al., 1998). Although ISR by beneficial microbes is often regulated through SA-independent mechanisms, several PGPR have been reported to trigger an SA-dependent type of ISR that resembles pathogen induced SAR (Pieterse et al., 2014a).

The first regulatory protein identified as being essential for ISR was NPR1 (Pieterse et al., 1998). While in SAR, *NPR1* functions as a transcriptional co-activator of SA-responsive *PR* genes, JA/ET-dependent ISR typically functions without *PR* gene activation. Hence, the role of *NPR1* in ISR seems to be different from that in SAR. In SA signaling, *NPR1* is clearly connected to a nuclear function (Fu and Dong, 2013), while in JA/ET signaling and ISR evidence is accumulating for a cytosolic function of *NPR1* (Pieterse and Van Wees, 2015).

Additionally a transcriptional regulator factor gene *MYB72*, was postulated to play an important role in the generation and/or translocation of a long-distance ISR signal (Segarra et al., 2009). Furthermore, Mathys et al. (2012) have shown that ISR did not negatively affect translation and photosynthesis processes. This may explain why ISR is relatively well-tolerated by the plants and does not cause any visually observable negative effects on their growth, contrarily to observation in SAR.

4. Priming

a. Introduction

Priming is a unique physiological state of plants. Indeed, primed plants display either faster, stronger or both activation of the various cellular defense responses that are induced following attack by pathogens, beneficial interactions with microorganism, or in response to abiotic stresses (Conrath et al., 2006). Stimuli caused by pests and diseases or beneficial microbes as well as chemicals and abiotic cues can trigger the establishment of priming by acting as warning signals (Mauch-Mani et al., 2017). Pathogen-derived molecules can be of

different biochemical natures (peptides, polysaccharides or lipids). Lipopolysaccharides and flg22 are probably the best-known priming PAMPs (Mauch-Mani et al., 2017).

b. Priming defense triggers

Plant-growth-promoting rhizobacteria and plant-growth-promoting fungi, both of which induce systemic resistance, can also trigger defense priming (Pieterse et al., 2014a). The most studied PGPRs belong to the genus *Pseudomonas*, followed by those belonging to *Serratia* and *Bacillus*. The most studied PGPFs are *Trichoderma* spp., nonpathogenic strains of *Fusarium* spp., *P. indica*, and AMF (Jung et al., 2012; Mauch-Mani et al., 2017). Inhibition of steps in primary metabolism (starch accumulation) can lead to the induction of a primed state that sensitize the tissue for improved activation of various cellular defense responses (Conrath et al., 2006). The release of plant volatile organic compounds (VOCs) can prime distal plant parts and neighboring plants (Frost et al., 2008). Priming has been associated with only marginal reductions in growth, whereas induction of direct defense may cause much stronger reductions in plant growth and even reduced seed production. Thus, induction of priming involves lower costs than direct defense (van Hulten et al., 2006).

c. Priming steps

Sequential steps of priming have been described. Stimuli coming from pathogenic or beneficial fungi, bacteria, rhizobacteria, arthropods, and abiotic stresses are perceived by the plant, leading to a slight induction of various compounds and activities in the priming phase. These compounds are referred as the priming fingerprint (involving notably ROS, genes activation, and cytosolic calcium). Upon challenge with an attacker, these primed plants display an enhanced perception of the attackers and therefore can express a more robust defense against it in the post-challenge primed state (i.e. PRs, callose, histone modifications) (Balmer et al., 2015; Mauch-Mani et al., 2017). The priming phase refers to the biological process of acquiring priming, which takes place from the initial stimulation through the exposure to a challenging stress. It includes all changes that occur in the plant after the perception of a stimulus and prepare the plant for enhanced response when a challenge occurs. These changes can take place at the physiological, molecular, and epigenetic levels. They can

occur within seconds or hours after stimulation and can be transient or maintained throughout the lifetime of a plant. Furthermore, these changes can even be inherited by subsequent generations. Different priming stimuli may cause similar changes as well as specific ones (Mauch-Mani et al., 2017). Transient changes in the level of intracellular calcium occur within a few seconds or minutes and are among the best-known early responses to stimulation (Campos-Soriano et al., 2011). Cytosolic calcium lead to membrane depolarization and the increase in intracellular calcium can precede the generation of reactive oxygen species (ROS) (Jeworutzki et al., 2010). It also lead to the accumulation of inactive unphosphorylated mitogen-activated protein kinase 3 or 6 (MPK3 and MPK6), which plays a pivotal role in the rapid activation of phosphorylation dependent defense mechanism (Conrath et al., 2015).

d. Epigenetic part of priming

Priming is based on the assumption that certain alterations serve as pivotal changes that affect a future response. Therefore, it has been hypothesized that changes at the epigenetic level could influence the way plants respond to biotic and abiotic stresses. Indeed, it has been shown that expression of the *WRKY70* gene, which encodes an SA-dependent transcription factor involved in crosstalk with JA, was dependent on trimethylation of histone H3 at lysine 4 (Alvarez-Venegas et al., 2007). Stimuli can change the epigenetic status by post-translational modifications and are not linked to direct gene expression (Jaskiewicz et al., 2011). Modifications of histones or DNA methylation levels can serve as pivotal mechanisms to facilitate transcription of defense-dependent genes upon subsequent challenges (Mauch-Mani et al., 2017).

e. Primed state

A key trait for resistance in plants is the fast and efficient perception of their surroundings. Recent discoveries suggested that the enhanced perception of attackers is an important aspect of defense priming (Mauch-Mani et al., 2017). This post-challenge primed state is suggested by the evidence that *Arabidopsis* plants treated with the SA analog BTH increased their levels of pattern recognition receptors and co-receptors (Tateda et al., 2014). Primed callose deposition is a widely used marker of successful enhanced responsiveness to fungi,

oomycetes, bacteria, or PAMP applications at the sites of attempted colonization or application (Kohler et al., 2002; Mauch-Mani et al., 2017). One of the main signaling components during defense, MPKs exhibit enhanced activation in primed plants upon pathogen challenge or abiotic stress (Conrath et al., 2015; Yi et al., 2015). Trans-generational priming is known to be associated with chromatin remodeling and DNA methylation (Slaughter et al., 2008; Luna and Ton, 2012).

The attractiveness of priming for agricultural protection is also associated with the fact that this phenomenon, unlike the direct activation of defenses, does not need major developmental costs (van Hulten et al., 2006).

5. Mycorrhiza induced resistance

a. Introduction

The benefits of the AMF symbiosis on plants fitness are well documented, including an improved mineral nutrition and increased ability to overcome abiotic or biotic stresses (Pozo and Azcón-Aguilar, 2007). Concerning the biotic stresses, AMF bio-protection has been shown principally against soil-borne pests and diseases (Whipps, 2004) but can also be effective against above-ground attackers such as insects or pathogens (Pozo and Azcón-Aguilar, 2007). Several mechanisms have been shown to play a role in plant protection by AMF: improved plant nutrition, damage compensation, competition for colonization site or photosynthates, changes in root physiology and exudation, qualitative and quantitative changes in rhizosphere microbial populations, locally and systemically priming of plant defense. It is obvious that several of these mechanisms can be involved simultaneously (Pozo and Azcón-Aguilar, 2007).

b. Mycorrhiza induced resistance in belowground plant parts

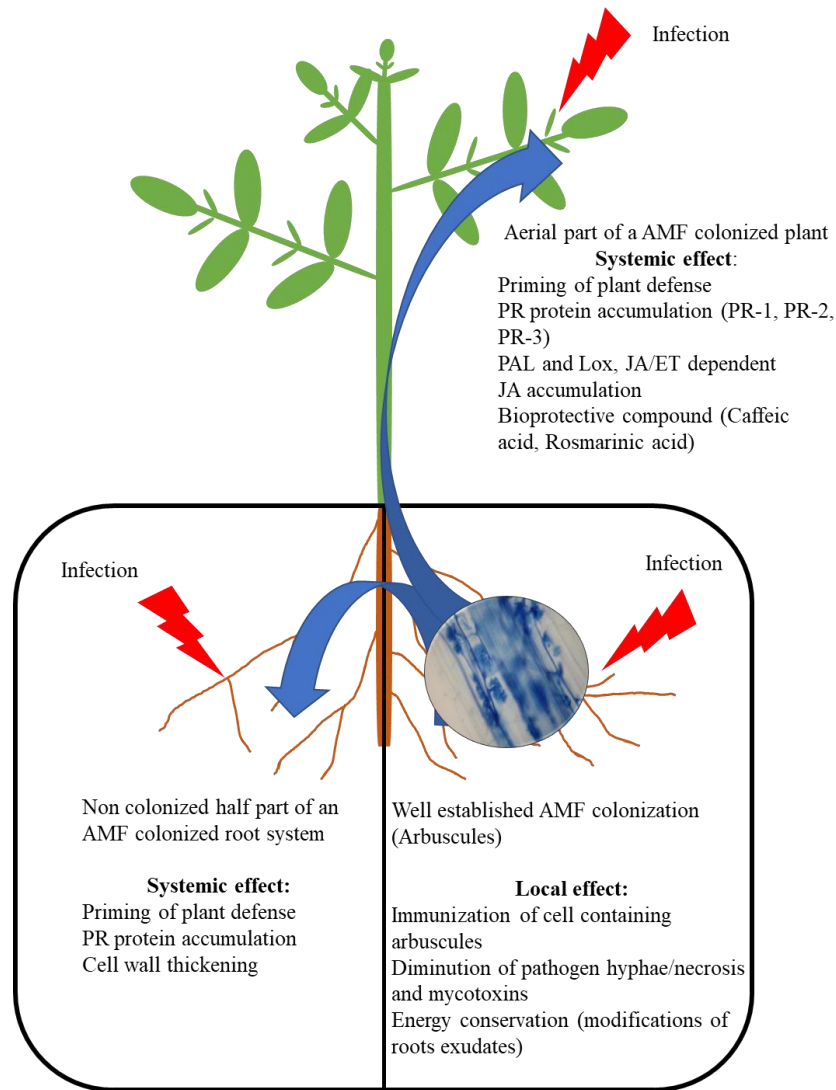


Figure 11. Schematic representation of Mycorrhiza Induced resistance in plants.

Mycorrhiza-induced resistance (MIR) provides systemic protection against a wide range of attackers and shares characteristics with systemic acquired resistance (SAR after pathogen infection and ISR following root colonization by non-pathogenic rhizobacteria)

(Cameron et al., 2013). Inoculation with *Glomus mosseae* (renamed *Funneliformis mosseae*) can compensate the negative impact of lower levels of phosphate fertilization in petunia plants growth and induce AMF bio-protection effects regardless of the phosphorus fertilization (Hayek et al., 2012). Furthermore, Fritz et al. (2006) demonstrated that MIR cannot be solely attributed to improved nutrition. Root protection by AMF has been shown to be more effective on plants harboring a complex root system (i.e. with a finely branched root system), compared to plant roots with simple system (Sikes et al., 2009). MIR (Fig. 11) have a local induced resistance component (Sikes et al., 2009) and also an ISR part (Pozo et al., 2002). Reduced pathogen development was reported in tomato plants associated with AMF (Cordier et al., 1996) as well as a decrease of root necrosis in AMF colonized tomato plants (Trotta et al., 1996). Control of a virulent fungal pathogen can lead to a reduction of mycotoxins (i.e. trichothecenes) in tubers and roots of potato plants (Ismail et al., 2013). Modification of root exudates induced by AMF does not seem to participate in the bio-protective effect of AMF (Lioussanne et al., 2009) but seems to be involved in energy conservation by suppressing specific root exudates such as proline (Ren et al., 2015). Immunization of tissues containing arbuscules have been shown in tomato plants (Fig. 11) and ISR in distant parts of the root system (i.e. PR protein, wall thickening) (Cordier et al., 1998; Zhang et al., 2018). Mycorrhizal colonization contributes significantly in maintaining the redox balance during oxidative stress, allowing a better management of HR (Alejo-Iturvide et al., 2008). Local bio-protection has been observed in *M. truncatula* mutants encoding for the allene oxide cyclase 1 (AOC1, a key enzyme in JA biosynthesis) suggesting that jasmonates do not play a major role in the local bio-protection effect of AMF in *M. truncatula* (Hilou et al., 2014). Elicitation of plant defense has been observed during the first step of AMF colonization, with locally and transitory activation of phytoalexine, glucanase, cysteine-rich proteins, a protease inhibitor, a cytochrome P450 and an isoflavonoid glucosyl transferase (Gianinazzi-Pearson, 1996; Gianinazzi-Pearson et al., 1996; Liu et al., 2007). MIR is induced if colonization by AMF is prior to infection (Rosendahl, 1985; Pozo et al., 1999; Dehariya et al., 2018) and transitory expression of gene defense has been observed around arbuscules (Dassi et al., 1998; Pozo et al., 1998). Arbuscules have been shown to be necessary to observe a bio-protection effect (Elsen et al., 2008). Phosphorus nutrition decrease root colonization and arbuscules formation, leading to a reduction of MIR in tomato plants (Fritz et al., 2006). MIR effect

is dependent of the AMF species considered (de la Noval et al., 2007; Lioussanne et al., 2009; Mustafa et al., 2016) but also on the plant cultivar (Castellanos-Morales et al., 2011; Steinkellner et al., 2012).

Shifts in the microbial communities of the rhizosphere may indirectly influence the outcome of plant interactions with other organisms including pathogens (Jung et al., 2012). AMF in tomato plants have been shown to modify the nutritional quality of herbivorous insects, leading to a better acceptance of this prey for the predator (Prieto et al., 2017).

c. Mycorrhiza induced resistance and systemic response

The systemic part of the MIR (Fig. 11) also induce changes in gene expression level in non-AMF colonized part of an inoculated root system (Liu et al., 2007). Grafting experiment on common bean have shown that grafting into mycorrhizal rootstocks induced MIR (Mora-Romero et al., 2015). This suggested that a signal moves from colonized roots to the upper parts of plants, enabling the induction of pathogen defense in non-mycorrhizal branches grafted to mycorrhizal rootstocks (Mora-Romero et al., 2015). MIR can also induce a faster and stronger plant defense gene expression in leaves during pathogen infection. Priming of *PR-1* and *PR-2* have been observed on potato plants challenged by *P. infestans* (Gallou et al., 2011), and *PR-3* against *Fusarium sambucinum* (Ismail and Hijri, 2012). Despite systemic priming of *PR* genes (Fig. 11), the systemic bio-protection effect does not seem to be dependent on the SA signaling pathway (Khaosaad et al., 2007). AMF pre-inoculation led to significant increases in activities of β -1,3-glucanase, chitinase, phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX) in tomato leaves upon pathogen inoculation and the JA signaling pathway was essential for mycorrhiza-primed disease resistance (Song et al., 2015). Additionally, the systemic increase in JA in response to AMF colonization plays a key role in expression of MIR against *Alternaria alternata*. Indeed JA levels increased twofold in AMF-colonized plants when subjected to *A. alternata* (Nair et al., 2015). MIR also induces production of bio-protection compound in leaves (i.e. caffeic acid and rosmarinic acid) (Toussaint et al., 2008). Priming seems to be the main mechanism operating in MIR (Pozo and Azcón-Aguilar, 2007). Gene over-expression is generally observed during the first hour post infection (Spagnoletti et al., 2017).

6. Common mycorrhizal network

a. Introduction

Plant roots are in constant contact with microorganisms (Le Fevre and Schornack, 2016) and soils contain extensive lengths of fungal hyphae. The density of the extraradical mycelium and its ability to form multiple entry points on the roots often leads to the formation of common mycorrhizal networks (CMN) that interconnect plants (Johnson and Gilbert, 2015). CMN can link plants belonging to the same species but also to different species (Rhodes, 2017). CMN formed by ectomycorrhiza fungi (ECM) allows nutrient transport and redistribution of minerals or water between plants of the same or different species (Selosse et al., 2006). CMN participate in processes allowing plants to recognize the degree of relatedness of neighboring plants. This “kin recognition” has been shown to be mediated by CMA or ECM (Simard, 2018).

The role of CMN formed by AMF in nutrient transfer from one plant to another has not been clearly demonstrated, but relocation of nutrient has been measured inside the CMN depending of C allocation by the plant and nutrient availability (Whiteside et al., 2019). The complexity of CMN formed by AMF is emphasized by the length of AMF extraradical hyphae. Indeed, in annual crops the length of ERM is estimated in the range of 2 to 8 m per g of soil and in grasslands, where there is minimal soil disturbance, between 68 and 101 m per g of soil (Leake et al., 2004).

b. Nutrients carrier

CMN formed by ECM have been shown to facilitate nutrients exchange (Simard et al., 1997), such as nitrogen, phosphorus (Selosse et al., 2006), or carbon (Selosse and Roy, 2009). Carbon transfer has been reported between photosynthetically active plants connected by the same ECM network. The magnitude of net C transfer was shown to be influenced by shading of the receiver plant. Indeed, plants grown under shaded conditions appear to be greater sinks for C (Simard et al., 1997). In controlled condition, CMN formed by AMF, have been shown to transfer C from a donor plant to hyphae connected to a receiver plant, but no C transfer to the aboveground parts of receiver plants was observed (Voets et al., 2008). AMF hyphae can transfer large

molecules such as lipids, amino acids and sugars (Bago et al., 2002). AMF hyphae employ cytoplasmic streaming as well as a full array of cytoskeletal proteins, such as “track-forming” microtubular arrays (Timonen et al., 2001), to translocate resources bi-directionally (Welte, 2009; Whiteside et al., 2019). AMF have been shown to regulate nutrient transfer depending of resource inequalities amongst the CMN (Welte, 2009; Whiteside et al., 2019).

c. Defense signal carrier

CMN formed by AMF have been shown to improve plant defense against insect herbivores (Babikova et al., 2013a; Song et al., 2014) and foliar necrotrophic fungi (Song et al., 2010) by acting as a “highway” for interplant signaling (Johnson and Gilbert, 2015). Signals have been shown to travel through CMNs of at least 20 cm in length (Babikova et al., 2013b). However, experiments so far have been carried out in pots or in situ leading to several limitations. Indeed, soil substrate makes it difficult to prevent effects of diffusion of molecules through the soil solution. It is also difficult to be sure that only one fungus connects the plants without other microorganisms, like bacteria (Gilbert and Johnson, 2017). The *in vitro* mycelium donor plant system developed by Voets et al. (2009) is promising to bypass such limitations.

The nature of signals transported by the CMN has been hypothesized to involve bacterial biofilm (Barto et al., 2012; Babikova et al., 2013a), molecule or electric signal (Johnson and Gilbert, 2015). CMNs may also act as conduit for transfer of semio-chemicals between connected plants (Dicke and Dijkman, 2001). The signals were detected in terms of measurable response by the receiver plant within 24 h of the donor being attacked by the pest, with peak response on the receiver plant between 48 h and 100 h (Gilbert and Johnson, 2015). Gene expression analyses of the receiver plant have revealed an up-regulation of *PR* genes (SA dependent) but also JA marker genes starting 18 h post infection of the donor plant (Song et al. (2010). Length of time over which signals are effective, meaning how long plant produces the defense response, remains unknown (Gilbert and Johnson, 2017).

RESEARCH OBJECTIVES

Arbuscular mycorrhizal fungi are widely distributed in arable soils. Their beneficial effects on plant growth and health are well established, but most of the observations so far have been made under controlled greenhouse conditions, while field results are more uncertain. This is linked to many reasons including the obligatory nature of these fungi (they require a host partner to complete their life cycle), their sensitivity to environmental conditions (e.g. agricultural practices – pesticides, fertilizers, monoculture) and competition with local flora. The obligate biotrophic nature of AMF make it difficult to successfully inoculate a specific strain to crops because local species will actively compete for the host. Similarly, linking an inoculant to the effects observed on plants is rather difficult since most techniques are based on the evaluation of root colonization by microscopy or on molecular techniques based on the genomic DNA, which are inadequate to identify AMF below the species level. In recent years, molecular markers based on the mitochondrial genome have been proposed and used to specifically identify AMF inoculants. These mitochondrial markers allow the identification of AMF at the strain level.

Arbuscular mycorrhizal fungi have been proposed as a potential alternative to chemicals (i.e. fertilizers and pesticides). They improve plant nutrition and increase their resistance/tolerance against abiotic and biotic stresses. Biocontrol induced by AMF has been amply described in different pathosystems but were mainly obtained in controlled environments (e.g. greenhouse) and against roots pathogens. Gallou et al. (2011) was the first to report a decrease in late blight symptoms in AMF-colonized potato plants grown *in vitro*, suggesting a potential benefit under field conditions. The potentiation of potato plant defense genes upon colonization by AMF involves mainly genes dependent or directly involved in signaling pathways like SA, JA and ET. Interestingly, the transfer of defense signals from diseased to healthy plants using common mycorrhizal networks (CMNs) as emerged in the last decade (Song et al., 2010; Babikova et al., 2013a; Babikova et al., 2013b; Song et al., 2014) as a potential mechanism to prepare healthy plants to resist to a pathogen. However, such observations were made in pots and could not completely exclude bacterial involvement via e.g. biofilm, further requiring *in vitro* studies. The first objective of the thesis was to develop a method for an adequate and effective colonization of potato plants in the field with a specific

AMF strain (i.e. *Rhizophagus irregularis* MUCL 41833). Indeed, AMF are ubiquitous organisms found naturally in arable soil and competition with indigenous AMF can often lead to unsuccessful development of introduced strains. Potato tubers were thus inoculated with a donor plant system in biodegradable pots in the greenhouse. The colonized plants were subsequently transferred to the field and inoculation success established with a molecular marker (MtLSU) allowing to discriminate the inoculant from any other indigenous AMF.

The second objective of the thesis was to study the effects of *R. irregularis* MUCL 41833 on the disease symptoms caused by *Phytophthora infestans* MUCL 54981 on potato plants (Bintje and Nicola) in a field experiment. In organic farming systems, disease management for late blight on potato is mainly achieved using copper. However, copper have detrimental environmental consequence and can negatively impact soil microorganisms. Alternative treatments must be promoted and evaluated, including the application of AMF. Inoculation was conducted over two cropping seasons and disease evaluated using adapted key of diseases assessment and rAUDPC.

The third objective was to evaluate whether a diseased plant can transfer warning (i.e. defense) signals to healthy plants via a common mycorrhizal network. Such observation was made recently under grow chamber/greenhouse conditions (Song et al., 2010; Babikova et al., 2013a; Babikova et al., 2013b; Song et al., 2014) avoiding to clearly establish the mechanisms involved. Using *in vitro* cultivation systems may help to understand the nature of the signal involved (chemical electrical or volatile).

To answer the objectives, three specific questions were addressed:

- Are molecular markers based on MtLSU specific to an inoculated AMF strain and can we link molecular quantification to roots colonization in greenhouse and field?
- Is *Rhizophagus irregularis* MUCL 41833 able to constrain late blight symptoms in the field and what are the environmental conditions more effective in the control of this disease by the AMF?
- Does extra radical hyphae connecting diseased to healthy plants transfer warning signals? What is the nature of the signals (chemical, electrical, volatile) and which are the transcriptional changes observed in the healthy plants?

MATERIAL AND METHODS

I. Organisms

i. Microorganisms

Two microorganisms were used in the experiments:

- The AMF *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) (Schüßler and Walker) as ['irregulare'] MUCL 41833. The strain was provided by the Glomeromycota *in vitro* collection (GINCO: <http://www.mycorrhiza.be/ginco-bel>). It was originally isolated from a soil in the Canary Island (Spain). Since then, it has been used *in vitro* for interaction studies with pathogens (e.g. Gallou et al. (2011); Gallou et al. (2012); Marquez et al. (2018a)) and in potato fields without (Loján et al. (2017)) or in combination with *Trichoderma harzianum* (e.g. Buysens et al. (2016)).
- The Oomycete *Phytophthora infestans* (mont.) de Bary MUCL 54981 – mating type A1. The strain was provided by the “Mycothèque de l’Université catholique de Louvain” (BCCM/MUCL - <http://bccm.belspo.be/about/mucl.php>). It was originally isolated by the “Centre Wallon de Recherches agronomiques” from a potato crop in Libramont (Belgium).

ii. Plants

Three plants were used in the experiments:

- The tuber crop *Solanum tuberosum* L. cultivars Bintje and Nicola. They were provided by the CRA-W, “Batiment Haute Belgique”, Libramont (Belgium). Bintje is a high yielding cultivar with numerous large caliber tubers. It represents 25% of potato cultivated in Belgium, in 2017 (Fiwap, 2018). However, this cultivar is highly susceptible to diseases such as late blight (caused by *P. infestans*), stem canker and black scurf (caused by *Rhizoctonia solani*) and common scab (caused by *Streptomyces* sp.). Nicola is a high yielding cultivar but with few large caliber tubers. Nicola is considered moderately resistant to late blight, and resistant to stem canker and black scurf and to common scab.

- The legume *Medicago truncatula* Gaertn. cv. Jemalong A 17 (SARDI, Australia). *Medicago truncatula* is a small annual plant with a short life cycle of two months. It is widely used for *in vitro* studies in association with AMF (e.g. Voets et al. (2008); de Boulois et al. (2009); Voets et al. (2009); Zhang et al. (2016); Gil-Cardesa et al. (2017)).
- The cereal crop *Zea mays* L. cultivar ES. Ballade (Euralis, France). This plant was used for mass production of the AMF *Rhizophagus irregularis* (MUCL 41833) (IJdo et al., 2011), due to a high colonization rate under controlled greenhouse conditions.

II. Study model and experimental set up

Most *in vitro* studies so far have been conducted with excised roots, the so-named root organ culture (ROC) system (see review by Fortin et al. (2002)). However, ROCs lack shoots and are thus inadequate to study the interaction between AMF and shoot pests or diseases. To overcome this problem, Voets et al. (2005) and Voets et al. (2009) developed whole plant *in vitro* cultivation systems, more precisely, the half-closed arbuscular mycorrhizal-plant (H-AMP) *in vitro* culture system and the mycelium donor plant (MDP) *in vitro* culture system. Both were used for instance by (Gallou et al., 2011 ; Marquez et al., 2018a; Marquez et al., 2018b) to study the interaction between AMF and *P. infestans*, *Macrophomina phaseolina* and *Fusarium virguliforme*, respectively. These systems are perfectly suited to investigate plant defense mechanisms and inter-plant communications. Indeed, they offer several advantages over the ROC system, which are the presence of photosynthetically-active tissues, representing a continuous carbon source for the AMF, and the absence of sugars and vitamins within the medium possibly impacting the physiology of the plant-fungus association (Fortin et al., 2002). Moreover, and similarly to the ROC, the whole plant *in vitro* culture systems allows non-destructive microscopic observations, the study of metabolism and transfer of nutrients from fungus to plant and vice versa and the study of molecular and physiological steps involved in the AMF roots colonization. The MDP system was used in Chapter III for inter-plant signaling studies via the common mycorrhiza network (CMN), and is detailed below:

1. Mycelium donor plant (MDP) *in vitro* culture system

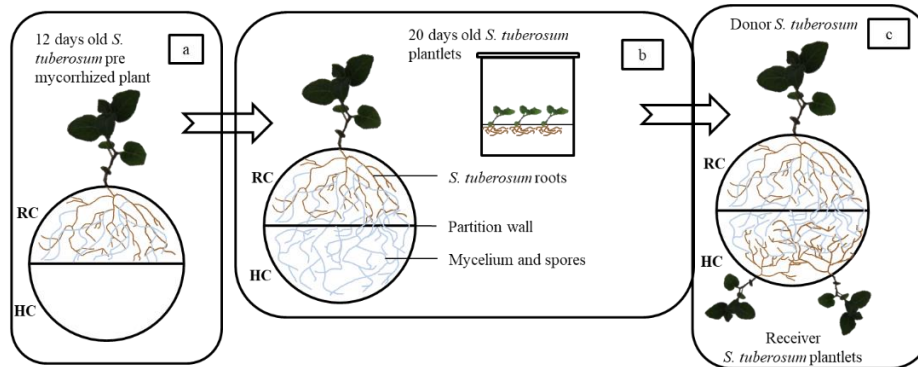


Figure 12. Schematic representation of the mycorrhizal donor plant *in vitro* culture system (adapted from Voets et al., 2009). RC (root compartment), HC (hyphal compartment). (a) Potato (donor) plant inoculated with *Rhizophagus irregularis* (MUCL 41833) in the RC of a bi-compartment Petri plate. The medium is the Modified Strullu Romand (MSR) without sucrose and vitamins. (b) After 5 weeks, the extra radical mycelium (ERM) of the fungus crosses the plastic barrier separating the RC from the HC and (c) Two newly (receiver) potato plantlets are plated in the HC with their roots in contact with the ERM and shoot extending outside the petri plate.

The MDP *in vitro* culture system represents an interesting alternative to the use of asymbiotic propagules (e.g. isolated spores), for the fast and homogenous colonization of plants (Voets et al., 2009). The MDP consist of a half-closed arbuscular mycorrhizal-plant (HAM-P) *in vitro* culture system with a root compartment (RC) separated from a hyphal compartment (HC). The plastic wall between both compartments prevents direct physical contact and exchanges of e.g. molecules, signals between RC and HC. The MDP *in vitro* culture system was used in Chapter III to investigate the transfer of signal between plants physically separated but connected by a common mycorrhiza network (CMN).

In chapter III, Potato cuttings were transferred in the MDP *in vitro* culture systems with their roots placed on the surface of the MSR medium without sugar and vitamins and shoots extending out of the Petri plates via a hole. The Petri plates were closed and sealed with Parafilm and the holes cautiously covered with sterilized silicon grease to avoid contaminations. The plants were associated with propagules of

the AMF and after a few weeks, the fungus colonized the roots producing a profuse mycelium with numerous spores developing in the RC. The hyphae started to cross the partition wall separating the RC from the HC to develop abundantly in the HC. At that time, new potato cuttings were plated on the surface of the HC with their roots in contact with the mycelium. A common mycorrhizal network (CMN) was rapidly (i.e. within 7 to 10 days) formed between the so-named potato donor plant (in the RC) and receiver potato plants (in the HC). This system linking both plants was used (Fig. 13) to determine whether an AMF is able to transfer defense signals from a late blight diseased potato (Bintje) plant to a healthy potato plant and describe which genes/pathways are concerned.

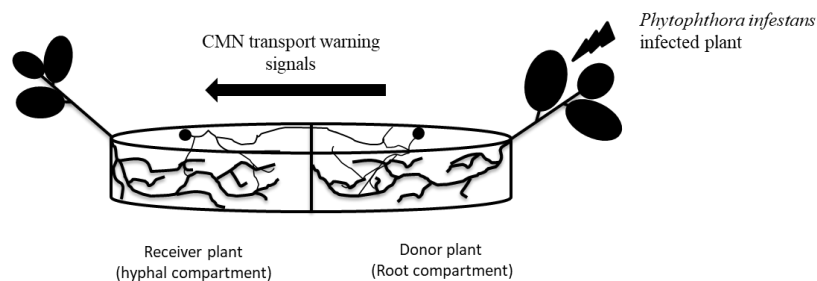


Figure 13. Schematic representation of the *in vitro* cultivation system associating two plants with a common mycorrhizal network (CMN). The two plants develop in physically separated compartments and are connected by the sole CMN. One plant (the donor plant in the root compartment) is infected by a pathogen (*P. infestans*) and signals investigated in the other plant (the receiver plant in the hyphal compartment).

2. Greenhouse and Field experiment

a. Pre-mycorrhization of potato sprouted tuber

Two experiments were conducted in the field to evaluate the effects of AMF on decreasing the symptoms caused by *P. infestans* on potato. To do so, and to ascertain colonization with the inoculant *R. irregularis* MUCL 41833, it was decided to inoculate the potato tubers under controlled greenhouse conditions before transfer of the AMF-colonized plants to the field. AMF-colonized donor plants (i.e. *M. truncatula*) were used as source of inoculum to colonize roots of sprouted potato tubers.



Figure 14. *Inoculation technique in the greenhouse on potato tubers for mycorrhization prior to field experiment: (A) Medicago truncatula (Black circle) mycorrhization, (B) Adapted MDP system for potato tubers, (C) Potato mycorrhization in CMN formed by donor plants (M. truncatula).*

Pre-germinated *M. truncatula* plants (donor plants) were grown in contact with a network of AMF developing on maize roots (Fig. 14A). After 6 weeks, the *M. truncatula* plants were heavily colonized and transferred into compressed peat pots (6 cm Diam., Jiffy®, Netherland) filled with a sterilized mix of sand/vermiculite in equal volumes (Fig. 14B). Each pot received three AMF-colonized *M. truncatula* plants and one sprouted potato tuber (Fig. 14B and Fig. 15).

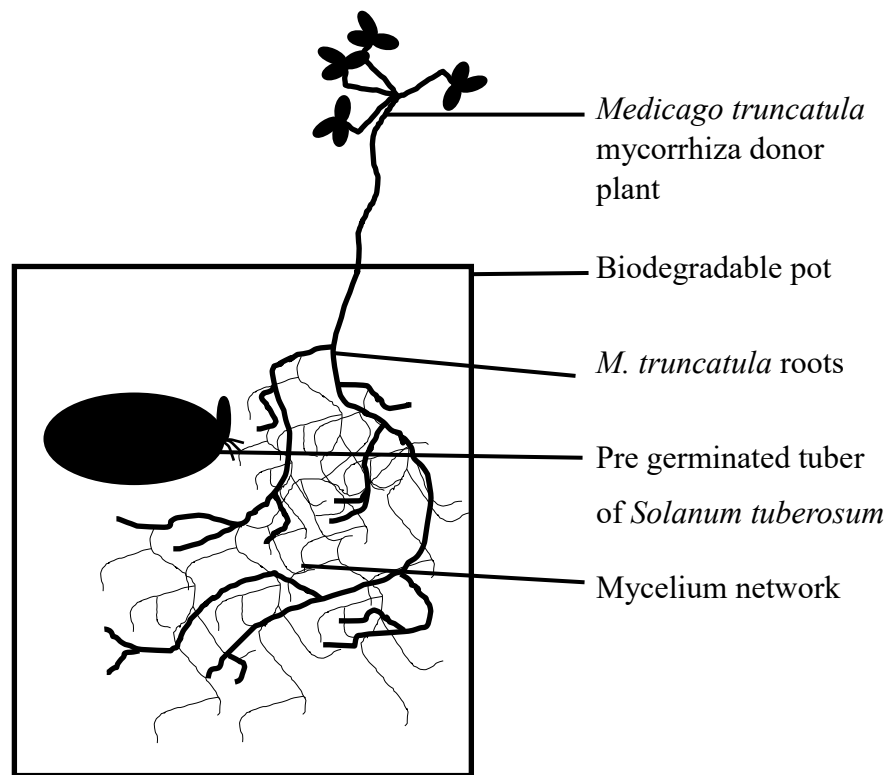


Figure 15. Schematic representation of the “Donor” plant system (inspired from Voets et al. (2009)). A rapid mycorrhization of sprouted potato tubers was obtained in biodegradable pots using *M. truncatula* as AMF donor plant. Sprouted tubers developed their roots in an active network of AMF.

The systems were maintained 4 weeks under greenhouse conditions (25 °C, 75 % RH, 16 /8 h (day/night) for colonization of the potato plants before transfer into the plots (Fig. 14C). A similar procedure was followed with non-AMF-colonized *M. truncatula* plants to produce control plantlets.

b. Field trials setups

Two field trials (over two growing seasons) were conducted at the CRA-W “Batiment haute Belgique” in Libramont (Belgium), , to evaluate the effects of AMF on decreasing the symptoms caused by *P. infestans*. During the first season the susceptible cultivar Bintje was used and during the second season the susceptible cultivar Bintje and moderately resistant cultivar Nicola were used. Potato plants colonized

as above and non-colonized controls were arranged in a completely randomized block design with four blocks per treatment. For both trials, preparation of the plots consisted in mechanical plowing (Fig. 5A) and application of chemical herbicides (Challenge 2 L ha⁻¹ + Proman 3 L ha⁻¹ + Artist 1, 25 kg ha⁻¹, Bayer, Germany). Nor fertilizers neither pesticides were used during the experiment. The late blight outbreak occurred naturally during both growing seasons.

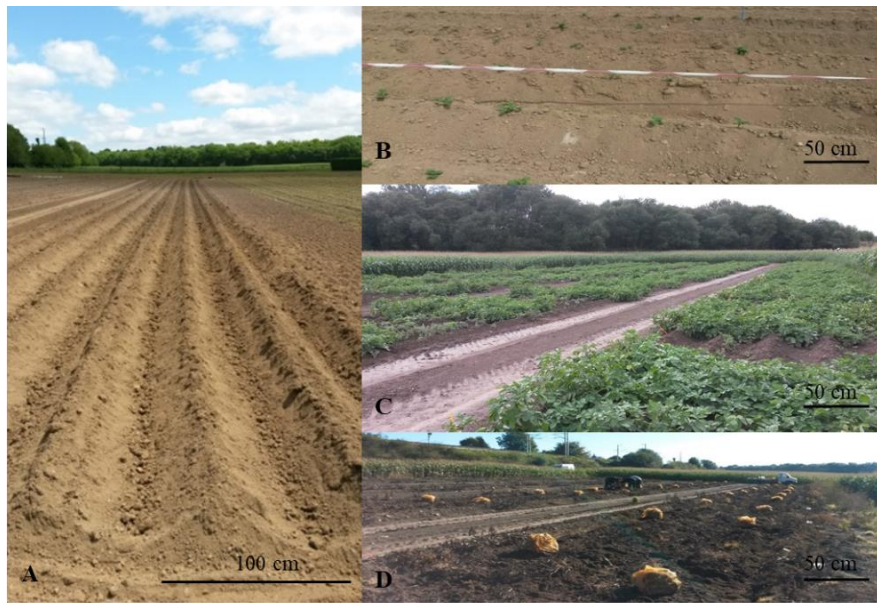


Figure 16. *Field experiment from soil preparation to harvest. Mounds organization (A), Potato systems in the mounds (B), Subplot for each treatment (C) and (D) Tuber harvest.*

Adapted donor plant systems for tuber mycorrhization were placed every 50 cm in the mounds (Fig. 16A and 16B). In both trials, each subplot measured 21 m² (length 3 m x width 7 m, forming 6 mounds) with 36 potato plants (Fig. 16C). At harvest, tubers yield (Fig. 16D) as well as roots and leaves fresh weight were measured. Samples were also kept for molecular analysis.

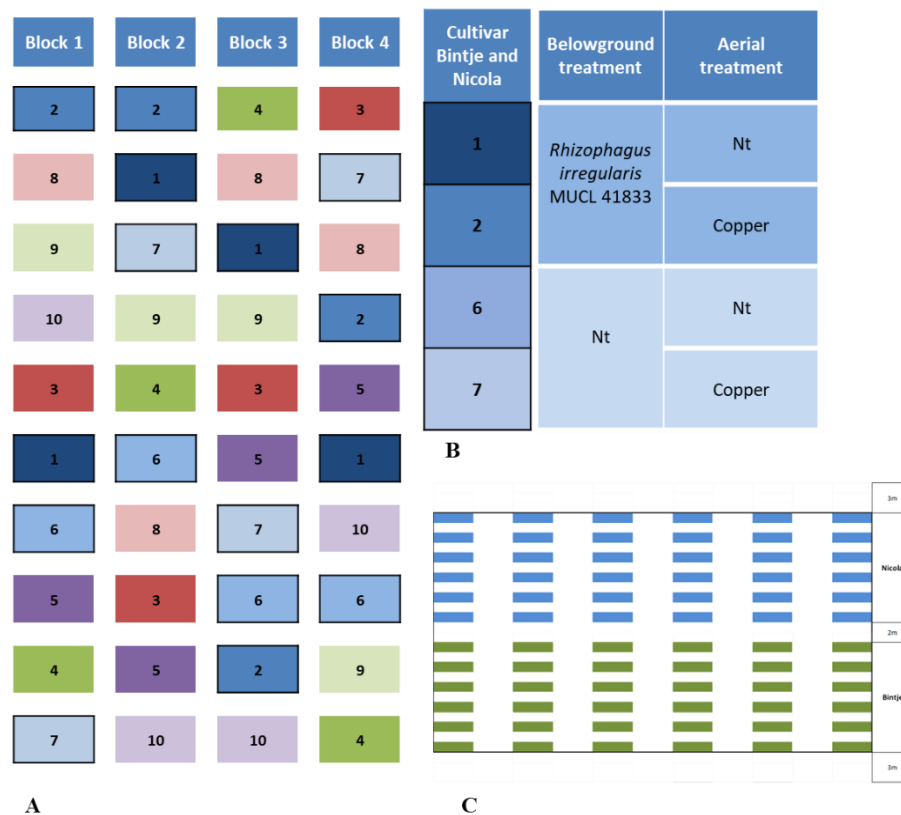


Figure 17. Field organization with four blocks. (A) Different treatments randomly distributed amongst field experiment and (B) Treatments analyzed (B). (C) Plot subdivision for two cultivars: in blue Nicola and in green Bintje. No treatment (Nt).

Four treatments were focusing on AMF effect on late blight, but this essay was part of a larger experiment with ten treatments in total (Fig. 17A). The blocks organization allowed the statistical analysis to account for eventual heterogeneity of the field or variation of local environmental conditions (Fig. 17B). Indeed, main factors of the mixed model were “AMF inoculation” and “Copper treatment” evaluating impact of those treatments on late blight development. But the blocks were used as random factor to cover for heterogeneity. During the second season, two cultivars were used and to facilitate technical itinerary each plot was subdivided in two subplots with Bintje and Nicola (Fig. 17C).

III. Organisms assessment

1. Density of AMF propagules evaluated by the Most Probable Number (MPN) method

The most probable number (MPN) method is widely used to estimate the number of microbial cells in a substrate, without fastidious counting of individual cells. McCrady (1915) was the first to present the MPN method for bacterial enumeration and it was only in the year 1979 that Porter (1979) used the method to evaluate the number of infective AMF propagules in a substrate. The MPN method is based on a serial dilution of the substrate and evaluation of the presence/absence of the targeted organisms in the different dilutions until the ultimate dilution where the organism is present.

The estimation of density is based on the application of the theory of probability and to certain assumptions (Andrango et al., 2016):

- The targeted organisms must be randomly distributed in the sample.
- Each part of the sample taken for dilution is certain to exhibit growth of the targeted organism whenever the part of the sample contains one or more organisms.
- Host plant used to measure the mycorrhizal potential must be highly mycotrophic.
- Plant with rapid root growth is required to provide a high root density.
- The soil nutrient status, especially the phosphorus level, must be appropriate.

In practice for AMF (Fig. 18), several dilutions of a soil are made, and a trap plant is grown on each dilution. After a defined time (generally 6 weeks), the plants are harvested, roots stained and presence/absence of the AMF evaluated until the ultimate dilution where traces of AMF is observed (Cochran, 1950).

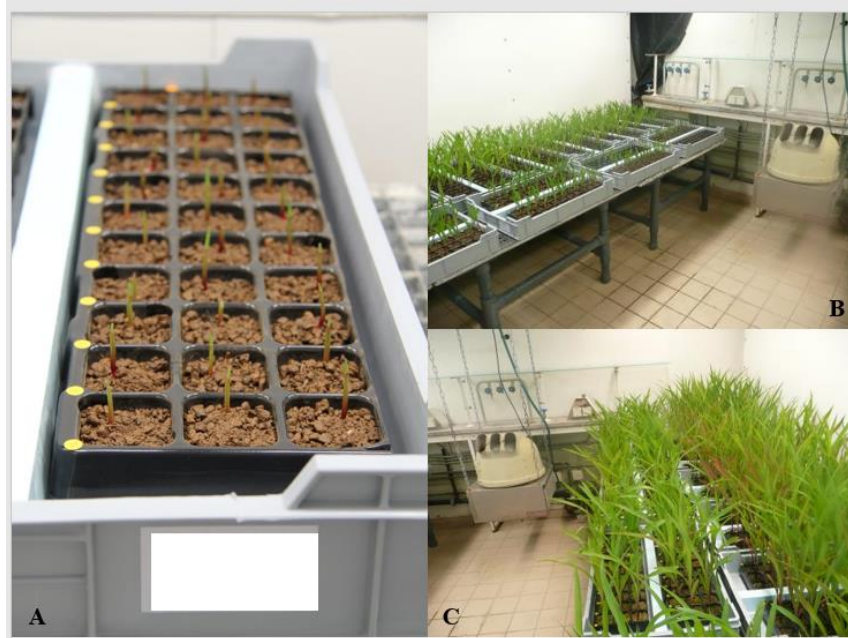


Figure 18. (A) MPN evaluation on sprouted maize plants used as trap plant, (B) Set up of the MPN experimental design in a growth chamber, (C) Maize plants after six weeks in the growth chamber.

In order to evaluate the number of AMF active propagule in the soil we have used the method from Cochran (1950). The reproductibility is highly affected by the parameters used. The parameters considered are (1) the level of dilution of the soil (generally for AMF it should range between 2 and 10), (2) the number of dilutions (depends on the preceding parameter and is higher with dilution 2 than with dilution 10), (3) the number of replicates (can vary between 3 and 9 depending on the number of soils to evaluate).

AMF evaluation of population density in our field experiments were made for each soil as follows:

- Soils were randomly sampled, within the first 20 cm, across maize fields and homogenized.
- Soils were then air dried and sieved using a 5 mm mesh sieve.
- A dilution series of $\frac{1}{4}$ was made by mixing the soil with the same soil sterilized by gamma irradiation (25 kilograys).

- Ten successive dilutions were made, with an additional control level, only sterile substrate. Nine replicates were considered for each level. (Table 4)
- Pre-germinated maize plants were then sown in each pot (Fig. 18A).
- Each pot contained 60g of substrate, a $\frac{1}{4}$ dilution was made at each level and replicated nine times (Table 4).

Table 4. MPN preparation made for a soil of unknown number of active propagules. Ten level of $\frac{1}{4}$ dilutions were used with an additional control level (only gamma sterilized soil).

Level	Dilution	Dilution factor	Weight inoculum/pots	Pots weight
1	1	1	60	60
2	4	0,25	15	60
3	16	0,0625	3,75	60
4	64	0,015625	0,9375	60
5	256	0,00390625	0,234375	60
6	1024	0,000976563	0,05859375	60
7	4096	0,000244141	0,014648438	60
8	16384	6,10352E-05	0,003662109	60
9	65536	1,52588E-05	0,000915527	60
10	262144	3,8147E-06	0,000228882	60
11	Control	Control	0	60

The target soil is diluted (Table 4) with the same soil sterilized by gamma irradiation (25 kilograys). Plants were watered two times per week and received modified Long Ashton (phosphorus concentration to one tenth) once per week after two weeks (Fig. 18B). Presence or absence of the AMF in the root of the trap plants (maize) was determined after 6 weeks (Fig. 18C) of growth in greenhouse conditions (25 °C/22°C, 75 % RH, 16 /8 h (day/night)) under binocular microscope (Olympus SZ40, Olympus Optical GmbH, Germany).

Jarvis et al. (2010) formula was used to estimate the MPN:

$$\sum_{i=1}^k \left(\frac{x_i d_i w_i}{1 - e^{-d_i w_i \mu}} \right) - n_i d_i w_i = 0$$

i: number of dilutions of each sample

x_i: number of positive observations at each level of dilution

d_i: dilution factor

w_i: weight of substrate in which the trap plant growth

μ: unknown MPN value

n_i: Number of replicates for each level of dilution

2. Root colonization of AMF evaluated by Mc Gonigle et al. (1990) method.

The structures of AMF within the roots are usually hidden by the natural pigments (i.e. tannins, polyphenols) and contents of root cells. To evaluate root colonization by AMF it is thus necessary first to clear the roots. Roots clearing aims to soften the cells wall, empty cortical cells from their cytoplasm, removes tannins to improve the penetration of ink within the cells. Clearing is done by bathing the roots in KOH 10% for 45 minutes at 60°C. The roots are then washed free from the KOH and acidified with HCl 1%. After a novel rinsing with water, the roots are stained with a solution of ink (Parker, blue ink) at 2%. The ink will be preferentially fixed on fungal walls (composed of chitin and callose) than on the bleached walls of root cells. Roots are then transferred in a solution of lactoglycerol and placed at 4 °C until analysis.

Root colonization was assessed following the method of McGonigle et al. (1990). This method is based on the observation of a representative length of randomly selected roots fragment, focusing on the presence of intra-radical structures, such as arbuscules, vesicles and hyphae. The presence of these structures is then used to infer the global rate of root system colonization. For each sample, 150 intersections were observed under a compound microscope at 40 X magnification (Olympus BH2, Olympus Optical, GmbH, Germany) and the percentage of total root colonization (%RC), percentage of arbuscules (%A) and percentage of vesicle (%V) estimated.

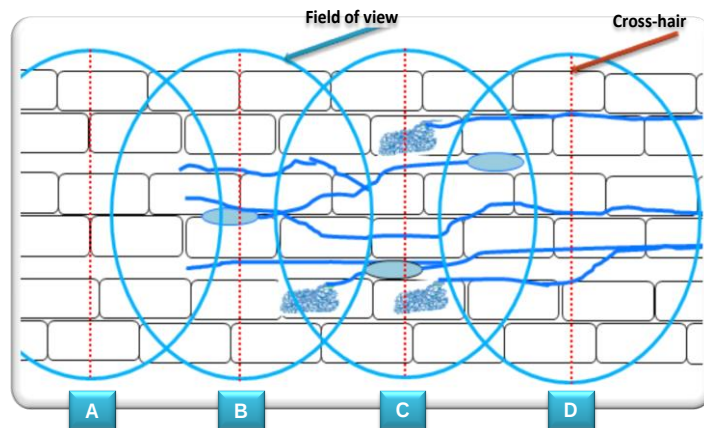


Figure 19. *Evaluation of the AMF root colonization using the method of McGonigle et al. (1990).*

During observation of stained roots fragments, different observations are possible (Fig. 19):

- In case of absence of AMF at cross-hair: note “negative-intersect” (Fig. 19A)
- In case of presence of spores/vesicles or hyphae: note “presence” of the observed structure (spores/vesicles, hyphae) (Fig. 19B).
- When at the same time spores/vesicles, arbuscules and hyphae are observed in the intersection (Fig. 19C): note “presence” of spores/vesicles and “presence” of arbuscules.
- When only hyphae are observed at the intersection (Fig. 19D): note “presence” of hyphae.
- Percentage of hyphae colonization (%H), percentage of arbuscules colonization (%A) and percentage of spores/vesicles colonization (%V) are then calculated by dividing the number of intersections with the presence of hyphae or arbuscules or spores/vesicles, respectively by the total number of intersections examined. Total colonization (%RC) is calculated as the proportion of non-negative intersections.

3. Late blight inoculation and evaluation of infection under controlled conditions

Assessing the impact of *P. infestans* on potato plants first need a proper method to inoculate the plants. Under controlled conditions (i.e. in the growth chamber), high humidity is needed.

Phytophthora infestans was applied in droplets on the leaves. Cellophane disks (5 mm diam.) were used to cover the droplets, avoiding their evaporation or fall-off the leaves (Fig. 20A); thus, maintaining the pathogen under optimal conditions for infection. The first necroses were observed after 48h (Fig. 20B) and developed rapidly, around the cellophane disk area (Fig. 20C). Disease assessment was estimated (Fig. 21) following the adapted key of James (1971) and EUCABLIGHT(www.eucablight.org/Lib/Eucablight/Protocol/FoliarBlight_V12.pdf) guidelines and converted into rAUDPC.

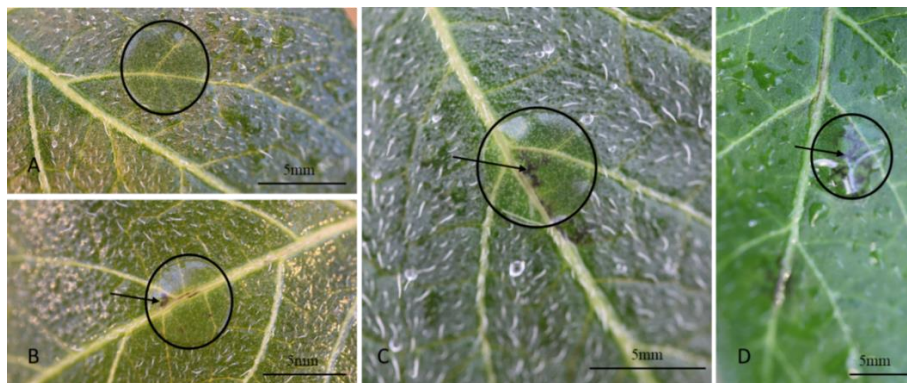


Figure 20. Late blight early development in controlled conditions (first necrosis - black arrow), using cellophane disk (black circle) to prevent droplets of *P. infestans* solution from drying and fall off. (A) *Phytophthora infestans* was applied in droplets on the leaves under cellophane disk. (B) The first necroses were observed after 48h and (C and D) Developed rapidly, around the cellophane disk area.

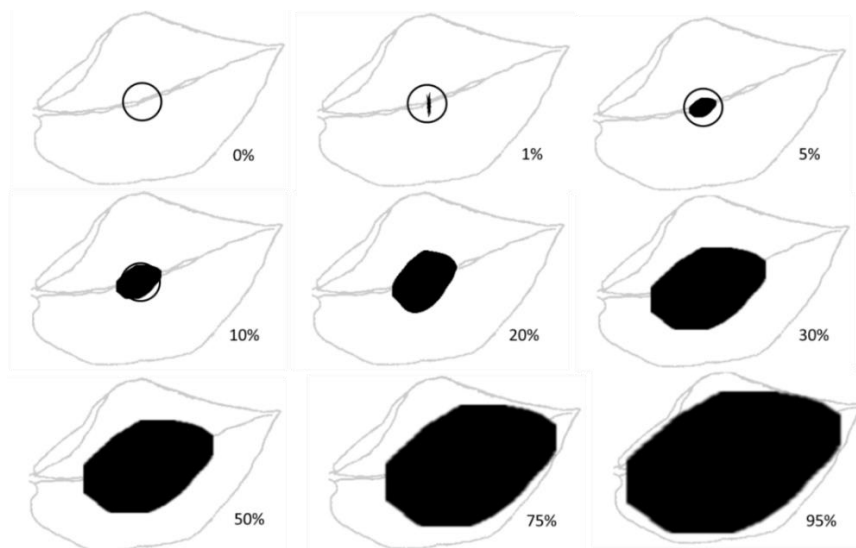


Figure 21. Adapted key (from James, W. C. (1971)) of *P. infestans* development for in vitro plant system from inoculation under cellophane disk (black circle) to 95% of leaf infection. Symptoms caused by *P. infestans* were monitored every day after the onset of the disease.

IV. Molecular methods

1. Selection of genes for RT-qPCR analyses

a. Reference genes

During the interplant signaling CMN experiments (Chapter III), we have used three reference genes. These genes are expressed constitutively, and their levels of expression are not affected by the different treatments or during time. The three reference genes were selected using Refinder software (<http://150.216.56.64/referencegene.php?type=reference>). These genes were used to make the normalization of genes expression obtained in the RT-qPCR.

Ubiquitin conjugating enzyme-like (Ubc - DQ241834): this gene is encoding for an enzyme, which has a function in the ubiquitin-mediated protein degradation pathway.

Glyceraldehyde 3-phosphate dehydrogenase (GADPH - AF527779): this gene is encoding for an enzyme involved in glycolysis and gluconeogenesis.

Elongation factor 1-alpha (EF1- α - AB061263): this gene is encoding for a protein which is responsible for the GTP-dependent binding of aminoacyl-tRNAs to the ribosomes.

b. Target genes

Fifteen target genes involved in plant defense pathway or signaling pathways were selected from literature and previous experiments.

- Seven target genes were selected as marker or involved in salicylic acid (SA) signaling pathways:

Mitogen-activated protein kinase (MAPK - AB206552): this gene is encoding for a wound-induced protein kinase (i.e. WIPK). The MAPKs are associated with physiological, developmental, hormonal, and stress responses. More particularly, some results indicate that WIPK and (SA)-induced protein kinases are convergent points in the signaling pathway of defense responses in plant-pathogen interactions (Yamamizo et al., 2006).

Pathogenesis related 1 (PR-1 - (Burra et al., 2015)): this gene is encoding for the PR1-a protein (i.e. acidic form), used as SA marker. This protein may act as an antifungal agent and has also been shown to be linked to a partial resistance to *P. infestans* in *Solanum* species (Burra et al., 2015).

Pathogenesis related 2 (PR-2- AJ009932): this gene is encoding for an acid β -1, 3-glucanase protein. This enzyme is involved in cell wall degradation by digesting β -1, 3-glucans in fungal cell walls. This gene is a classical marker of the systemic acquired resistance (SAR) (Van Loon and Van Strien, 1999).

Pathogenesis related 9 (PR9 - AJ401150): This gene is encoding peroxidases. It can contribute to plant disease resistance in several ways including the strengthening of plant cell walls through the deposition of lignin, which is thought to be a general defense mechanism against a broad spectrum of pathogens and the production of toxic radicals such as hydrogen peroxides.

Glutathione-S-transferase 1 (GST1 - J03679): this gene is encoding for an auxin-responsive glutathione-S-transferase 1, which is involved in the cellular detoxification.

Putative regulatory protein Non-expressor of PR genes 1 (NPR1 - XM_006357647.2): The transcriptional co-activator NPR1 is essential for SAR and is required for the predominant part of SA downstream responses, including activation of defense gene expression (Shah and Zeier, 2013). In SA signaling, NPR1 is clearly connected to a nuclear function.

WRKY transcription factor 6 gene (WRKY6 - LOC102577893): The WRKY TFs are among the largest families of transcriptional regulators in plants and this specific gene was found up-regulated in potato infested by *Phytophthora infestans*.

Pathogenesis related 5 (Osmotin like - (Ruiz et al., 2005)): The osmotin-like genes are functional candidates for quantitative resistance to late blight (Ruiz et al., 2005).

- Two target genes were selected as marker or involved in jasmonic acid and ethylene (JA/ET) signaling pathways:

Phenylalanine ammonia lyase (PAL - X63104): a key enzyme catalyzing the first reaction in the biosynthesis from L-phenylalanine of a wide variety of natural products based on the phenylpropane skeleton. Moreover, this protein is believed to be activated by the JA/ET signaling pathway in the context of induced plant defenses.

Pathogenesis related 1 (PR-1c - AJ250136): this gene is encoding for the PR1-b protein (i.e. basic form). This protein may act as an antifungal agent and was also shown to be linked to a partial resistance to *P. infestans* in *Solanum* species.

- One target gene was selected as a marker of ET signaling pathways:

Ethylene response factor 3 (ERF3 - EF091875): this gene is encoding for a transcription regulator, previously named ethylene responsive element binding proteins (EREBPs), with a promotor element essential for ethylene responsiveness.

- Three genes were selected as marker or involved in jasmonic acid (JA) signaling pathways:

Lipoxygenase (Lox - AY135641): this gene is encoding for the lipoxygenase 1 enzyme, which produced roughly equimolar amounts of 13- and 9-hydroperoxides from linoleate.

Allene oxide cyclase (AOC - AY135641): allene oxide cyclase: AOC produce a JA precursor 12-oxo-phytodienoic acid (OPDA) which

is transported to peroxisomes where it is reduced by the peroxisomal reductase OPR3 and subject to β -oxidation to produce JA.

OPDA reductase 3 (OPR3 - (Halim et al., 2009)): After translocation from chloroplasts to peroxisomes, 12-oxophytodienoic acid (OPDA) is reduced by an OPDA reductase.

2. Relative quantification in RT-qPCR

Once references and targets primer genes have been designed *in silico*, their efficiency and specificity have been analysed by RT-qPCR. Material (i.e. cDNA) from different treatments (control, mycorrhized plants, infected plants, from different time point) were mixed to serve in the standard curve. Six levels of dilution were used, from pure mix to a dilution of 1/10000. To increase the stability of the standard curve, chain dilution was made, starting with a 1/5 dilution, then 1/2 and followed by three 1/10 dilution. For all primer genes tested, three technical replicates of each level of standard curve were made (Fig. 22A, blue). Additionally, three replicates of negative control (i.e. water instead of DNA template, Fig. 11A red) were used. Every standard curve plate also had a positive control (Fig. 22A, green) of run quality: one level of the standard curve of reference gene (e.g. UBC) placed in every plate. Efficacy of the reaction was assessed for each gene, normally around 2 with curve slope of -3,3.

Selected primers were then used on all tested treatments, one plate was used by gene at each time point (Fig. 22B): biological replicate tested (Fig. 22B, grey), negative control (Fig. 22B, red), positive control (Fig. 22B, green).

Relative quantification analysis results are expressed as a ratio: the relative amount (fold change) of a target nucleic acid (test) compared to a control sample (calibrator). The advantage of using a reference gene (such as GADPH, β tub) is that this method prevents the need for accurate quantification and loading, of the starting material. The drawback is that this method requires the availability of a known reference gene or genes, with constant expression in all samples tested and whose expression is not changed by the treatment under study. To increase accuracy of the calculus, multiple reference genes were used, and geometrical means of reference gene was used for each sample. Control treatment was selected at each time point as the calibrator. At each time point, data of target gene (Ct test) on the target sample and calibrator (Ct calibrator) were obtained and equivalent for reference gene (Ct test and Ct calibrator). Efficacy (E) of each gene was

calculated using the standard curve. Ratios (fold change compared to a calibrator) were then calculated.

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_T, \text{target (calibrator - test)}}}{(E_{\text{ref}})^{\Delta C_T, \text{ref (calibrator - test)}}}$$

*	1	2	3	4	5	6	7	8	9	10	11	12
A	S 1 Btub	S 1/5 Btub	S 1/10 Btub	S 1/100 Btub	S 1/1000 Btub	S 1/10000 Btub	S 1 Lox	S 1/5 Lox	S 1/10 Lox	S 1/100 Lox	S 1/1000 Lox	S 1/10000 Lox
B	S 1 Btub	S 1/5 Btub	S 1/10 Btub	S 1/100 Btub	S 1/1000 Btub	S 1/10000 Btub	S 1 Lox	S 1/5 Lox	S 1/10 Lox	S 1/100 Lox	S 1/1000 Lox	S 1/10000 Lox
C	S 1 Btub	S 1/5 Btub	S 1/10 Btub	S 1/100 Btub	S 1/1000 Btub	S 1/10000 Btub	S 1 Lox	S 1/5 Lox	S 1/10 Lox	S 1/100 Lox	S 1/1000 Lox	S 1/10000 Lox
D	S 1 GADPH	S 1/5 GADPH	S 1/10 GADPH	S 1/100 GADPH	S 1/1000 GADPH	S 1/10000 GADPH						
E	S 1 GADPH	S 1/5 GADPH	S 1/10 GADPH	S 1/100 GADPH	S 1/1000 GADPH	S 1/10000 GADPH						
F	S 1 GADPH	S 1/5 GADPH	S 1/10 GADPH	S 1/100 GADPH	S 1/1000 GADPH	S 1/10000 GADPH						
G	+	+	+	-	-	-						
	1/5 UBC	1/5 UBC	1/5 UBC	H2O Lox	H2O Lox	H2O Lox						
H	-	-	-	-	-	-	-	-	-			
	H2O UBC	H2O UBC	H2O UBC	H2O Btub	H2O Btub	H2O Btub	H2O GADPH	H2O GADPH	H2O GADPH			A

*	1	2	3	4	5	6	7	8	9	10	11	12
A	U NM1 ERF3	U NM1 ERF3	U NM1 ERF3	U NM2 ERF3	U NM2 ERF3	U NM2 ERF3	U NM3 ERF3	U NM3 ERF3	U NM3 ERF3	U NM4 ERF3	U NM4 ERF3	- H2O ERF3
B	U NMc1 ERF3	U NMc1 ERF3	U NMc1 ERF3	U NMc2 ERF3	U NMc2 ERF3	U NMc2 ERF3	U NMc3 ERF3	U NMc3 ERF3	U NMc3 ERF3	U NMc4 ERF3	U NMc4 ERF3	- H2O ERF3
C	U NM+P1 ERF3	U NM+P1 ERF3	U NM+P1 ERF3	U NM+P2 ERF3	U NM+P2 ERF3	U NM+P2 ERF3	U NM+P3 ERF3	U NM+P3 ERF3	U NM+P3 ERF3	U NM+P4 ERF3	U NM+P4 ERF3	+
D	U NMc+P1 ERF3	U NMc+P1 ERF3	U NMc+P1 ERF3	U NMc+P2 ERF3	U NMc+P2 ERF3	U NMc+P2 ERF3	U NMc+P3 ERF3	U NMc+P3 ERF3	U NMc+P3 ERF3	U NMc+P4 ERF3	U NMc+P4 ERF3	+
E	U Mr1 ERF3	U Mr1 ERF3	U Mr1 ERF3	U Mr2 ERF3	U Mr2 ERF3	U Mr2 ERF3	U Mr3 ERF3	U Mr3 ERF3	U Mr3 ERF3	U Mr4 ERF3	U Mr4 ERF3	U Mr4 ERF3
F	U Mc1 ERF3	U Mc1 ERF3	U Mc1 ERF3	U Mc2 ERF3	U Mc2 ERF3	U Mc2 ERF3	U Mc3 ERF3	U Mc3 ERF3	U Mc3 ERF3	U Mc4 ERF3	U Mc4 ERF3	U Mc4 ERF3
G	U Mr+P1 ERF3	U Mr+P1 ERF3	U Mr+P1 ERF3	U Mr+P2 ERF3	U Mr+P2 ERF3	U Mr+P2 ERF3	U Mr+P3 ERF3	U Mr+P3 ERF3	U Mr+P3 ERF3	U Mr+P4 ERF3	U Mr+P4 ERF3	U Mr+P4 ERF3
H	U Mc+P1 ERF3	U Mc+P1 ERF3	U Mc+P1 ERF3	U Mc+P2 ERF3	U Mc+P2 ERF3	U Mc+P2 ERF3	U Mc+P3 ERF3	U Mc+P3 ERF3	U Mc+P3 ERF3	U Mc+P4 ERF3	U Mc+P4 ERF3	U Mc+P4 ERF3

B

Figure 22. Plate organization: (A) Standard curve and (B) Gene analyses with a square for each technical replicate, in blue standard level, in green positive control and in red negative control.

RESEARCH RESULTS

Chapter I

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

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Preface

Arbuscular mycorrhizal fungi are organism naturally occurring in arable soils. They are obligate biotrophs with beneficial effects on plants often reported when effective and functional root colonization is set. Several factors may affect root colonization, among which the cultural practices, the edapho-climatic conditions and the competition with soil microflora and indigenous AMF. Moreover, to evaluate the potential beneficial effects of AMF inoculants it is necessary to ascertain its root colonization. Nowadays, most studies within the field do not strictly report on the effects of inoculated AMF on plant growth because they are most often not traced in the field and thus their contribution to root colonization cannot be evaluated or separated from the native AMF. In summary, there is no clear indication that growth promotion, if any, is strictly related to the inoculated strains.

In chapter I, potato plantlets were pre-colonized within the greenhouse via the so-named mycorrhizal donor plant system. A molecular tool was first developed, based on the mitochondrial genome (MtLSU), to trace and quantify the AMF inoculant (i.e. *Rhizophagus irregularis* MUCL41833) versus the developmental stages of the fungus. In a second step, three potato varieties (Bintje, Charlotte and Nicolas), were planted in the field and inoculated with the above-mentioned AMF inoculant via the encapsulation technology. The objective was to validate the MtLSU markers developed above to detect/trace and quantify the AMF inoculant in the roots of potato plants naturally-colonized by the local AMF community. Therefore, differentiate the AMF inoculant (*R. irregularis* MUCL41833) from the native *Rhizophagus irregularis* strains.

Abstract

Crop inoculation with arbuscular mycorrhizal fungi (AMF) is a promising option to increase plant yield. However, in most cases, the inoculated strains could not be traced in the field and their contribution to root colonization separated from native AMF. Therefore, there is no clear indication that growth promotion is strictly related to the inoculated isolates. Here, *Rhizophagus irregularis* MUCL 41833 was inoculated on three potato cultivars (Bintje, Nicola, Charlotte) under field conditions in Belgium. Mitochondrial Large SubUnit (MtLSU) of inoculated *R. irregularis* MUCL 41833 was characterized to design haplotype- (inoculated *R. irregularis* haplotype) and species-specific (native or inoculated *R. irregularis*) markers. The magnitude of detection of the markers, determined by Real-Time quantitative PCR, was linked to the stage of colonization of potato cv. Bintje grown under greenhouse conditions. Under field conditions, the inoculant *R. irregularis* MUCL 41833 was detected at a very low level (between 10^{-5} and 10^{-7} ng/ ng total DNA) in a marginal number of plants, in contrast to native *R. irregularis* strains that were detected at higher levels (between 10^{-4} and 10^{-6} ng/ ng total DNA) in all plants of the three cultivars. This suggested that the inoculated strain was almost absent in the plants due either to the environmental conditions, competition with indigenous AMF or inadequate placement of inoculum. This was corroborated by the absence of growth promotion and differences in root colonization between inoculated versus non-inoculated potato plants. This study validated the MtLSU markers to detect/trace and quantify AMF inoculants as native strains in plants grown under field conditions and further supported that potato cultivars in the same field conditions differed in root colonization.

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 Jr et al. (2007)). Their major benefits to plants include increased acquisition and accumulation of nutrients (e.g. P, N) (Smith and Read, 2008) and improved tolerance/resistance 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 to plants in the field remains scarce as compared to the numerous results obtained in greenhouse or growth chamber conditions. It is obvious that under field conditions, inoculated AMF have to compete with native AMF communities (Verbruggen et al., 2013) and may be influenced by numerous agricultural as well as environmental factors (see Gosling et al. (2006) impacting their capacity to improve plant yield and health.

Rhizophagus irregularis is a worldwide distributed AMF, commonly 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). A recent analysis of 231 field experiments in North America and Europe where potatoes were inoculated with *R. irregularis* DAOM 197198 (syn. MUCL 43194), using a liquid suspension of AMF spores sprayed onto potato seeds (71 spores/seed piece), showed a significant increase in the production of marketable potato tubers (Hijri, 2016). An increase in potato yield was also observed in a potato culture succeeding to a cover crop of *Medicago sativa* inoculated with *R. irregularis* MUCL 41833 and *Trichoderma harzianum* MUCL 29707 encapsulated into alginate beads (20 AMF propagules and 5 conidia/bead – ± 30 beads/potato seed). However, even if it was supposed that growth promotion was related to the inoculated isolates, it could not be ruled out that other AMF belonging to the same species or different species could be involved. Indeed, *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 as opposed to indigenous AMF species, in particular if the local communities harbor similar species to the inoculated ones.

Microscopically, it is difficult to distinguish different AMF species or different AMF isolates of the same species based on fungal structures within 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 et al. (2000)). Intraspecific markers were also developed to detect different isolates of *R. irregularis* by amplifying the mitochondrial Large SubUnit (MtLSU) (Croll et al. (2008); Koch et al. (2004); Raab et al. (2005)). 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 qPCR 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* under greenhouse conditions. 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 is the most important tuber crop worldwide (FAO, 2013). This crop is characterized by heavy mechanization and huge applications of fertilizers and pesticides. The effect of a direct inoculation of AMF on potato was investigated by Douds Jr et al. (2007), Bayrami et al. (2012) and Hijri (2016) under different fertilization treatments. These authors noticed that inoculation with *R. intraradices*, *R. irregularis* 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, these authors could not separate the contribution of native from 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 used as inoculant in our field experiment; (2) to validate the markers on inoculated potato plants under greenhouse conditions (3) to evaluate field inoculation success at potato planting by quantifying introduced *R. irregularis* MUCL 41833 isolate as opposed to the native *R.*

irregularis; and (4) to compare AMF root colonization abundance by microscopic and molecular techniques between different potato cultivars.

Materials and methods

Biological material

Solanum tuberosum L. cv. Bintje, cv. Nicola and cv. Charlotte (Euroseeds, Belgium) were used. 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) seeds were used in the mycorrhizal infective potential (MIP) test (see 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 pulverized lava (DCM, Belgium) supplemented with Osmocote®.

Six other *R. irregularis* strains (MUCL 46241; MUCL 46239; MUCL 43194; MUCL 43204; MUCL 46240; MUCL 43195) and four closely related species to *R. irregularis* (*Rhizophagus diaphanus* MUCL 43196; *Rhizophagus intraradices* MUCL 49410; *Rhizophagus clarus* MUCL 46238; *Rhizophagus fasciculatus* MUCL 46100) were supplied by the Glomeromycota *in vitro* collection for the tests of specificity.

Entrapment of AMF and mycorrhizal infective potential

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 20 AMF propagules (i.e. isolated spores or root fragments containing spores/vesicles). Beads were air-dried to 35 % of their initial weight.

The MIP of the beads was evaluated using the method described in Declerck et al. (1996) slightly modified. The method was based on a dose-response relationship and involved the cultivation of a population

of mycotrophic plantlets on a range of inoculum density under controlled conditions. Briefly, beads (1, 3, 10, 30 and 100 – logarithmic scale), were thoroughly mixed in 200 cm³ 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$. 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)(Walker, 2005). The presence/absence of AMF was evaluated in each plantlet under stereomicroscope (Olympus SZ40, Olympus Optical GmbH, Germany) at X 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 number of beads needed to colonize 90% of the plants (i.e. ID₉₀). This number was 13.

Molecular characterization of *R. irregularis* MUCL 41833

Fungal DNA was extracted from a mix of ± 1000 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 as explained below). Spores/mycelium of 2-month-old cultures were extracted from the Modified Strullu Romand (MSR) medium Declerck et al. (1998) following solubilization of the gelling agent by citrate-buffer (Doner and Bécard, 1991). They were further 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 with the buffer. Extracted DNA was then subjected to PCR amplification of the 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 supplementary material Table S1). 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 MgCl₂ (see Table 1). 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 (see supplementary material Table S1). One sequence type was obtained (accession number KY347874).

Primer design

Two primer pairs targeted to the MtLSU region (Table 5) were designed. One primer pair was designed to amplify MtLSU of all *R. irregularis* strains and another to amplify MtLSU of the specific target strain. Specific primers were designed by aligning the sequence obtained 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/>). After a first specificity assessment by checking *in silico* the absence of significant similarity of our primers with other known DNA sequences using BLAST in the NCBI GenBank public database, tests in qPCR were performed using gDNA of various AMF taxa from the Glomeromycota *in vitro* collection or root samples (colonized or not by AMF) from *in vitro* or *in vivo* collection as templates (see supplementary material Table S2). 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 5. *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)	Amplification size (bp)	Amplification efficiency [†] (SD)
MtLSU, <i>R. irregularis</i>	BF7/BR5	TGGTCTA AACATGG TTGAAAA AT/ ACGCTAT CGCTAAA AGGTGG	0.2/0.2	62	67	96.2 (5.3)
MtLSU, <i>R. irregularis</i> MUCL 41833 haplotype	BF8/BR6	AAGTCCT CTAGGTC GTAGCA/ ACAGGTA TTTATCA AATCCTT CCC	0.2/0.2	62	121	93.2 (4.5)

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

Optimization of qPCR assay

To prepare standards for the qPCR experiments, DNA extract from *R. irregularis* MUCL 41833 spores and mycelium grown *in vitro* was 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.

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, 10 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 were expressed as ng of *R. irregularis* DNA or ng of *R. irregularis* MUCL 41833 haplotype DNA/ng total DNA.

Greenhouse validation of molecular markers to trace and quantify *R. irregularis* MUCL 41833 haplotype or *R. irregularis* species in potato roots

A greenhouse experiment was set up to validate molecular markers and to quantify the MtLSU genes at different stages of potato root colonization by *R. irregularis* MUCL 41833. Potato tubers of the 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 an *in vitro* cultured suspension of spores of *R. irregularis* MUCL 41833 and cultured for two months in pots of 8 dm³ filled with sand and vermiculite (1v/1v) to set up a profuse and active extra radical 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 plants. A pre-germinated tuber and 3 donors *M. truncatula* plants were placed in biodegradable pots (6 cm, Jiffy®, Netherland) 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 with an annual mean precipitation of 1191 mm year⁻¹ and an annual mean temperature of 8.3 °C (Pameseb asbl. 2014). The soil was a Dystric Cambisol (IUSS Working Group WRB, 2014). The soil had a pH of 6.3 and 5.3 in H₂O and KCl, respectively. Soil organic matter and C content were 48 g kg⁻¹ and 28 g kg⁻¹, respectively.

The soil was covered with a mown 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 system, but no fertilizers were added. The timeline of the trial and the conventional agricultural practices and treatments applied are presented in supplementary material Table S3.

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 (referred as Ri41833) and beads without AMF (i.e. the control). Each potato tuber was inoculated with 120 beads. Inoculation was done on the 19th of May 2014 in the planting hole close to the tuber. Each individual subplot had a surface of $\pm 21 \text{ m}^2$ ($3 \times 7 \text{ m}$) 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 before tuber harvest 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), just before defoliation (the 27th of August 2014) and at harvest (the 24th of September 2014). Whole plants were harvested but only the roots were analyzed. These plants were not considered for the final harvest. 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 rows 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 stained following the method of Walker (2005). 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 X magnifications. Total root colonization

(% RC), abundance of arbuscules (% A), intraradical spores/vesicles (% V) and/or hyphae only (% H) were determined.

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

Frozen root samples of the potatoes were crushed in liquid nitrogen, and DNA of 500 mg crushed root samples 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 crushed roots for the lysis). The concentration of DNA was 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 was used as the template in real time qPCR.

Real time qPCR was performed as described above. Standard curves constructed from 5-fold dilutions of genomic standard (i.e. DNA extract from *R. irregularis* MUCL 41833 spores and mycelium grown *in vitro*) 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 were 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 Tukey’s test ($P \leq 0.05$).

Results

R. irregularis specific Real-Time qPCR markers

Specificity of the primer pairs is detailed in the supplementary material Table S2. The first primer pair was able to amplify all *R. irregularis* strains tested (but also one *Rhizophagus fasciculatus* and one *Rhizophagus diaphanus* strain) and the second primer pair was able to amplify the specific target strain MUCL 41833 and MUCL 43204 but not the other *R. irregularis* strains. This last marker detected also *Rhizophagus diaphanus* MUCL 43196. More specific primers could not be found in the MtLSU region sequenced.

Validation of qPCR assay

Standard curve, qPCR amplifications and melting peak for each primer pair are illustrated in the supplementary material Fig. S1 and Fig S2. qPCR amplification with each primer pair was confirmed by Sanger sequencing. CT values of potato roots in the greenhouse and in the field ranged from 21 to 36 and fit with the standard curves. Amplification efficiency was calculated from ten independent 5-fold dilutions of DNA template and corresponded to 96.2 % for MtLSU *R. irregularis* species-specific primer pair and 93.2 % for MtLSU *R. irregularis* MUCL 41833 haplotype primer pair (see Table 1).

Greenhouse validation of molecular markers to trace and quantify the haplotype *R. irregularis* MUCL 41833 or species-specific *R. irregularis* in potato roots

Three different stages of colonization (early, intermediate and late stage – according to Gallou et al. (2010)) could be distinguished in the thirteen potato plants sampled from the greenhouse experiment. Three samples (harvested at week 2) were in the early stage of root colonization, characterized by the presence of few hyphae (i.e. 18 ± 9 %) and absence of arbuscules and vesicles/spores. Four samples (harvested at week 2, 5 and 6) were in the intermediate stage, with hyphae (i.e. 18 ± 4 %), a few arbuscules (i.e. 2.2 ± 0.3 %) and no vesicles/spores. Similarly, four samples (harvested at week 3 and 4) were in the intermediate stage with the presence of hyphae (i.e. 22 ± 7 %), some arbuscules (i.e. 10 ± 3 %) and the first vesicles/spores (i.e. 1 ± 0 %). The two intermediate stages were thus discriminated by the presence of vesicles/spores. Finally, two samples (harvested at week 5) were in the late stage of colonization with higher amounts of hyphae

(i.e. 25 ± 13 %), arbuscules (i.e. 15 ± 3 %) and vesicles/spores (i.e. 8 ± 0 %).

Whatever the colonization stage, no significant differences were noticed in % RC or % H, even if values increased from early to late stage. In contrast, significant differences were noticed in % A and % V between colonization stages with the highest values observed in the second intermediate and late stage for % A and late stage for % V. With the mitochondrial markers, significant differences were noticed in MtLSU detection with the lowest concentration ($3.9 \cdot 10^{-6} \pm 2.1 \cdot 10^{-6}$ ng MtLSU_MUCL41833/ ng DNA vs. $3.5 \cdot 10^{-6} \pm 2.0 \cdot 10^{-6}$ ng MtLSU_RI/ ng DNA) in the early stage of root colonization, and with the highest concentration ($7.4 \cdot 10^{-3} \pm 5.1 \cdot 10^{-3}$ ng MtLSU_MUCL41833/ ng DNA vs. $7.8 \cdot 10^{-3} \pm 4.1 \cdot 10^{-3}$ ng MtLSU_RI/ ng DNA) in the late stage. An intermediate concentration of mitochondrial markers ($5.7 \cdot 10^{-5} \pm 1.7 \cdot 10^{-5}$ ng MtLSU_MUCL41833/ ng DNA vs. $4.6 \cdot 10^{-5} \pm 1.8 \cdot 10^{-5}$ ng MtLSU_RI/ ng DNA and $3.0 \cdot 10^{-4} \pm 1.2 \cdot 10^{-4}$ ng MtLSU_MUCL41833/ ng DNA vs. $2.8 \cdot 10^{-4} \pm 1.4 \cdot 10^{-4}$ ng MtLSU_RI/ ng DNA) was found in the intermediate stages. Pearson's correlation coefficients (r) between MtLSU of *R. irregularis* MUCL 41833 and AMF root colonization parameters (% RC, % A, % V and % H) were calculated for the thirteen samples. A significant positive correlation was observed between MtLSU_MUCL41833 and % RC ($r = 0.5967$; $P = 0.0240$), % A ($r = 0.6228$; $P = 0.0168$) and % V ($r = 0.7797$; $P = 0.0004$) but not between MtLSU_MUCL41833 and % H.

Field experiment

Potato tuber weight, size and number:

The average tubers weight per plant ranged from 1.91 ± 0.12 kg to 2.31 ± 0.04 kg among the different treatments and a significant effect of the factor "cultivar" was observed ($P < 0.01$). 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 %) as compared to Charlotte.

The % tubers > 60 mm ranged from 14 ± 2 % to 30 ± 2 % among the different treatments. 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 as compared to cultivar Nicola ($P < 0.001$) or Charlotte ($P < 0.0001$) and % tubers > 60 mm was significantly higher for cultivar Nicola as compared to cultivar Charlotte ($P < 0.05$).

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

Root colonization by AMF:

Potato root colonization at flowering stage, before defoliation and at harvest is presented in Fig. 23 and statistical analysis in supplementary material Table S4. 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” and the interaction of both factors had no significant effect on % RC, % A or % V. The % RC, % A and % V were calculated for the “Ri41833” and “Control” treatment combined. Significant ($P < 0.0001$) higher % RC and % A were noticed for cultivars Nicola and Charlotte in comparison to cultivar Bintje, while no differences were observed between Nicola and Charlotte. In addition, significant higher % V was noticed for cultivars Nicola ($P < 0.0001$) and Charlotte ($P < 0.05$) in comparison to Bintje, while no differences were observed between Nicola and Charlotte.

Before defoliation the % RC ranged from 29.9 ± 2.8 % to 50.2 ± 3.2 %, % A from 17.8 ± 2.7 to 34.6 ± 3.0 and % V from 0.8 ± 0.3 % to 5.6 ± 0.9 %. Similarly, to the flowering stage, 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. 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. Significant ($P < 0.0001$) lower % RC, % A and % V were noticed for cultivars Bintje in comparison to cultivar Nicola. Significant ($P < 0.05$) lower % RC, % A and % V were noticed for cultivars Charlotte in comparison to cultivar Nicola and significant ($P < 0.05$) lower % RC, % A and % V were noticed for cultivar Bintje in comparison to cultivar Charlotte.

At harvest the % RC ranged from 14.1 ± 1.3 % to 37.4 ± 4.5 %, % A from 7.3 ± 0.7 % to 21.6 ± 3.3 % and % V 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. Significant ($P < 0.0001$) lower % RC and % A were noticed for cultivars Bintje and Charlotte in comparison to cultivar Nicola, while no differences were observed between Bintje and Charlotte. Moreover, significant different % V were noticed between the three cultivars, 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 %).

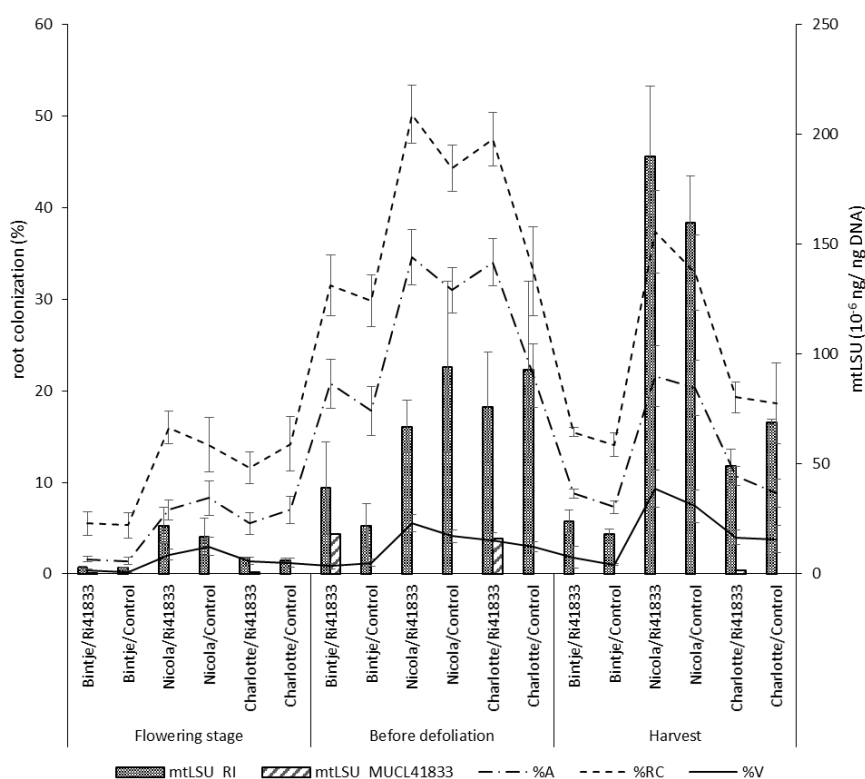


Figure 23. Root colonization (Total root colonization (%RC), Arbuscules (%A) and Vesicles/Spores (%V)) by AMF of potato cultivars Bintje, Nicola and Charlotte and MtLSU DNA concentration of *R. irregularis* (MtLSU-RI) or *R. irregularis* haplotype MUCL 41833 (MtLSU_MUCL41833).

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

MtLSU detection levels of all *R. irregularis* strains or just the target isolate at flowering stage, before defoliation and at harvest are presented in Fig. 23. 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 ($2.2 \times 10^{-7} \pm 1.7 \times 10^{-7}$ ng/ ng DNA) and Charlotte ($7.0 \times 10^{-7} \pm 1.2 \times 10^{-7}$ ng/ ng DNA) inoculated with this strain. Before defoliation, detection was again noticed in only one plant of the inoculated cultivar Bintje ($1.8 \times 10^{-5} \pm 8.0 \times 10^{-6}$ ng/ ng DNA) and inoculated cultivar Charlotte ($1.6 \times 10^{-5} \pm 1.5 \times 10^{-5}$ ng/ ng DNA). At harvest, detection was only noticed in one inoculated plant of cultivar Charlotte ($1.5 \times 10^{-6} \pm 2.9 \times 10^{-7}$ ng/ ng DNA). MtLSU of haplotype

MUCL 41833 was never detected in cultivar Nicola, whatever the stage of development. Inoculated strain was never detected in the control plants.

The species-specific MtLSU region of *R. irregularis* (MtLSU_RI) was detected in all the potato plants (Fig. 23). Statistical analyses of these results are presented in the supplementary material Table S5. 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. At harvest, detection of MtLSU_RI of cultivar Nicola was significantly higher than for cultivar Charlotte ($P < 0.001$) and cultivar Bintje ($P < 0.001$). In addition, detection of MtLSU_RI of cultivar Charlotte was significantly higher ($P < 0.0001$) than for cultivar Bintje.

Pearson’s correlation coefficients (r) between MtLSU of *R. irregularis* and AMF root colonization parameters (% RC, % A and % V) were calculated for all the treatments. A significant positive correlation was observed between MtLSU and % RC ($r = 0.6544$; $P = 0.0019$), % A ($r = 0.59750$; $P = 0.0062$) and % V ($r = 0.9184$; $P < 0.0001$).

Discussion

This study is the first, to our knowledge, that trace and quantify native and inoculated *R. irregularis* by Real-Time quantitative PCR of MtLSU under field conditions. The study was conducted on three different potato cultivars. MtLSU of the inoculant *R. irregularis* MUCL 41833 was characterized and two different markers designed. The first marker was a haplotype-specific marker (to trace the field-inoculated isolate) and the second marker a *R. irregularis* species-specific marker (to trace either the field-inoculated isolate or all other native isolates belonging to the species *R. irregularis*). Prior to field experiment, the markers were validated on potato plants grown under greenhouse conditions and the quantification of MtLSU paralleled with the root colonization stage as defined by Gallou et al. (2010). Even if in our field experiment markers were specific enough to trace inoculated or native strains, complete specificity of these primers could not be found in the MtLSU region sequenced. Other regions of mitochondrial genome should be further explored for more specific primers.

Significant correlation was noticed between MtLSU_MUCL41833 and total, arbuscular or vesicular colonization within roots of potato c.v. Bintje inoculated with *R. irregularis* MUCL 41833 in the greenhouse. This suggested that the haplotype marker was a good indicator of inoculation success of this specific isolate as well as a good indicator of the stage of root colonization. Krak et al. (2012) compared this MtDNA-based quantification to nrDNA-based quantification, which is used in numerous studies (Filion et al., 2003; Alkan et al., 2004; 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 nrLSU (Börstler et al., 2010) increased the chances of distinguishing an introduced isolate from a native background. The MtLSU marker was already used for the characterization of *R. irregularis* strains in pure cultures (Raab et al., 2005; Börstler et al., 2008) as well as for diversity studies (Börstler et al., 2010) or tracing of introduced isolates (Sýkorová et al., 2012) in the field.

The absolute quantification of the MtLSU gene of an introduced isolate was to our knowledge never performed on field samples. In our field trial, the haplotype *R. irregularis* MUCL 41833 was not detected in the non-inoculated controls suggesting that this haplotype was not

present in the field before the trial. Moreover, the haplotype-specific molecular marker revealed the low detection of the inoculated *R. irregularis* MUCL 41833 isolate in the cultivars Charlotte and Bintje and its non-detection in the cultivar Nicola. By contrast, the species-specific marker allowed to detect *R. irregularis* in all potato roots. This indicated that *R. irregularis* was indigenous to the experimental field since it was never inoculated in this specific location. The low detection of the inoculant *R. irregularis* MUCL 41833 could be explained by various factors such as the viability/infectivity of introduced isolate or its incompatibility with the soil environment or plant host (Oehl et al., 2010; Öpik and Moora, 2012). The competition with other microorganisms including the native AMF, lack of time for adequate and significant root colonization (Dickie et al., 2012) or the inadequate localization of the inoculant versus the tuber may similarly explain the low to absence of root colonization by *R. irregularis* MUCL 41833. From our experiment, it appears that the viability/infectivity of inoculum was high and probably not responsible for the low to absent colonization of the inoculated strain. The number of beads necessary to infect 90% of the plants was 13 (estimated by the MIP trial) and 120 beads were applied per potato plant. Competition between isolates of the same species is another factor that was proposed by Krak et al. (2012). These authors observed a decrease in MtLSU levels for one of two coexisting isolates from the same species. This hypothesis could not be verified in our experiment. The distance of inoculum to the roots of the germinating tubers was possibly responsible for the low colonization. Beads were placed next to the potato tuber but the distance with the roots was probably not optimal for good establishment. Propagule germination is generally restricted to less than one mm (D'Souza et al., 2013) and it is not excluded that the first large roots, produced by reserves from the tuber, are less adequate to be colonized by AMF than secondary roots that are at a higher distance from the tuber. However, in other studies (Hijri, 2016) potato seeds were sprayed with a liquid suspension of spores and apparently, this way of inoculation led to increased AMF root colonization and subsequent increased tuber yield. As described above a lot of factors can influence inoculation success. For example, inoculation of *Medicago sativa* cover crop with *R. irregularis* combined with *Trichoderma harzianum* increases AMF colonization and yield of subsequently-grown potato under low nutrient conditions (Buysens et al., 2016).

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 at flowering stage, before defoliation or at harvest was in most cases the highest for Nicola, followed by Charlotte and Bintje. 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 also showed that crop breeding programs selecting for high yield varieties under fertilized conditions may have inadvertently select genotypes that are less responsive to mycorrhiza (Johnson and Pfleger, 1992). But a meta-analysis on studies from 1981 to 2010 (Lehmann et al., 2012) observed that new cultivars were less intensively colonized but were more mycorrhiza-responsive (and possibly dependent) compared to ancestral genotypes. Although this could not be proved in our experiment it may support the interest to consider the association with AMF within breeding strategies (Rengel, 2002).

Conclusion

In conclusion, our study demonstrated, for the first time, that MtLSU-based Real-Time qPCR assays could be used in the field for quantification of native and inoculated AMF strains in addition or potentially in substitution to root colonization estimation by microscopic observations. MtLSU amount was correlated with colonization level (especially % of arbuscules) and is thus a good indicator of colonization success. This study showed that the effectiveness of AMF inoculation under field conditions is complex and depends on several factors. This molecular traceability method should be further used in studies searching for better inoculation methods. Even if the markers used in the present were specific enough, specificity of the markers could be further enhanced by sequencing whole mitochondrial genome to find more specific regions (Badri et al., 2016). In addition, this study showed that MtLSU-based Real-Time qPCR assays are adapted to quantify selected AMF strains in potato roots in order to compare different potato cultivars and find the most colonized cultivars by AMF (e.g. Nicola > Charlotte > Bintje).

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Chapter II

Impact of *Rhizophagus irregularis* MUCL 41833 on disease symptoms caused by *Phytophthora infestans* in potato grown under field conditions

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Preface

Potato organic farming relies mostly on copper for late blight management. However, the environmental impact of copper is not neutral, and may strongly impact soil microbial communities. Several EU directives have been implemented with the aim to achieve a copper-free control of late blight. However, no completely satisfactory alternative has emerged, and copper treatment therefore remains unavoidable. Recently, Gallou et al. (2011) demonstrated that the AMF *R. irregularis* MUCL 41833 was able to stimulate potato defense mechanisms against *Phytophthora infestans*. However, the study was conducted *in vitro*, and field trialing are needed to ascertain the potential of AMF to participate in late blight management. It appears thus important to determine the conditions under which the AMF inoculant may be used in the field.

In chapter I, we developed a molecular tool based on the mitochondrial genome MtLSU to trace the AMF inoculant (i.e. *Rhizophagus irregularis* MUCL 41833) into the roots of potato plants grown in the field. In chapter II, the objective was to study the effects of this specific AMF on potato (var. Bintje and Nicolas) over two contrasting (dry and wet) cropping seasons. The study was focused on the detection of *R. irregularis* MUCL 41833 within the roots of potato plants to ascertain its presence; potential effects on plant growth parameters (e.g. tuber yield) and late blight disease development. On above-ground plant parts the disease was scored by percentage of leaf infection and the relative area under the disease progress curve (rAUDPC) was calculated.

Abstract

In organic potato production in Europe, only copper-based fungicides allow to directly control *Phytophthora infestans* (the causal agent of late blight). Due to environmental concerns caused by the repeated and excessive use of Cu before the nineties, the EU legislation has promoted alternatives approaches such as the use of biocontrol agents (e.g. arbuscular mycorrhizal fungi – AMF). Here, two field trials were conducted over two climatic-contrasting growing seasons. Trial 1 was characterized by a dry and hot cultural season with low pressure of *P. infestans*, while trial 2 was conducted under high humidity and relatively low temperatures with high pressure of the pathogen. In both trials, sprouted potato tubers were inoculated with AMF in the greenhouse before transplanting to the field. A Real-Time quantitative PCR assay was designed to target the inoculant strain *Rhizophagus irregularis* MUCL 41833 as well as the native *Rhizophagus irregularis* strains. In both trials, the inoculated AMF was detected in the roots at harvest, demonstrating the capacity of the inoculated strain to incorporate the microbiome of the potato plants. In the first trial, disease severity in AMF pre-colonized potato plants was markedly decreased and the onset of late blight symptoms was delayed by 10 days. In contrast, in the second trial no differences were noticed between AMF pre-colonized and control plants. In both trials, no mycorrhizal effect was noticed on tuber yield. As a conclusion, disease severity of *P. infestans*, measured by symptoms development on leaves, was decreased in AMF pre-colonized plants under conditions of low pressure of late blight and over a short period of time, while under conditions more adequate to the pathogen, no reduction in symptoms was noticed.

Keywords

Arbuscular mycorrhizal fungi; *Rhizophagus irregularis*; *Phytophthora infestans*; late blight management; Real-Time quantitative PCR; traceability.

Introduction

Potato is the fourth most important crop produced in Europe and the fifth worldwide (FAO, 2014). In Belgium, it occupies 80000 Ha, with Bintje representing around 50% of the surface (FIWAP, 2010). Hundreds of varieties are produced worldwide. However, because of their low genetic base, most varieties are susceptible to many devastating pests and diseases (Consortium, 2011).

Phytophthora infestans, the causal agent of late blight, is the most devastating potato pathogen worldwide (Haas et al., 2009). Damages caused by this oomycetes as well as the measures to control the disease accounts for more than 1 billion € per year in Europe (Vos and Kazan, 2016). This pathogen is a hemibiotroph that behave as a biotroph during the early stages of potato infection turning to a necrotroph in the later stage (Gallou et al., 2011). Genome sequence and analyses showed rapid turnover and extensive expansion of secreted disease effector proteins that alter host physiology (Haas et al., 2009).

Late blight control is mostly achieved via the repeated (10 to 16 per growing season) applications of fungicides (Haverkort et al., 2009), which may have serious side-effects on the environment and human health (Wilson and Tisdell, 2001; Beketov et al., 2013). Copper application has been used intensively in pest management since the discovery of its effects on diseases by the end of the 19th century. However, the repeated application of Cu at doses 50% to 100% higher than the admitted norm have resulted in Cu accumulation in soils with significant impacts on below-ground organisms (Graham et al., 1986; Brun et al., 1998). In 1991, the EU issued a directive (Regulation No.2092/91 – (Regulation, 1991) limiting the use of copper in organic farming to 6 kg/ha per year. In 2009, another directive (the EU directive 2009/128/CE – (directive, 2009) established a framework to achieve a sustainable use of pesticides by reducing the risks associated with their use, particularly on human health and environment. This directive promotes the use of integrated pest management and of alternative approaches or techniques such as the application of nature-based compounds (NBCs) or biological control agents (BCAs). To replace Cu-based fungicides, NBCs including plant extracts or BCAs such as

Trichoderma spp., arbuscular mycorrhizal fungi (AMF) or Bacillus spp. have been tested and were shown effective against *P. infestans* or other *Phytophthora* species under laboratory or greenhouse conditions (Dorn et al., 2007).

Arbuscular mycorrhizal fungi are obligate root symbionts that form associations with an estimate of 80% of terrestrial plant species (Smith and Read, 2008), including most crops such as potato, wheat, maize. They improve plant mineral nutrition (especially phosphorus) and thus growth and yield. They also play major roles in protecting the plants against biotic and abiotic stresses (Parniske, 2008; Smith and Read, 2008).

Several studies have reported an increasing resistance of AMF-colonized plants against above and below-ground pathogens (Whipps, 2004). The effects were either local (at the site of infection of the pathogen) (Trotta et al., 1996; Hayek et al., 2014) or systemic (Cordier et al., 1998; Khaosaad et al., 2007) and mostly concerned root pests and diseases. The impact on above-ground pathogens were less numerous and conclusive (Pozo and Azcón-Aguilar, 2007), even if systemic effects have been reported on tomato against *Fusarium sambucinum* or *Alternaria solani* (Song et al., 2010; Ismail and Hijri, 2012) and on potato against *P. infestans* (Gallou et al., 2011). Most studies were conducted under controlled conditions (in the greenhouse or *in vitro*). Results in the field are less abundant and more controversial due among others to the competition with the indigenous microorganisms that could impact the establishment and development of the AMF inoculants (Berruti et al., 2015).

Rhizophagus irregularis is amongst the most widely used AMF in commercial products (Buysens et al., 2017). It is a ubiquitous, generalist symbiont (Berruti et al., 2014) that can colonize a wide variety of plants, including potato (Buysens et al., 2016). Large scale experiments conducted in Canada showed an increase in yield of marketable potato tubers in plants inoculated with *R. irregularis* DAOM 197198 (Hijri, 2016). However, in most studies, the authors could not separate root colonization by the inoculant from the indigenous AMF and thus could not firmly attribute the increased yield to the introduced fungus.

To identify closely related members of the same AMF species, the mitochondrial genome is a promising target (Badri et al., 2016). It was used to relate crop health improvement to inoculants in largescale field experiments (Börstler et al., 2008; Badri et al., 2016; Nadimi et al., 2016). Development of specific molecular markers for field application is particularly interesting when the strain has already shown promising result on *P. infestans* in controlled conditions (Gallou et al., 2011).

Molecular toolkits, based on mitochondrial genome, were developed on spore samples (Badri et al., 2016) and for field assays (Buysens et al., 2017). They are, to the best of our knowledge, the only one-step PCR molecular analysis for AMF strain traceability. During the field experiment of Buysens et al. (2017), *R. irregularis* MUCL 41833 was inoculated on potato and traced via the mitochondrial Large SubUnit (MtLSU). The inoculated AMF strain could be detected but at low levels and no significant difference in total root colonization was noticed between the control (i.e. non-inoculated) and inoculated potato plantlets. This was probably related to various factors among which the mode of inoculation, the viability/infectivity of the introduced isolate, the compatibility of the AMF with the soil environment, host plant or agricultural practices (e.g. frequency of fungicides application) (Buysens et al., 2017; Loján et al., 2017).

The aim of the present study was to evaluate the effects of *Rhizophagus irregularis* MUCL 41833 on the development of late blight in potatoes grown under field condition. Potato tubers were pre-inoculated in the greenhouse and subsequently transferred to the field. Although, we are aware that this method of inoculation offers few perspectives for potato field production, it allows to ascertain the resilience of the inoculant (i.e. *R. irregularis* MUCL 41833) in the field (i.e. its capacity to develop within roots and to compete with the local AMF community). A Copper-based fungicide was use as positive control. The AMF root colonization was estimated visually and by using mitochondrial markers specific to the inoculated haplotype or to the local *R. irregularis* species in the field, while symptoms of late blight were rated on the leaves. Trials were conducted over two climatic-contrasting growing seasons.

Materials and method

Biological Material

Solanum tuberosum cv. Bintje and Nicola (susceptible and semi resistant to foliar late blight, respectively) were provided by the “Station de Haute Belgique”, Libramont (Belgium). Tubers (size 28-35 mm) were placed in a growth chamber set à 20°C during 15 days to break dormancy.

Seeds of *Medicago truncatula* Gaertn. cv. Jemalong A 17 (SARDI, Australia) and *Zea mays* L.cv. ES. Ballade (Euralis, France) were surface-disinfected following (Gallou et al., 2012) and further pre-germinated on wet paper. The maize seedlings were used for mass production of the AMF (IJdo et al., 2011).

The AMF strain *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) Schüßler and Walker (2010) as ['irregulare'] MUCL 41833 was provided by the Glomeromycota *in vitro* collection (GINCO: <http://www.mycorrhiza.be/ginco-bel>) on the Modified Strullu-Romand (MSR) medium (Declerck et al., 1998). Pieces of gel containing AMF-colonized roots and spores were inoculated on tubers of Bintje in 1.4 L pots containing a mix of sterile sand/vermiculite in equal volume, for AMF mass-production. Plants were grown in a greenhouse (25°C, 75 % Relative Humidity (RH) and 16/8 h (day/night) photoperiod under natural light conditions) for 4 months. They received Osmocote® (NPK (Mg): 17/11/10 (2) at regular intervals.

Pre-mycorrhization of potato tubers

AMF-colonized roots were sampled from the potato plants, chopped in 1 cm fragments and placed in a 4 L container (40x60x10 cm, ETS Dubois Frère S.A, Belgium) between two layers of sterile sand/vermiculite in equal volume. Pre-germinated seedlings of maize were planted on top of the inoculum layer and covered by 2 cm of sterile sand/vermiculite mix and watered 2 times per week with deionized water. The containers were subsequently placed in the greenhouse (25 °C, 75 % RH, 16 /8 h (day/night) under natural light conditions) for 1 month. Control containers without AMF were identically set up. Pre-

germinated seedlings of *M. truncatula* were transferred in the containers hosting the AMF-colonized or control maize plants for fast and homogenous colonization. The plants received 6.5 g Osmocote® (NPK 17/11/10) and were co-cultured for 6 weeks.

The pre-germinated *M. truncatula* plantlets were colonized within a period of 6 weeks. The plantlets were subsequently transferred into compressed peat pots (6 cm Diam., ref: 306 122 90, Jiffy®, Netherland) filled with sterile mix of sand/vermiculite in equal volumes. Each pot received three AMF-colonized *M. truncatula* plantlets and one sprouted potato tuber (Sup Fig. S3). The systems were maintained 4 weeks under the same greenhouses conditions as above for pre-mycorrhization of the potato plantlets before manual transplantation into the plots. A similar procedure was followed with non AMF-colonized *M. truncatula* plantlets for the control plantlets.

Experimental design and field management

Two field trials were conducted in Libramont (Belgium) at the Walloon Agricultural Unit. Field trial 1 (49°55'25.3"N 5°21'51.0"E) was conducted from early June to end of September 2015 and field trial 2 (49°55'37.9"N 5°22'05.4"E) from late June to early September 2016. The climate of the region is temperate with an annual mean precipitation of 1191 mm year⁻¹ and an annual mean temperature of 8.3 °C (PAMESEB 2012 Belgium network, <http://www.pameseb.be/>). The average daily rainfall was 2.3 L.m⁻² and 2.1 L.m⁻² during the 4 months of field trial 1 and 2, respectively (PAMESEB 2015 and 2016, Belgium network, respectively). Environmental conditions were recorded daily (Sup. Fig. S4). Soil minerals content is presented in Sup. Table S6. For trial 1, crop rotation the last 3 years was spelt (2014, 2013) and grassland (2012). For trial 2, it was spelt (2015, 2014) and grassland (2013). For both trials, preparation of the plot consisted of mechanical plowing and application of chemical herbicide (Challenge 2 L ha⁻¹ + Proman 3 L ha⁻¹ + Artist 1, 25 kg ha⁻¹, Bayer, Germany) (Sup. Table S7). No fertilizers were used during the experiment.

In trial 1, the potato cultivar Bintje was used and individual subplots were arranged in a completely randomized block design with four blocks per treatment. Copper treatment consisted of 10 applications of 600 g ha⁻¹ of Cu (Cuperit, Bayer, Germany). The different treatments are combinations of two factors: with (+AMF) or without (-AMF) pre-mycorrhization of the potato plants and with (+Cu)

or without (-Cu) copper application in the field. Four treatments were thus considered: (+AMF -Cu), (+AMF +Cu), (-AMF -Cu) and (-AMF +Cu). In trial 2, the same individual subplots and treatments were considered but two potato cultivars (Bintje and Nicola) were considered. In both trials, each individual subplot measured 21 m² (length 3 m x width 7 m or 6 mounds) with 36 potato plants of cultivar Bintje or Nicola. Subplots were separated from each other by a distance of 2 m.

Relative abundance of AMF (MPN)

Before the experiments, the density of AMF within the plots was estimated by the most probable number (MPN) method (Cochran (1950) and following Jarvis et al. (2010) for calculation. An average of 5 (1.7 < 5 > 15) and 2.8 (1.2 < 2.8 > 6.9) propagules g⁻¹ soil was estimated in the soil of trial 1 and 2, respectively.

Disease assessment

Symptoms caused by *P. infestans* were monitored every 3 to 4 days after onset of the disease. *P. infestans* infection occurred naturally in the fields, in late August in 2015 (trial 1) and late July in 2016 (trial 2). In both trials, the disease symptoms were evaluated until potato harvest. Disease assessment was estimated following James (1971) and EUCABLIGH (www.eucablight.org/Lib/Eucablight/Protocol/FoliarBlight_V12.pdf) guidelines and converted into percentage of leaf infection (EUCABLIGH guidelines – see Sup Table S8) or relative area under the disease progress curve (rAUDPC). The AUDPC was calculated according to the equation of Madden and Campbell (1990): $AUDPC = \sum_{i=1}^{n-1} (y_i + y_{i+1})/2 \times (t_{i+1} - t_i)$, where n = number of observations, y = the leaf infection index and t = the day post emergence. Relative AUDPC (rAUDPC) was calculated by dividing the AUDPC of each treatment by the number of days of effective *P. infestans* infection (i.e. first sporulation lesion in the plot as described in EUCABLIGH guideline). No late blight symptoms was measured on tuber during both trial.

Phytophthora infestans identification

In both trials, samples of the pathogen were collected and processed in order to characterize the genotype and mating type (Sup Table S9) according to EUCABLIGHT guidelines (www.eucablight.org/Lib/Eucablight/Protocol/BlightSampling_V1.0.pdf). Specie characterization was made by morphological analysis and genotypes were assessed using simple sequence repeats or microsatellites (SSR) (<http://www.eucablight.org/EucaBlight.asp>).

Estimation of potato tuber weight, size and number

In both trials, potato tubers were harvested in the four central lines. Transplantation success was assessed after three weeks. In trial 1, only the number and size of tubers were considered and ranked in three categories from size (< 35 mm, between 35 and 50 mm and > 50 mm). In trial 2, potato yield was measured over all the tubers as well as number of tubers per plant and weight of aerial part.

AMF root colonization

Root colonization was assessed at plantation on five randomly selected plants of each cultivar. Similarly, at harvest, root colonization was evaluated on five plants from the four central lines of each subplot in the two trials. The root systems were cleaned from soil debris and dried under laminar flow at room temperature. Half of the root system was stored at -20°C for molecular analysis (see below) and the other half was used for root colonization assessment.

Roots were prepared as in (Vierheilig et al., 1998) for colonization assessment. Roots were then transferred in a solution of lactoglycerol (equal volume – lactic acid: glycerol: H₂O) and placed at 4 °C until analysis. Root colonization was assessed following the technique of McGonigle et al. (1990). For each sample, 150 intersections were observed under a compound microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 40 X magnification and

percentage of total root colonization (%RC) and percentage of arbuscules (%A) estimated.

Detection of *R. irregularis* MUCL 41833

In both trials, the frozen root samples were ground using liquid nitrogen and DNA were extracted following innuPREP Plant DNA kit (Analytikjena, Germany) protocol with slight modifications (for details see Buysens et al. (2017)). The same protocol was conducted for the extraction of AMF spores used for qPCR standards curves.

Previous to the field trials, soil samples were collected to confirm the absence of the inoculated strain in the native community of AMF. The soil was dried and then sieved using a 5 and 1 mm mesh. Soil extraction was performed using FastDNA™ SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacturer instructions.

Concentrations of DNA extracted from the different samples were measured with Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, USA) using Fluoroscanner Ascent FL (LabSystems, USA). DNA concentrations were adjusted at 4 ng μl^{-1} , allowing a 10 ng DNA template in each qPCR reaction.

The qPCR was made using LightCycler® FastStart Essential DNA Green Master in 10 μl volume of reaction formed as follow; 5 μl Master mix, 0.5 μl of each primer from the pair, 1.5 μl H₂O PCR grade and 2.5 μl DNA. The cycling condition were: 10 min at 95 °C, 45 cycles of denaturation (95 °C, 10 s)/annealing (62 °C, 10 s)/extension (72 °C, 15 s) and finalized by a standard melting curve analysis. Two pairs of primers (BF7/BR5 and BF8/BR6) were used (Buysens et al., 2017). The first one (BF7/BF5) is *R. irregularis* specie-specific and the second one is *R. irregularis* MUCL 41833 haplotype-specific.

Absolute quantification of specific target was made using the LightCycler® 96 software 1.1 (Roche, Switzerland). The results were expressed as ng of the target mitochondrial marker (*R. irregularis* or haplotype) DNA for 10 ng of template DNA.

Data analysis and statistics

Data analyses were performed with JMP® Pro statistical software version 12.1.0 (SAS Inc., Canada) with a linear mixed model, where “AMF inoculation” and “Copper application” were regarded as fixed factors and block as a random factor. Mixed procedure used to analyze dynamics of infection were performed with the SAS university edition (SAS Institute Inc., Cary, USA) and Wilcoxon/Kruskal-Wallis on JMP® Pro to identify the significant differences between treatments (+AMF–Cu or –AMF–Cu) at each time point. Mixed procedure was used to analyze different slopes with 3 fixed effects; “AMF inoculation” (+AMF–Cu or –AMF–Cu), “Time” (9 and 7 time points for trial 1 and 2, respectively) and interactions between the factors for each cultivar. The number of points corresponded with the number of observations of *P. infestans* symptom over the cropping season. During the first trial the cropping season was longer and thus 2 more observations were made.

Results

Field trial 1

AMF root colonization

Table 6. Trial 1, percentage roots (% RC) and arbuscules (% A) colonization and MtLSU DNA of *R. irregularis* (MtLSU-RI) and *R. irregularis* haplotype MUCL 41833 (MtLSU-41833) in roots of potato cv. Bintje, at harvest for the different treatments that are combinations of two factors: “AMF inoculation” (with (+AMF) or without (-AMF) inoculation) and “Copper application” (with Copper (+Cu) or without Copper (-Cu) application).

Treatment	% RC	% A	MtLSU-RI ng/ng DNA	MtLSU-41833 ng/ng DNA
-AMF -Cu	14.2±7.5	6.7±3.9	4.2x10 ⁻⁸ ±2x10 ⁻⁸	nt
-AMF +Cu	9.1±2.4	3.8±1.2	5.9x10 ⁻⁷ ±6.6x10 ⁻⁷	nt
+AMF -Cu	35.6 ±6.2	17.1±4.5	2.07x10 ⁻⁷ ±3.5x10 ⁻⁷	8.33x10 ⁻⁰⁷ ±1.33x10 ⁻⁰⁶
+AMF +Cu	26.9 ±14.1	12.7±6.9	1.31x10 ⁻⁶ ±1.31x10 ⁻⁶	7.25x10 ⁻⁰⁷ ±1.25x10 ⁻⁰⁶
Mixed model	p-values			
AMF inoculation	0.0029	0.0050	0.2654	nt
Cu application	0.1905	0.1982	0.0546	nt
AMF inoculation vs Cu application	0.7234	0.7868	0.4756	nt

Data are means ± standard error of 4 blocks (24 plants per block). Main effects and interactions between the factors “AMF inoculation” and “Copper application” are presented, with significant effect (in bold) and significance values were set at $P < 0.05$ (mixed model). Not tested (nt).

At plantation in the field, the %RC and %A was 34.0 ± 19.7 % and 6.3 ± 7.7 %, respectively. These values did not differ from those recorded at harvest. At harvest (Table 6), a significant effect was noticed for the factor “AMF inoculation” on %RC and %A with values nearly three times higher for the inoculated (+AMF) versus the control (-AMF) plants, irrespective of the application of Cu. No significant effect of the factor “Copper application” or interactions “AMF inoculation” vs “Copper application” was observed on %RC or %A.

Quantification of native and inoculated *R. irregularis* in potato field

The MtLSU DNA concentration of *R. irregularis* and *R. irregularis* haplotype MUCL 41833 was evaluated at harvest (Table 6). MtLSU of haplotype MUCL 41833 was detected in the roots of the inoculated plants and absent in the control plants. The MtLSU concentration of haplotype MUCL 41833 was significantly higher ($P < 0.0082$) at plantation in the field than at harvest. No significant difference was noticed between the (+AMF) inoculated plants treated or not with Cu. The species-specific MtLSU region of *R. irregularis* was detected in all potato plants. No effect of the factor “AMF inoculation” or “Copper application” or interaction between both factors was observed on the amount of MtLSU-RI detected in the roots.

Leaf infection dynamics of *P. infestans*

The rate of infection by *P. infestans* of pre-mycorrhized and control potato plants in absence of Cu application was plotted against time (Figure 24). The rate of infection followed a similar dynamic, but the progression of disease was significantly ($P < 0.0179$) slower in the +AMF-Cu versus -AMF-Cu treatment. The first traces of infection were observed 86 days after plantation in the field. The first significant differences between the -AMF-Cu and +AMF+Cu treatments were observed 3 days following disease appearance ($P < 0.05$, Wilcoxon/Kruskal-Wallis). The rate of infection increased rapidly but no plateau was reached at harvest.

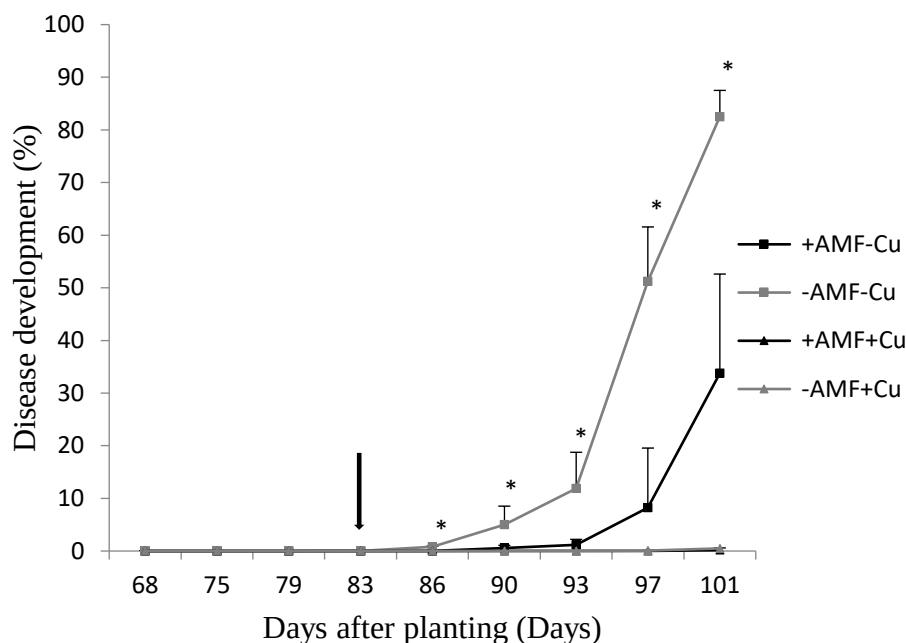


Figure 24. Trial 1, percentage of cv. Bintje leaf infection by *P. infestans* of the different treatments that are combinations of two factors: “AMF inoculation” (with (+AMF) or without (-AMF) inoculation) and “Copper application” (with Copper (+Cu) or without Copper (-Cu) application). Measurements were made starting 68 days after plantation until harvest (102 days after plantation) and disease onset was observed 83 days after planting. Data are means $\pm$ standard error of four blocks (36 plants per block). For each time, main effects “AMF inoculation” was tested, data for copper were not included. Significance values (*) were set at $P < 0.05$ (Wilcoxon/ Kruskal-Wallis). The dark arrow indicates the first observation of symptoms of *P. infestans* (i.e. 83 days after planting).

In presence of Cu, the development of *P. infestans* was almost inexistent (data not reported in Fig. 24) with values for rAUDPC close to zero. In absence of Cu, the rAUDPC was 0.073 ± 0.048 and 0.262 ± 0.037 for the +AMF-Cu and -AMF-Cu treatments, respectively. A significant effect of the factor “AMF inoculation” ($P < 0.0004$), “Copper application” ($P < 0.0001$) and interactions between the factors

($P < 0.0005$) was noticed on the rAUDPC. The rAUDPC was more than three times lower in the +AMF-Cu versus -AMF+Cu treatment.

Potato tuber weight, size and number

No significant effect of the factor “AMF inoculation”, “Copper application” or interactions between both factors was noticed on potato weight and number of tubers per treatment (Table 7).

Table 7. Trial 1, Tuber weight and number of potatoes tubers (cv. Bintje) per subplot at harvest (sorted by size). Different treatments are combinations of two factors: “AMF inoculation” (with (+AMF) or without (-AMF) inoculation) and “Copper application” (with copper (+Cu) or without copper (-Cu) application).

Treatment	Potato Tubers					
	Average weight (kg) per treatment			Average number per treatment		
	C 2			C 2		
	C 1 <35 mm	35 mm > <50 mm	C3 >50 mm	C 1 <35 mm	35 mm > <50 mm	C 3 >50 mm
-AMF -Cu	2.9± 0.9	9.5± 3.8	18.2± 2	170±5 1.4	151.4 ±59.7	98±19.4
-AMF +Cu	2.3± 0.5	7.6± 1.1	20.6± 5.5	133.4 ±40.4	120±1 8.4	95.5±25.6
+AMF -Cu	1.8± 0.5	5.8± 0.8	18.6± 1.3	108.7 ±30.2	92.2± 17.8	85± 5.5
+AMF +Cu	2.2± 0.3	7.3± 1.6	22.6± 1.3	129.8 ±16.9	115.5 ±30.7	109±13.7
Mixed model	p-values					
AMF inoculation	0.093	0.140	0.415	0.151	0.148	0.979
Cu application	0.733	0.877	0.055	0.718	0.845	0.245
AMF inoculation vs Cu application	0.165	0.185	0.607	0.197	0.206	0.159

Data are means ± standard error of 4 blocks (24 plants per block). Main effects and interactions between the factors “AMF inoculation” and “Copper application” are presented. Significance values were set at $P<0.05$ (Mixed model).

Field trial 2

AMF root colonization

Potato root colonization was evaluated at harvest (Table 8). For cv. Bintje, no significant difference was detected in %RC and %A between plantation in the field and harvest. To the contrary, for cv. Nicola, %RC and %A were significantly lower at plantation in the field ($11.5 \pm 5\%$ and $7.5 \pm 3.9\%$, respectively) as compared to harvest. For both cultivars, a significant effect was noticed for the factor “AMF inoculation” at harvest. For cv. Bintje, the %RC and %A were significantly higher for the plants in the +AMF versus -AMF treatment. Similarly, for cv. Nicola, the %RC and %A were significantly higher for the plants in the +AMF versus -AMF treatments. For both cultivars, no significant effect was observed for the factor “Copper application” or for interactions between both factors.

Table 8. Trial 2, percentage roots (% RC) and arbuscules (% A) colonization and MtLSU DNA concentration of *R. irregularis* (MtLSU-RI) and *R. irregularis* haplotype MUCL 41833 (MtLSU-41833) in roots of potato cv. Bintje or cv. Nicola, at harvest for the different treatments that are combinations of two factors: “AMF inoculation” (with (+AMF) or without (-AMF) inoculation) and “Copper application” (with copper (+Cu) or without copper (-Cu) application).

Treatment	Bintje				Nicola			
	% RC	% A	MtLSU-RI ng/ng DNA	MtLSU-41833 ng/ng DNA	% RC	% A	MtLSU-RI ng/ng DNA	MtLSU-41833 ng/ng DNA
-AMF -Cu	8±3.1	4.6±2.2	7.2x10 ⁻⁷ ±2.6x ⁻⁷		17.4 ±10.7	10.1±6.7	3x10 ⁻⁵ ±2x10 ⁻⁶	
-AMF +Cu	9.3±5.3	4.5±2.3	1.3x10 ⁻⁵ ±1.3x10 ⁻⁶		22 ±14.5	11.8±7.5	3.4x10 ⁻⁵ ±3.7x10 ⁻⁶	
+AMF -Cu	19.2 ±6.6	7.4±2.5	1.7x10 ⁻³ ±1.4x10 ⁻⁴	2.6x10 ⁻³ ±1.3x10 ⁻⁴	30.7 ±14.3	17.2±8.4	4.3x10 ⁻⁴ ±3.8x10 ⁻⁵	4.9x10 ⁻⁴ ±2.3x10 ⁻⁵
+AMF +Cu	21.3 ±8.2	9.4±3.8	3.3x10 ⁻⁴ ±2.8x10 ⁻⁵	4.3x10 ⁻⁴ ±3.3x10 ⁻⁵	36 ±12.3	18.7±6.2	1.1x10 ⁻³ ±4.2x10 ⁻⁵	1.2x10 ⁻³ ±5.6x10 ⁻⁵
Mixed model	p-values				p-values			
AMF inoculation	0.0001	0.0001	0.0405	nt	0.0002	0.0014	0.0020	nt
Cu application	0.3504	0.1963	0.1594	nt	0.1124	0.4326	0.1490	nt
AMF inoculation vs Cu application	0.8149	0.2369	0.1590	nt	0.8775	0.9629	0.1536	nt

Data are means ± standard error of 4 blocks (24 plants per block). Main effects and interactions between the factors “AMF inoculation” and “Copper application” are presented, with significant effect (in bold) and significance values were set at $P < 0.05$ (Mixed model). Not tested (nt).

Quantification of native and inoculated *R. irregularis* in potato field

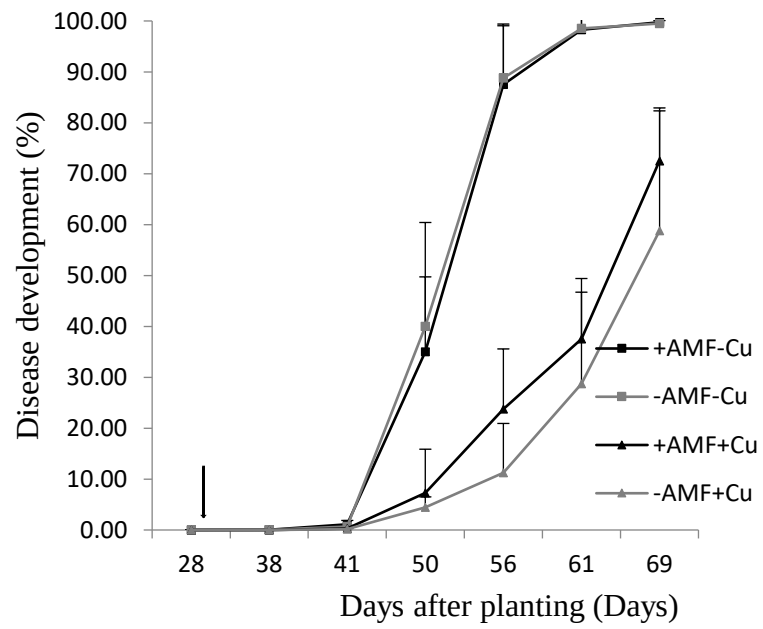
For both cultivars, the concentration of MtLSU of haplotype MUCL 41833 did not differ significantly between plantation and harvest (Table 8). Irrespective of the Cu application, the MtLSU of haplotype MUCL 41833 was detected in the roots of the plants in the +AMF treatment and absent in the plants of the -AMF treatment. No significant difference was further noticed between the plants from the +AMF treatment irrespective of the Cu application.

The species-specific MtLSU region of *R. irregularis* was detected in all the potato plants. For cv. Bintje, the MtLSU-RI concentration was significantly higher in the plants of the +AMF versus –AMF treatment. Similarly, for var. Nicola, the MtLSU-RI concentration was significantly higher in the plants of the +AMF versus –AMF treatment. There was no effect of the factor “copper application” or interaction between both factors on the amount of MtLSU-RI detected in the roots. No MtLSU-RI and MtLSU41833 was detected in the soil at the field plots and the factor “cultivar” (Bintje or Nicola) did not affect the level of detection of AMF.

Leaf infection dynamics of *P. infestans*

The rate of infection by *P. infestans* in the plants of the +AMF treatment or control potato plants in absence of Cu application was plotted against time (Figure 25. a and b). Differences in leaf infection between the two cultivars were significant starting at day 38 of culture. At harvest, the percentage of leaf infected was significantly higher ($P < 0.0007$) for cv. Bintje (i.e. 99.6 ± 0.5) as compared to cv. Nicola (49.4 ± 12.7).

25. a



25. b

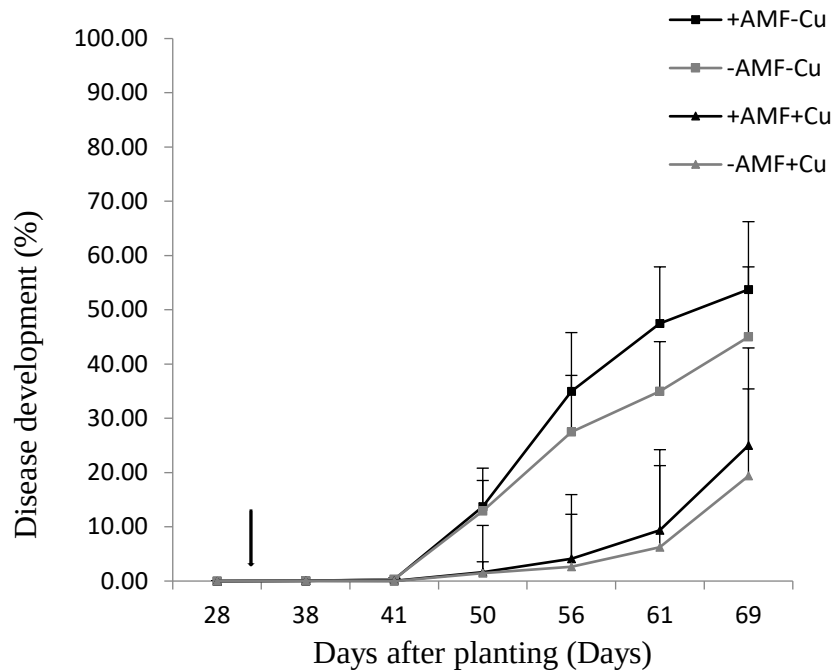


Figure 25. Trial 2, percentage of leaf infection by *P. infestans* of the different treatments that are combinations of two factors: “AMF inoculation” (with (+AMF) or without (-AMF) inoculation) and “Copper application” (with Copper (+Cu) or without Copper (-Cu) application), (a) Bintje, (b) Nicola. Measurements were made starting 28 days after plantation until harvest (69 days after plantation) and disease onset was observed 38 days after planting. Data are means $\pm$ standard error of 4 blocks (36 plants per block). For each time, main effects “AMF inoculation” was tested, data for copper were not included. Significance values (*) were set at $P < 0.05$ (Wilcoxon/Kruskal-Wallis). The dark arrow indicates the first observation of symptoms of *P. infestans* (38 days after planting).

The development of *P. infestans* was only significantly reduced ($P < 0.001$ for both cultivars) in presence of Cu. The rAUDPC of the two treatments (+Cu) were 0.233 ± 0.092 and 0.067 ± 0.035 for cv. Bintje and Nicola, respectively. In absence of Cu application, the rAUDPC of

cv. Bintje or Nicola was not significantly different in presence or absence of AMF-inoculation (0.590 ± 0.051 or 0.243 ± 0.06 , respectively).

Potato tuber weight, size and number

No significant effect was noticed for the factors “AMF inoculation” and “Copper application” on tuber weight and number of tubers/plant and on shoot weight for the cultivar Nicola. For cv. Bintje, the factor “Copper application” had a significant effect on the tuber weight and number of tubers, ($P = 0.0024$ and $P = 0.0041$, respectively) and the shoot weight ($P < 0.0041$). For cv. Bintje, yield, shoot weight and number of tubers/plant were 142.9 ± 41.7 g, 13.6 ± 10.1 g, 11.4 ± 2.7 , respectively as compared to 57.3 ± 25.0 g, and 7.8 ± 6.5 , 6.7 ± 2.4 g tubers/plant for the Cu free treatments. For both cultivars, no significant effect was noticed for the interaction between both factors on yield.

Discussion

Arbuscular mycorrhizal fungi have been repeatedly reported to increase the resistance of plants against pests and diseases (Whipps, 2004). However, studies conducted in the field only represent a fraction of the total number of studies involving AMF and, in most cases, the inoculated strains could not be traced and their contribution to root colonization and potential biocontrol effect separated from native AMF. Here we investigated for the first time the effects of inoculation of a specific AMF (i.e. *R. irregularis* MUCL 41833) on the progress of disease symptoms of *P. infestans* in potato grown under field conditions. We developed a method for efficient colonization of the plantlets and used an adequate tracing tool to quantify the presence of the fungus in the potato roots.

In the study conducted by Buysens et al. (2017), field inoculation with *R. irregularis* MUCL 41833 did not increase significantly % RC of cv. Bintje as compared to the non-inoculated control. The inoculated fungus was detected at extremely low concentration with the mitochondrial markers suggesting that colonization was mostly due to the local AMF species. The same result was obtained by Loján et al. (2017) in a field experiment conducted in Ecuador. It was thus decided to use the “plant donor” system of Voets et al. (2009) for the rapid and homogenous colonization of potato plantlets prior to field transfer. *R. irregularis* MUCL41833 was subsequently traced and quantified with the strain-specific primer developed by Buysens et al. (2017) to ascertain its presence during the field trials and evaluate its potential impact on the pathogen. The same field experimental design was conducted in two-closely related places over 2 growing seasons but under contrasting climatic conditions and thus pressure of the pathogen. In field experiment 1, *P. infestans* was detected late in the season (August 28th 2015), and consisted of a pool of different genotypes (13_A2 and other_A1), while in field experiment 2, the pathogen was detected earlier (July 26th 2016) with only one virulent genotype detected (13_A2).

In both trials, the %RC and %A at harvest were significantly higher for the pre-mycorrhized plants as compared to the non-pre-mycorrhized ones. Colonization by *R. irregularis* MUCL 41833 was detected using the MtLSU_MUCL41833 specific primer, while any species belonging to *R. irregularis* (inclusive strain MUCL 41833)

were detected with the pair of primers MtLSU_RI that discriminates at the species level. *R. irregularis* MUCL 41833 was detected only in the AMF-inoculated treatments, while the species-specific primers were detected in all the treatments. In trial 1, the species-specific primers level of detection tended to be higher in the plants of the AMF-inoculated treatment as compared to the non-inoculated treatment, but no significant differences were measured. To the contrary, in trial 2, the species-specific primer level of detection was significantly higher in the plants of the AMF-inoculated treatments for both cultivars as compared to the plants in the non-inoculated treatments. These differences in %RC and %A and in molecular detection in trial 2 and, for both growing seasons, was due to the pre-inoculation with the AMF, strongly suggesting that the inoculated strain remained active and incorporated the microbiome of the host during both trials. Interestingly in trial 2, the %RC and %A were significantly higher in cultivar Nicola than in cultivar Bintje. This corroborated the earlier findings of Buysens et al. (2017) and analyses of Bhattarai and Mishra (1984) suggesting that colonization of AMF was faster and development more abundant in the resistant cultivars.

In both trials, the application of Cu (10 times during the growing season in the Cu-treatments) did not, at least visibly, impact AMF root colonization either by the inoculated fungus or by the indigenous fungi. However, our experiment was conducted over a short period of time and it is not excluded that long-term experiments may lead to the accumulation of Cu in the field due to repeated applications over seasons and may thus impact soil microorganisms and more precisely AMF, as suggested by (Gosling et al., 2006). Accumulation of Cu in soil is particularly problematic in long term monoculture systems such as avocado (Van-Zwieten et al., 2004).

Potato production was not improved by the AMF inoculant. These results corroborated earlier findings by Hayek et al. (2012) and Mustafa et al. (2016) under controlled conditions, but differed from the study of Hijri (2016) reporting a global yield increase in a field experiment conducted in Canada with another *Rhizophagus irregularis* strain (DAOM 197198). It is obvious that results in the field may widely vary with AMF strain, potato cultivar and general environmental conditions and are thus a major issue for farmers desirous to use microbial inoculants. In trial 1, yield was in accordance with the study

of Buysens et al. (2017) conducted in the same area, while in trial 2, yield was strongly decreased. This strong decline was mainly attributed to the shorter period of culture and faster emergence of *P. infestans*. As compared to trial 1, the growing season was 1.5 months shorter and the first symptoms of late blight appeared 45 days earlier.

Climate was a key parameter of disease development of *P. infestans* in both trials. The length of a wet climate combined with temperatures has been used by Magarey et al. (2005) for developing infection models. In this model, *P. infestans* infection is optimal with a minimum of 6 h of wetness at 15°C. Furthermore, pressure of the pathogen were classified in groups, notably low or high, by Bangemann et al. (2014) depending as well on the temperature and relative humidity. Under a low or moderate pathogen pressure, copper adequately control the disease, while under high pressure, the symptoms are only partially decreased. This was observed in our trials Trial 1 was characterized by a dry and hot period of culture (PAMESEB 2015 Belgium network) very unfavorable to the pathogen. The first symptoms of late blight appeared at a very late stage of culture on well-developed potato plants gradually starting their senescence process. The combinations of temperature between 20°C during the day, 15 °C at night and precipitation were observed only at the very end of the cropping season. Under such conditions, Cu treatment completely controlled the development of the disease. Importantly, in the treatments without Cu application, the symptoms appeared later and were less severe in the pre-mycorrhized plants as compared to the non-pre-mycorrhized ones. The symptoms appearance was delayed by ten days and at harvest were more than 10-fold lower than in the non-pre-mycorrhized plants. Conversely, trial 2 was conducted under high humidity and relatively low temperatures (PAMESEB 2016 Belgium network) which were particularly favourable to *P. infestans*. These environmental conditions were observed early in the season and the first symptoms of late blight were detected already one month after potato plantation in the field. Copper treatments could only reduce by half the severity of symptoms and no effect of pre-mycorrhization could be observed, even for the moderated resistant cultivar Nicola.

The infection by *P. infestans* occurred naturally over the two growing seasons. However, the consequence drastically differed, due to the climatic conditions prevailing in the two trials but also because of

the genotypes. In trial 1, three genotypes were detected and only one in trial 2 (see Sup Table S9). Significant variations of aggressiveness have been noticed among isolates of the same geographic area (Carlisle et al., 2002). In our study, two weakly aggressive genotypes were present in trial 1 and one aggressive genotype was detected in both trials. We could hardly hypothesize that competition between genotypes associated with unfavorable climatic conditions could have decreased the pressure of *P. infestans* during trial 1.

We did not investigate the mechanisms responsible for the lower symptoms observed in the pre-mycorrhized plants in trial 1. However, it is well known that plants can develop an increased defensive capacity in response to colonization by AMF. This mycorrhiza-induced resistance (MIR) allows the colonized plant to respond faster and more efficiently to a pathogen infection (Pozo and Azcón-Aguilar, 2007) without inducing constant and costly production of defence compound (van Hulten et al., 2006; Pieterse et al., 2014a). Supposedly, the control plants and pre-mycorrhized plants were confronted to the same AMF community, therefore suggesting that the higher resistance on the pre-mycorrhized plants may be attributed to the inoculated strain. Indeed, Bødker et al. (2002) have suggested that indigenous strains are not necessarily the most effective in inducing resistance mechanisms in plants. Strain/host interaction are most likely specific and therefore may result in different bio-protective outcome, from enhancing resistance to no effect (Kobra et al., 2009; Hayek et al., 2012) or even negative effect (Whipps, 2004). Under elevated pathogen pressure, as in trial 2, *P. infestans* could strongly decrease the AMF beneficial plant defence effects in the susceptible (Bintje) as well as in the semi-resistant (Nicola) cultivars. Indeed, priming rarely provides complete protection against one dominant pathogen or pathogen race (Ahmad et al., 2010). During the first trial, the onset of disease symptoms was delayed as earlier reported with the same AMF under *in vitro* culture conditions (Gallou et al., 2011) before outbreak. Delay and transitory reduction of tomato wilt caused by *Ralstonia solanaceum* was also observed in *in vitro* conditions, using *R. irregularis* MUCL41833 (Chave et al., 2017). The MIR in this case seemed to be very effective in the first stages of plant/AMF/pathogen interaction. During the second trial, no impact on the late blight development was noticed and so MIR was not effective under high pathogen pressure.

As a conclusion, root colonization estimated by microscopic observations and MtLSU-based Real-Time qPCR assays confirmed the success of pre-mycorrhization with *R. irregularis* MUCL 41833 in both trials. Even though, potato yield was not improved. The MtLSU-based Real-Time qPCR is a promising approach to link a specific inoculated AMF strain to root colonization. The AMF *R. irregularis* MUCL 41833 partly and transitorily reduced the symptoms of the disease under low pressure of the pathogen. This suggested a potential role of AMF in field applications under specific conditions of pathogen pressure. The application of AMF should be further tested in combination with other agricultural practices in an integrated approach allowing to reduce the amount of pesticides used or their frequency of application during the cropping season.

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Chapter III

Interplant signaling via common mycorrhizal networks might warn uninfected potato plants from late blight outbreak

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Preface

Within soil, most plants are interconnected by fungal hyphae, the so-named “common mycorrhizal networks” (CMNs) (Gilbert and Johnson, 2017). A number of studies have suggested that these CMNs are involved in the distribution of nutrients (e.g. P) (Welte, 2009; Whiteside et al., 2019) as well as transmission of warning signals, as shown by Song et al. (2010) with AMF-colonized tomato plants infected by *Alternaria solani*, a necrotrophic fungus. Since then, other pathosystems have been investigated and CMNs have become a major issue in the elicitation of defense mechanisms in non-infected plants connected to diseased plants. However, all the experiments to date have been conducted in pots and the impact of unwanted microbes (e.g. bacteria and fungi) could not be discarded with certainty. Similarly, the type of signal (i.e. electric, chemical, volatile) transferred from diseased to healthy plants remains questionable. An alternative is thus to apply CMNs under *in vitro* cultivation systems, since it was shown *in vitro* that *Rhizophagus irregularis* MUCL 41833 was able to reduce the incidence of *P. infestans* in potato (cv. Bintje) resulting from a stronger plant defense response (Gallou et al., 2011).

In chapter II, it was observed that *R. irregularis* MUCL 41833 was able to reduce the incidence of *P. infestans* on Bintje in the field under dry season. A number of mechanisms have been proposed but did not consider the potential role of CMN. In chapter III, it was the objective to investigate (1) whether CMNs were able to elicit defense genes responses in healthy plants connected to diseased plants (2) which are the mechanisms possibly involved in the transmission of the signals.

Abstract

Most plants are connected belowground via common mycorrhizal networks (CMNs). In their presence, the transmission of warning signals from diseased to uninfected plants has been reported. However, current studies have all been conducted in pots making it difficult to discriminate direct from indirect contribution of hyphae to the transmission of the signals. Here, we conducted an *in vitro* study with potato plantlets connected by a CMN of the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. The plantlets were grown in physically separated compartments and their connection ensured only by the CMN. The donor potato plantlets were infected by *Phytophthora infestans* and defense genes analyzed 24, 48 and 120 h post-infection (hpi) in the uninfected receiver potato plantlets. Twenty-four hpi by the pathogen, *PAL*, *PR-1b*, *ERF3* and *LOX* genes were significantly up-regulated, whereas no significant transcript variation was noticed 48 and 120 hpi. The exact nature of the warning signals remains unknown but was not associated to below-ground microbes, diffusion mechanisms through the growth medium or induced by volatile compounds. The defense response appeared to be transitory and associated with the Jasmonic acid or Ethylene pathway. These findings demonstrate the direct involvement of hyphae in the transmission of warning signals from diseased to uninfected potato plantlets and their indubitable role in providing a route for activating defense responses in uninfected plants.

Keywords

Arbuscular mycorrhizal fungi; *Rhizophagus irregularis*; *Phytophthora infestans*; RT-qPCR; Genes expression; Common mycorrhiza network.

Introduction

Plant roots establish complex interactions with microbial communities (Le Fevre and Schornack, 2016). Whereas soil bacterial and fungal communities interact with plants in many different ways (e.g. promoting or impacting plant productivity and health), the roles of arbuscular mycorrhizal fungi (AMF) are mostly associated with the improvement of plant nutrition (especially phosphorus) and alleviation of abiotic and biotic stresses (Pozo and Azcón-Aguilar, 2007; Pozo et al., 2010; Plouznikoff et al., 2016). The concept that these fungi may form common mycorrhizal networks (CMNs) interconnecting plants is nowadays widely recognized (Simard et al., 1997; Rhodes, 2017; Simard, 2018) suggesting their potential roles in nutrients exchange. Indeed, for ectomycorrhizal fungi these belowground interconnections have been reported to favor nitrogen, phosphorus (He et al., 2003; Selosse et al., 2006) and carbon (Selosse and Roy, 2009) exchanges. However for AMF, the roles of CMNs in nutrients (e.g. phosphorus or nitrogen) or carbon transport among plants remains a matter of debate (Voets et al., 2008; Bücking et al., 2016).

In the recent decade, CMNs formed by AMF have been reported to improve plant defense by acting as channels for warning signals transmitted from plants impacted by pests or diseases to healthy neighbors (Johnson and Gilbert, 2015). Indeed, the production of volatile repellent compounds or elicitation of defense genes have been observed in healthy plants connected to plants attacked by insect herbivores (Babikova et al., 2013a; Song et al., 2014) or foliar necrotrophic fungi (Song et al., 2010). However, the nature of the signals conveyed by the CMNs remains speculative. Bacterial biofilms or microbial community shift (Barto et al., 2012; Babikova et al., 2013a) as well as chemicals or electric signals (Johnson and Gilbert, 2015) have been suggested, while no evidences of a role played by volatile organic compounds (VOCs) or root exudates (Song et al., 2010; Babikova et al., 2013a) have been reported.

Current studies on CMNs have all been conducted in pots with plants physically separated by 15/20 cm. The maximum response in the neighbor uninfected plants occurred usually between 48 and 100 h following attack or infection of the donor plant (Gilbert and Johnson, 2017) but first defense responses were recorded 18 h (Song et al., 2010) and even 6 h (Song et al., 2014) post infection by the pathogen. It is obvious that the benefit to the uninfected plants will be higher if the

signal transmission is fast, enabling them to set up defense mechanisms prior to attack (Babikova et al., 2013b).

Pot culture systems have obvious limitations, namely the possible presence of undesirable microbes that may affect the infected plant or uninfected neighbor plant or even the CMN. Diffusion of molecules and/or volatile compounds through the soil solution (e.g. not via the only mycelium connecting the plants) is also a factor that could impact the response of the uninfected neighbor plant (Gilbert and Johnson, 2017). Therefore, pot culture systems could make it difficult to discriminate the direct contribution of hyphae to the transmission of warning signals from the indirect effects related to external factors (e.g. changes in microbial communities or transfer of chemical or volatile signals through the substrate). The utilization of contaminant-free *in vitro* cultivation systems with CMNs connecting plants separated by a physical wall may overcome part of these problems. Indeed, *in vitro* cultivation systems, although artificial, offer several advantages which are mainly, the absence of unwanted belowground contaminants potentially impacting the CMN or signal transfer and the strict physical separation between the donor and received compartments only connected by the hyphae crossing a partition wall, thus excluding transmission via diffusion of chemical or volatile compounds through the medium. Using *in vitro* cultivation systems may thus discriminate direct from indirect effects of the CMN on the transmission of warning signals.

In the last decade, *in vitro* cultivation systems have been used to investigate the interplant transport of carbon (Voets et al., 2008) or cesium (Gyuricza et al., 2010) between *Medicago truncatula* plantlets connected by CMNs. Interestingly, Gallou et al. (2011), used the Mycelium Donor Plant (MDP) *in vitro* culture system, following the name given by Voets et al. (2009), for the fast and homogenous colonization of potato plantlets and for studying their resistance against late blight caused by *Phytophthora infestans*. This study demonstrated the elicitation of defense genes in infected potato plantlets connected to potato donor plantlets free of the pathogen. Priming of *PR-1* and *PR-2* genes was observed in the receiver potato plantlets starting 48 hpi (Gallou et al., 2011) and a reduction of leaf infection and area under disease progress curves (AUDPC) were observed starting 24 hpi. Both observations clearly demonstrated that *R. irregularis* MUCL 41833 triggered a systemic resistance in the leaves of potato plantlets, especially, during the first stage of infection. Similar results were

obtained with soybean root pathogens (Marquez et al., 2018a; Marquez et al., 2018b). However, none of these studies considered the defense response in uninfected receiver plantlets connected to infected plantlets.

Phytophthora infestans, the causal agent of late blight, is an hemibiotroph pathogen known to produce several effectors during its biotrophic phase of infection, first suppressing plant defense responses and later inducing larger necrosis (Lee and Rose, 2010). In the present study, a CMN formed by the AMF *R. irregularis* MUCL 41833 was established *in vitro* between two potato plantlets, one donor infected by *P. infestans* and one receiver uninfected by the pathogen. We investigated whether the signal transmission was directly associated to the connecting hyphae and postulated that if the fungus can transmit signals from an infected to an uninfected potato plantlet, the latter will set up defense mechanisms likely similar to that of a direct infection by the pathogen. Therefore, the experiment was designed to study plant defense genes expression in an uninfected receiver potato plantlet connected by a CMN to an infected potato donor plantlet.

Materials and methods

Biological material

In vitro produced plantlets of *Solanum tuberosum* L. var. Bintje were provided by the “Station de Haute Belgique” (Libramont, Belgium). The plantlets were micropropagated every 4 weeks in culture microboxes, sealed with breathing filters in the lid (ref: 0118/120+OD118, Combines, Belgium). The growth medium was Murashige and Skoog (MS) (Duchefa, The Netherlands) supplemented with 20 g l⁻¹ sucrose and solidified with 4.2 g l⁻¹ Gelrite™. The microboxes were placed in a growth chamber (Snijders Scientific B. V, Netherlands), under a relative humidity (RH) of 75 %, a photoperiod of 16 h/8 h (day/night), a photosynthetic photon flux (PPF) of 24.5 µmol sec⁻¹ m⁻² and a temperature of 20 °C/18 °C (day/night).

An *in vitro* culture of the AMF *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) (Schüßler and Walker, 2010) as [‘irregulare’] MUCL 41833 was provided by the Glomeromycota *in vitro* collection (GINCO: <http://www.mycorrhiza.be/ginco-bel>) on the Modified Strullu-Romand (MSR) medium (Declerck et al., 1998). The fungus was maintained on plantlets of *Medicago truncatula* Gaertn. cv. Jemalong A 17 (SARDI, Australia) in the mycelium donor plant (MDP) *in vitro* culture system (for details see Voets et al. (2009)) until use.

An *in vitro* culture of the Oomycete *Phytophthora infestans* (mont.) de Bary MUCL 54981 – mating type A1 – was provided by the Mycothèque de l’Université catholique de Louvain (BCCM/MUCL - <http://bccm.belspo.be/about-us/bccm-mucl>). It was initially isolated in July 2012 by the Centre Wallon de Recherches Agronomiques (CRAW, Libramont, Belgium) from a potato field in Temploux (Belgium). The strain was sub-cultured every two weeks in Petri plates (90 mm diam.) containing 20 ml of potato dextrose agar (PDA, Oxoid: CM0139) medium. Leaves from *in vitro* micropropagated potato plantlets (see above) were placed on the surface of actively growing colonies of *P. infestans* to stimulate the production of sporangia. The Petri plates were sealed with cellophane, wrapped in aluminum foil and placed in the dark in a culture chamber set at 20 °C ten days before use.

Experimental design/set up

Potato plantlets were grown in the MDP *in vitro* culture system for root colonization by the AMF (see for details Voets et al. (2009)). After 12 days, the plantlets were heavily colonized and transferred in the root compartment (RC) of bi-compartmented Petri plates (90 mm diam.) filled with 23 ml MSR medium without sucrose and vitamins and solidified with 3.2 g l⁻¹ Gelrite™. The AMF hyphae started to grow profusely out of the roots of the donor plantlets (named the mycorrhizal donor – D^{+M} – plantlets) colonizing the RC. The hyphae started to cross the partition wall separating the RC from a hyphal compartment (HC – containing 25 ml MSR medium) within 3 weeks. The growth front of the mycelium in the HC was homogenized by removing the MSR medium 2 cm away from the plastic wall and replacing it with fresh MSR medium (10 ml). After one week (i.e. seven weeks of growth in the MDP *in vitro* culture system), a 20 days old potato plantlet (i.e. named the mycorrhizal receiver – R^{+M} – plantlet) was placed in the HC with roots in contact with the mycelium growing front and shoot extending outside the Petri plate via a hole. A common mycelium network (CMN) linking both plantlets was established within a few days (i.e. the (DR)^{+M} treatment). Control Petri plates (i.e. without AMF, the (DR)^{-M} treatment) were prepared strictly as above. The systems were placed in a growth chamber set at 75 % RH, a photoperiod of 16 h/8 h (day/night), a PPF of 24.5 µmol sec⁻¹ m⁻² and a °T of 20 °C/18 °C (day/night). Forty-eight plantlets were considered for the (DR)^{+M} and (DR)^{-M} treatments.

After 8 days, the plantlets in the (DR)^{+M} treatment were randomly separated in two groups. In the first group, a piece of 0.5 cm length of MSR medium was removed (i.e. cut – +C) along the partition wall of the RC, cutting CMN from the HC (i.e. (D^{+C}R)^{+M} treatment). In the second group, no medium piece was cut (i.e. non-cut – -C) along the RC (i.e. (D^{-C}R)^{+M} treatment). After an additional 4 days, half of the donor potato plantlets in each group was inoculated (+Pinf) or not (-Pinf) with *P. infestans*. Inoculation was done with sporangia of *P. infestans* collected from 10 days-old detached leaves of potato (see above). They were delicately washed-off the leaves with deionized sterilized (121 °C for 15 min) water and their concentration adjusted to 100 per µl⁻¹. Sterilized cellophane disks of 0.5 mm diameter were placed on the adaxial part of the five oldest leaves of each donor potato plantlet, preventing evaporation and drop fall. Then, 20 µl of the sporangia solution was disposed between the cellophane and the leaf

with a micropipette (Sartorius Stedim Biotec, France). Four treatments were thus considered (Fig. 26), $(D^{+C+Pinf} R)^{+M}$, $(D^{-C+Pinf} R)^{+M}$, $(D^{+C-Pinf} R)^{+M}$, $(D^{-C-Pinf} R)^{+M}$. Each treatment was finally separated in three groups of four replicates corresponding to three-harvesting times: 24, 48 and 120 hour post inoculation (hpi) by *P. infestans*, which corresponded, to sporangia germination, first hyphae division (initiation of tissue invasion) and apparition of the first necrotic lesions, respectively (supplementary Fig. S5). Twenty-four, 48 or 120 hpi by the pathogen, the leaves were removed from each receiver plantlet and transferred within 3 min to liquid nitrogen. The non-mycorrhizal potato plantlets were prepared strictly as above, and four treatments with four replicates per each harvesting time were identically set up (Fig. 26): $(D^{-C-Pinf} R)^{-M}$, $(D^{+C-Pinf} R)^{-M}$, $(D^{+C+Pinf} R)^{-M}$ and $(D^{-C+Pinf} R)^{-M}$.

Finally, 5 additional plantlets from the $(D^{-C-Pinf} R)^{+M}$ treatment were prepared to evaluate root colonization in the receiver plantlets at the start of the experiment (i.e. before infection by *P. infestans*). The complete experiment was conducted twice to evaluate repeatability of the results.

Plant growth parameters

At the end of the experiment, the number of leaves of the receiver plantlets was enumerated and their fresh weight measured prior to immediate storage in liquid nitrogen (see above). Roots were then separated from the gel, their fresh weight measured, and AMF root colonization assessed (see below).

P. infestans assessment on donor plant

Leaf infection by *P. infestans* was assessed at 24, 48 and 120 hpi by staining with lactophenol cotton blue. Briefly, at each time point, two inoculated leaves of each infected donor plantlet were cut and 20 μ l of lactophenol cotton blue solution added under the cellophane disk. After 24 h, the leaves were cut in 0.5 cm² fragments and placed on slides for observation under a compound microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 80 X magnifications. The observations were compared to an infection scale developed on Bintje placed in the MDP *in vitro* culture system. Three stages were identified: stage 1 (24 hpi) sporangia germination, stage 2 (48 hpi) first hyphae branching and plant tissue infection and stage 3 (120 hpi), spreading of

the hyphae on the leaf surface with the first symptom observed (supplementary Fig. S5).

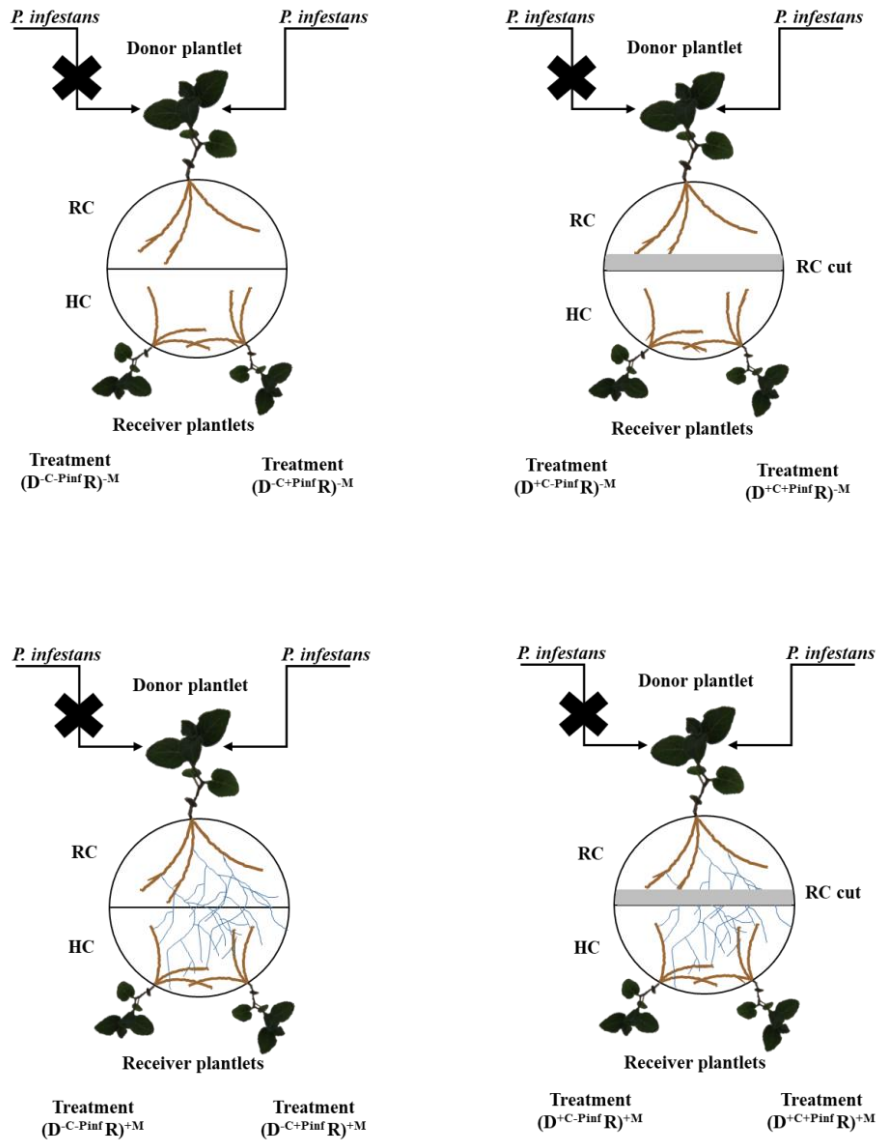


Figure 26. Schematic representation of the *in vitro* experimental system. Warning signal transmission was investigated from a donor (D) potato plantlet infected by *Phytophthora infestans* MUCL 54981 to two uninfected receiver (R) potato plantlets linked by a common mycorrhizal network of the arbuscular mycorrhizal fungus, *Rhizophagus irregularis* MUCL 41833. The plantlets developed in

separate compartments (a root compartment, RC and a hyphal compartment, HC) physically separated by a plastic wall, while the AMF crossed the wall and interconnected the plants. Several treatments were considered, depending on whether the roots of the donor (D) plantlet were colonized or not by the AMF (+M, -M), the shoot infected or not by *P. infestans* (+Pinf, -Pinf) and the medium in the RC cut or not (+C, -C): $(D^{-C-Pinf} R)^{+M}$, $(D^{+C-Pinf} R)^{+M}$, $(D^{+C+Pinf} R)^{+M}$ and $(D^{-C+Pinf} R)^{+M}$, $(D^{+C-Pinf} R)^{-M}$, $(D^{+C+Pinf} R)^{-M}$, $(D^{-C+Pinf} R)^{-M}$, $(D^{-C-Pinf} R)^{-M}$.

Root colonization by AMF

The roots of the receiver plantlets were cut in 3 cm long pieces, cleared with KOH (10 %) at 60 °C for 45 min, rinsed with deionized water and then with 1 % HCl before staining with a solution of 1 % HCl and 2 % blue ink (Parker®, USA). The roots were subsequently transferred in a solution of lactoglycerol (1v:1v:1v – lactic acid/glycerol/H₂O) and kept at 4 °C. Root colonization was assessed following McGonigle et al. (1990). A mean of 150 intersections was observed under a compound microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 100 X magnifications. Percentages of total root colonization (%RC) and arbuscules (%A) were assessed.

RNA extraction

The frozen leaves of each replicate were ground in liquid nitrogen (-196 °C). Total RNA extraction was done using the RNeasy Plant Mini Kit (Qiagen, USA) following manufacturer recommendations with a slight modification (i.e. for water elution, a 5 min step of incubation on the column at room temperature was performed instead of 1 min). The total RNA was treated with TURBO DNA-free™ Kit (Thermo Fisher Scientific, USA), according to the manufacturer protocol. Concentration of each sample was measured using NanoDrop®-ND 1000 UV–vis Spectrophotometer (NanoDrop Technologies, USA) and RNA purity estimated from the A260/A280 absorbance ratio.

Following total RNA extraction, reverse transcription (RT) of 0.5 mg of total RNA was performed with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Montreal, Canada), according to the manufacturer instructions. For each RNA sample, a reaction without

RT enzyme was performed as a control for contamination by genomic DNA.

RT-qPCR

Three reference genes (Table 9) were selected (i.e. abbreviations and GenBank ID, respectively) using Refinder software (<http://150.216.56.64/referencegene.php?type=reference>): ubiquitin conjugating enzyme-like (*Ubc*), elongation factor 1-alpha (*EF1-α*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Fifteen target genes involved in plant defense or signaling pathways were selected (Table 9): a mitogen-activated protein kinase (*MAPK*), pathogenesis related 3 (*PR-3*), pathogenesis related 1 (*PR-1*), pathogenesis related 2 (*PR-2*), pathogenesis related 9 (*PR9*), glutathione-S-transferase 1 (*GST1*), Putative regulatory protein Non-expressor of PR genes 1 (*NPR1*), WRKY transcription factor 6 gene (*WRKY6*), osmotine like (*OSM*), phenylalanine ammonia lyase (*PAL*), pathogenesis related 1 basic (*PR-1b*), ethylene response factor 3 (*ERF3*), lipoxygenase (*LOX*), allene oxide cyclase (*AOC*), 12-Oxophytodienoate reductase 3 (*OPR3*). The qPCR was made using LightCycler® FastStart Essential DNA Green Master in 10 µl volume of reaction formed as follow; 5 µl Master mix, 0.5 µl of each primer from the pair, 1.5 µl H₂O PCR grade and 2.5 µl DNA. The cycling conditions were: 10 min at 95 °C, 40 cycles of denaturation (95 °C, 10 s)/annealing (60 °C, 10 s)/extension (72 °C, 15 s) and finalized by a standard melting curve analysis (95 °C). Normalization was achieved for each experiment separately using the geometric means of the reference genes (i.e. *Ubc*, *EF1-α*, *GAPDH*) and the “Pfaffl” method (Pfaffl, 2001). The results were then expressed as means of fold change in target genes expression in each of the following treatments ((D^{+C-Pinf} R)^{-M}, (D^{+C+Pinf} R)^{-M}, (D^{-C+Pinf} R)^{-M}, (D^{-C-Pinf} R)^{+M}, (D^{+C-Pinf} R)^{+M}, (D^{+C+Pinf} R)^{+M} and (D^{-C+Pinf} R)^{+M}) as compared to the (D^{-C-Pinf} R)^{-M} treatment. Two experiments were conducted independently for 24 hpi and 48 hpi and one was prolonged to 120 hpi. Significance values of genes fold change were set at P <0.05, with an additional cut off value of 2.5-fold change.

Table 9. *Primers used in the study*

Pathways	Name	Accession number or reference	Primer sequence 5'-3' (Forward)	Primer sequence 5'-3' (Reverse)
reference genes	<i>Ubc</i>	DQ241834	TGATGGTTACCCA TTTGAGCC	ACTGGTCCTTCAG GATGTC
	<i>GADPH</i>	AF527779	GGACATTGTCTCC AACGC	ATGAGACCCTCCA CAATGC
	<i>EF1-α</i>	AB061263	ATTGGAAACGGAT ATGCTCCA	TCCTTACCTGAAC GCCTGTCA
SA	<i>MAPK</i>	AB206552	CCAAGTAACCTCT TGCTAAATGC	CTGTCATATTCTC GTTCTCTAGG
	<i>PR-1</i>	(Burra et al., 2015)	AACACTCTGGTGG CCCTTAC	AGCACAACCGAG ACGTACTG
	<i>PR-2</i>	AJ009932	TATCATCAGCAGG GTTGCAAA	TCGCGAAAAATGC TATTTCTAGG
	<i>PR-3</i>	(Büchter et al., 1997)	GGCTGCCTTTTTC GGTCAAA	CCTTGTCCAGCTC GTTTCGTA
	<i>PR-9</i>	AJ401150	AAGAAACAACAC CAGGGCAC	TGCCCTCAAGCTG AAGAAAT
	<i>GST1</i>	J03679	TTCTAGCCACCAG ATTTGACC	ACATATTCCCTAT ATTTTTGGAGTGA GTA
	<i>NPR1</i>	XM_0063576 47.2	GGTGCACCGATGC ATTTTGT	TCAGTCCTTGAG TTCCAGC
	<i>WRKY6</i>	LOC1025778 93	ATCAAAATTCCAA AGACCCTCC	ATGTTATGTCATC TGGGGTTTAC
	<i>OSM</i>	(Ruiz et al., 2005)	GAGGTACGCAAC ACCTGTCCATAC	GCTAGGGTGTGTTG GCGATTTAC
	<i>PAL</i>	X63104	GGATATGCCATCG AACTTTGAGA	ACAAATAATGGC ATGGATGAGG
JA/ET	<i>PR-1b</i>	AJ250136	TGGTGATTTCACG GGGAGGGCA	TCCGCACACTTGT CCGCTTGCA
	<i>ERF3</i>	EF091875	CCTGTAAAAAATG AAATCAATCGGA GTCC	CGGCGATGATGA ATCAACCATAAC
JA	<i>LOX</i>	Y18548	GAGTTCTCCTCAT GGTGTTTCGTTTA	AGTAGTCTGACAC CCAACCTT
	<i>AOC</i>	AY135641	GCTACCCTCTGCC TTCCAAA	GGAAGCAGTAGT GGAGGTGG
	<i>OPR3</i>	(Halim et al., 2009)	AATCCACTCAGCC TTGGCTTAGCAG	GTCCATTGCTTCC ATTTCTTGAA

Statistical analyses

Data analyses were performed with JMP® Pro statistical software version 14.0.0 (SAS Inc., Canada) with a linear mixed model, where “Treatment” and “Time” were regarded as fixed factors and “Experiment” as random factor to account for potential differences between experiments. In the case of non-normality and/or unequal variances, data were log or ln transformed prior to running the linear mixed model. Differences between means were subsequently tested by Tukey’s test, significant values were set at $P < 0.05$. Analysis of the variability between experiments for each target genes were made, and proportion of variability explain by the factor experiment was never significant, $P < 0.05$ (supplementary table S11), therefore results are shown as means of both experiments.

Results

Arbuscular mycorrhizal colonization

Root colonization of the potato plantlets in the HC (i.e. the uninfected R plantlets) was assessed prior (i.e. time 0 h) or 24, 48 and 120 hpi by *P. infestans* of the leaves of potato plantlets in the RC (i.e. the infected D plantlets). This corresponded to 12, 13, 14 and 17 days of contact of the R plantlets with the extra radical mycelium network (Table 10).

At the time of inoculation of *P. infestans* (0h), the %RC and %A of the R plantlets grown in the HC were $19.9 \pm 17.2\%$ and $6.3 \pm 6.1\%$, respectively and did not differ significantly from the values at 24, 48 and 120 hpi by *P. infestans* ($P > 0.05$), whatever the treatment. No significant effect of the factor “Treatment”, “Time” or interactions between both factors was observed on %RC and %A (Table 10). No traces of AMF colonization were observed in the R potato plantlets grown in the non-AMF treatments.

Table 10. Percentage of total roots (% RC) and arbuscules (% A) colonization of the potato receiver plantlets – R (i.e. plantlets grown in the HC) connected to the potato donor plantlets – D (i.e. plantlets grown in the RC) via the extra radical mycelium of *R. irregularis* MUCL 41833. The different treatments were D plantlets colonized or not by the AMF (+M, -M), their shoot infected or not by *P. infestans* (+Pinf, -Pinf) and the medium in the RC cut or not (+C, -C). The measurements were made 24, 48 and 120 h post-infection (hpi) by *P. infestans* of the D plantlets.

Treatment	%RC			%A		
	24h	48h	120h	24h	48h	120h
(D ^{-C-Pinf} R) ^{+M}	19.0 ±18.7	31.7 ±19.1	32.9 ±30.4	6.7 ±9.5	11.6 ±7	13.0 ±13.4
(D ^{+C-Pinf} R) ^{+M}	18.7 ±10.4	13.5 ±7	38.4 ±30.8	5.2 ±2.9	4.4 ±3.3	17.0 ±15.7
(D ^{+C+Pinf} R) ^{+M}	18.9 ±10.3	13.7 ±9.9	29.6 ±25.9	6.6 ±4.2	4.3 ±3.2	8.9 ±11
(D ^{-C+Pinf} R) ^{+M}	14.7±1 0	23.2 ±14.9	13.6 ±15.3	4.8 ±3.9	7.3 ±5.7	4.9 ±6.7
Mixed model			p-values			
Treatment		0.24024		0.18423		
Time		0.08084		0.15094		
Treatment vs Time		0.19568		0.22407		

Data are means ± standard error of eight replicates at 24h, 48h and four replicates at 120h. Main effects and interactions between the factors “Treatment” and “Time” are presented with significance values set at $P < 0.05$ (mixed model).

Plant growth parameters

Root fresh weight (RFW) and leaf fresh weight (LFW) as well as number of leaves of the R plantlets grown in the HC were measured 24, 48 and 120 hpi by *P. infestans* of the D plantlets grown in the RC (Table 11). A significant effect of the factor “Time” was noticed on RFW, LFW and number of leaves of the R plantlets grown in the HC. Whatever the treatment a significant increase of the three parameters was noticed at 48 and 120 hpi as compared to 24 hpi by *P. infestans* ($P < 0.05$). Conversely, no effect of the factor “Treatment” or interactions between ‘Time x Treatment’ was observed whatever the time of observation and parameter ($P > 0.05$).

Table 11. Root fresh weight (RFW), leaf fresh weight (LFW) and number of leaves of the receiver potato plantlets – *R* (i.e. plantlets grown in the HC) connected to the donor potato plantlets – *D* (i.e. plantlets grown in the RC) via the extra radical mycelium of *R. irregularis* MUCL 41833. The different treatments were *D* plantlets colonized or not by the AMF (+M, -M), their shoot infected or not by *P. infestans* (+Pinf, -Pinf) and the medium in the RC cut or not (+C, -C.) The measurements were made 24, 48 and 120 h post-infection (hpi) by *P. infestans* of the *D* plantlets

Treatment	RFW (mg)			LFW (mg)			Number of leaves		
	24h	48h	120h	24h	48h	120h	24h	48h	120h
(D ^{-C-Pinf} R) ^M	321.3±198.7	303.8±196.5	380.0±202.1	151.3±63.3	190.0±89.3	211.3±198.7	8.5±3.9	9.1±3.4	9.5±3.8
(D ^{-C-Pinf} R) ^M	280.0±233.7	356.3±226	296.3±131.7	185.0±111.4	205.0±96.4	208.8±233.7	8.5±3.3	9.0±3.8	9.0±3.5
(D ^{-C-Pinf} R) ^M	240.0±175.4	197.5±86.1	280.0±118.7	168.8±87.1	191.3±84.4	172.5±175.4	8.3±3.7	8.5±3.3	8.8±3.3
(D ^{+C-Pinf} R) ^M	190.0±123.2	341.3±92.8	412.5±167.3	175.0±85.5	192.5±59.9	216.3±123.2	8.4±3.1	8.6±2.9	8.9±3.1
(D ^{-C-Pinf} R) ^M	282.5±246.6	327.5±287.4	298.8±51.9	141.3±68.5	190.0±104.9	190.0±246.6	8.0±2.9	9.1±4.4	9.1±3.2
(D ^{-C-Pinf} R) ^M	252.5±159.3	195.0±89	287.5±54.7	164.4±69	161.3±74.1	183.8±159.3	8.1±3	8.4±3.4	8.6±3.8
(D ^{+C-Pinf} R) ^M	203.8±49.8	193.±75	285.0±137.4	186.3±46	196.3±32	201.3±49.8	9.0±3.4	8.5±3	9.0±3.5
(D ^{-C-Pinf} R) ^M	341.3±225.9	323.2±172	418.8±160.9	173.8±71.9	205.0±95.5	211.3±225.9	8.1±3.4	9.0±3.6	9.3±4.2
Mixed Model									
p-values									
Treatment	0.2161			0.3219			0.3677		
Time	0.0001			0.0004			0.0009		
Treatment vs Time	0.4080			0.9933			0.8773		

Data are means ± standard error of eight replicates at 24h, 48h and four replicates at 120h, in mg. Main effects and interactions between the factors “Treatment” and “Time” are presented with significance values in bold and set at $P < 0.05$ (mixed model).

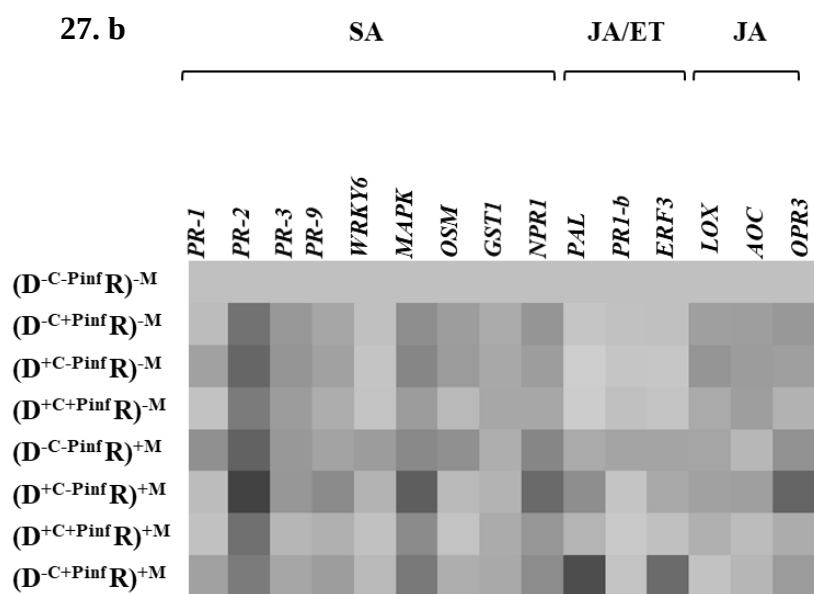
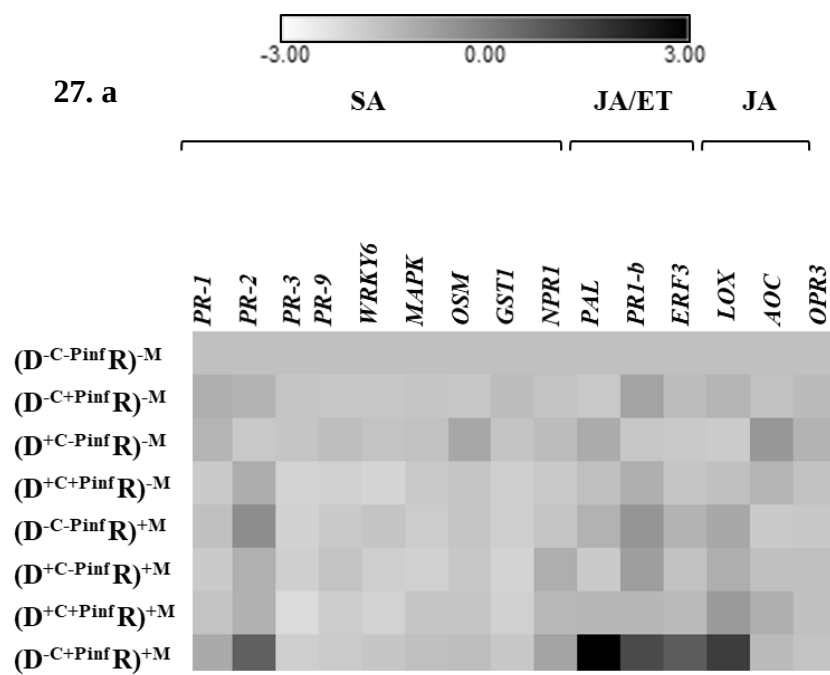
Leaf infection of the donor plantlets by *P. infestans*

The successful infection of the donor plantlets by *P. infestans* (i.e. in the treatments: (D^{-C+Pinf} R)^{-M}; (D^{+C+Pinf} R)^{-M}; (D^{+C+Pinf} R)^{+M}; (D^{+C+Pinf} R)^{+M}) was evaluated on two leaves per plantlet, using lactophenol cotton blue staining. The infection stage was compared to a determination key developed for *P. infestans* under similar growth conditions (supplementary Fig. S5). Whatever the treatment all the plantlets inoculated by the pathogen were infected and corresponded to the three stages (i.e. 24, 48 or 120 hpi) from the key scale. No impact on the stage of development of the different treatments was observed.

Gene expression analysis in the receiver plants

The transcript of fifteen target genes (Fig. 27) in the leaves of the R plantlets in the HC was assessed 24, 48 and 120 hpi by *P. infestans* on the leaves of the D plantlets in the RC (Fig. 26).

At 24 hpi (Fig. 27a), four defense genes were significantly up-regulated in the (D^{-C+Pinf} R)^{+M} treatment. Indeed, *PR-1b*, *ERF3*, *LOX* and *PAL* were up-regulated by 5.8, 4.8, 9.8, 26.5-fold, respectively. No significant variation of the genes relative expression was observed in the treatments without AMF regardless of *P. infestans* inoculation of the D plantlets (i.e. in the (D^{-C-Pinf} R)^{-M}; (D^{+C-Pinf} R)^{-M}; (D^{-C+Pinf} R)^{-M}; (D^{+C+Pinf} R)^{-M} treatments). Similarly, no significant variation of the genes relative expression was induced by the AMF in the (D^{-C-Pinf} R)^{+M}; (D^{+C-Pinf} R)^{+M}; (D^{-C+Pinf} R)^{+M}; (D^{+C+Pinf} R)^{+M} treatments, or by the cut in the RC compartment in the (D^{+C-Pinf} R)^{-M}; (D^{+C+Pinf} R)^{-M}; (D^{+C-Pinf} R)^{+M}; (D^{+C+Pinf} R)^{+M} treatments. The genes expressions analyses in the leaves of the R plantlets showed no significant modification in treatment without an active CMN linking infected donor to receiver plantlets. Furthermore, transcript analysis did not reveal any variation in the relative expression ratio of the target genes regardless of the treatments at 48 or 120 hpi by *P. infestans* (Fig. 27b and 27c).



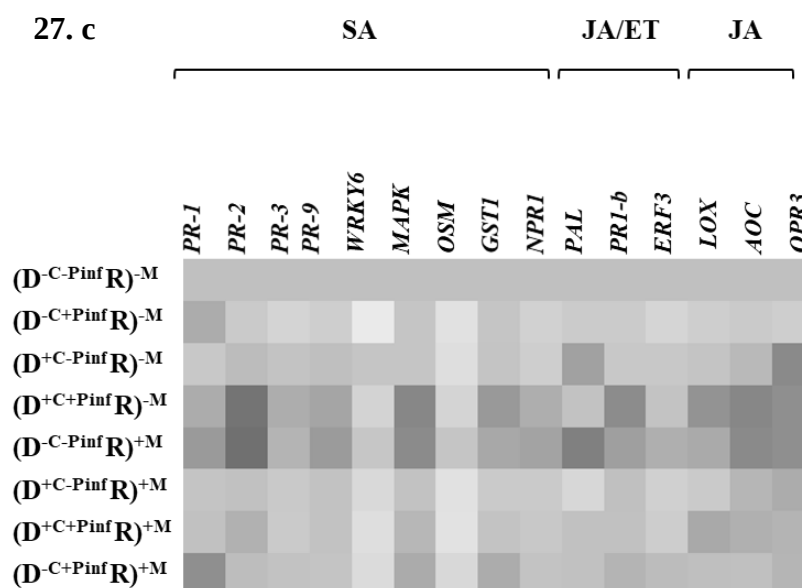


Figure 27. Heat map of the 15 genes relative expression; up and down regulated ratio (in ln) in the leaves of potato receiver plantlets – R (i.e. plantlets grown in the HC), (a) 24h, (b) 48h, and (c) 120 h post inoculation of a donor – D plantlet by *P. infestans* (i.e. plantlets grown in the RC). The different treatments were D plantlets colonized or not by the AMF (+M, -M), their shoot infected or not by *P. infestans* (+Pinf, -Pinf) and the medium in the RC cut or not (+C, -C). Fold change of gene expression of the treatment used for the normalization ($D^{-C-Pinf}R^{-M}$) is equal to 1. Quantitative real time RT-PCR was used to detect the transcripts of 15 target genes. Seven Salicylic acid (SA) dependent genes; pathogenesis related 1 (PR-1), pathogenesis related 2 (PR-2), pathogenesis related 3 (PR-3), pathogenesis related 9 (PR9), WRKY transcription factor 6 gene (WRKY6), mitogen-activated protein kinase (MAPK), osmotine like (OSM), glutathione-S-transferase 1 (GST1), Putative regulatory protein Non-expressor of PR genes 1 (NPR1). Two Jasmonic acid (JA) and Ethylene (ET) dependent genes; phenylalanine ammonia lyase (PAL), pathogenesis related 1 basic (PR-1b). One ET dependent gene: ethylene response factor 3 (ERF3). Three JA dependent genes: lipoxygenase (LOX), allene oxide cyclase (AOC), OPDA reductase 3 (OPR3). Every square are means of eight replicates at 24 h and 48 hpi and four replicates at 120 hpi (Supplementary Table S10 Expression fold change).

Discussion

It is only in the recent decade that the role of CMNs in the transmission of warning signals from infected to uninfected plants has been firmly demonstrated (Song et al., 2010; Babikova et al., 2013a; Gilbert and Johnson, 2017). However, the direct contribution of connecting hyphae can only be ascertained in a context where indirect effects, attributed to shift in microbial communities or biofilm formations or to diffusion of chemical or volatile signals through the substrate, can be excluded. Here, a strict *in vitro* culture system was used to investigate warning signals transmission from donor potato plantlets infected by *P. infestans* to uninfected receiver potato plantlets linked by a CMN of the AMF *R. irregularis*. The plantlets were grown in contiguous compartments, physically separated by a plastic wall, while the AMF crossed the wall and interconnected the plantlets. No significant elicitation of any of the genes considered in this study was noticed in the receiver plantlets at 48 and 120 hpi by *P. infestans* of the donor plantlets (Fig. 27b and 27c), while a significant over-expression of *PR-1b*, *PAL* and *ERF3* (JA/ET pathway) and *LOX* (JA pathway) transcripts was observed at 24 hpi (Fig. 27a). This demonstrated the fast and transitory transmission of warning signals from infected to uninfected plantlets via the sole ERM and supported the indubitable role of CMN in providing a route for activating defense responses in uninfected plantlets.

The bi-compartmented experimental system was adequate to investigate signal transmission from infected to uninfected plantlets. Indeed, the CMN was established within 12 days as earlier reported (Gallou et al., 2010) and root colonization of the receiver plantlets reached a mean value close to 20%, at the time of infection by *P. infestans*. Similarly, leave infection of the donor plantlets by *P. infestans* was clearly observed at 24, 48 and 120 hpi by the pathogen with sporangia germination, first hyphae branching and plant tissue infection, and spreading of the hyphae on the leaf surface with the first symptom observed, respectively. Within the experiment time frame, neither *P. infestans* nor the cutting of the medium in the RC impacted root colonization, as earlier observed Gallou et al. (2011), or plant growth parameters of the received plantlets. Only the factor “time” induced an increase in plant growth parameters with significant higher values at 48 and 120 hpi as compared to 24 hpi, suggesting an actively development of the plantlets.

The *in vitro* approach used allowed to discard the role of microbial communities on the transmission of warning signals. Indeed, bacterial biofilms have been hypothesized as a potential signal carrier (Barto et al., 2012) and shift in communities has been associated to AMF (Marschner et al., 2001). Here, nor biofilm formation neither contamination of the growth medium was noticed. Diffusion of chemical compounds (Gilbert and Johnson, 2017) via the growth medium could also be discarded because both compartments were physically separated by a plastic wall with only hyphae connecting the plantlets. However it is not excluded that signals can be transmitted by capillarity along the hyphae, which is another direct pathway along the hyphae (Barto et al., 2012). Furthermore, the root systems of the donor and receiver plantlets were potentially exposed to the same VOCs accumulated inside the Petri plates. To investigate this pathway, a piece of medium was removed along the RC in the AMF or non-AMF treatments with and without *P. infestans*. Similarly, the potential involvement of VOCs released by the aerial parts of the plantlets was controlled by placing AMF-colonized plantlets (intact CMN without infection of donor, $(D^{-c-Pinf} R)^{+M}$ treatment) between the infected intact CMN treatment (i.e. $(D^{-c+Pinf} R)^{+M}$). In both cases, no response was measured in the receiver plantlets, indicating that VOCs were most likely not involved in the signaling process as earlier suggested (Song et al., 2010). From these observations, it appears that the most plausible mechanism for the transmission of warning signals is by chemicals along or with more certainty inside the hyphae.

Plant defense genes activation was observed 24 hpi by *P. infestans*, while no effect was noticed 48 and 120 hpi. Earlier studies have reported response in uninfected plants already 18 hpi by *Alternaria solani* (Song et al., 2010) or even 6 hpi by *Spodoptera litura* (Song et al., 2014) and wounding signal transmission from leaves to roots via the JA pathway has been shown within 90 min (Zhang and Baldwin, 1997). Mitochondria, nucleus and fat droplets speed movement in hyphae was evaluated at 15 cm day^{-1} (Giovannetti et al., 1999) but cytoplasmic flux was calculated at 12.6 cm h^{-1} in pot conditions (Cox et al., 1980). In a recent study conducted *in vitro*, the cytoplasmic/protoplasmic flux was estimated between 0.7 and 18 cm h^{-1} (Whiteside et al., 2019) and in our experiment it was estimated around 0.9 cm h^{-1} . Therefore, movement of cytoplasmic/protoplasmic material between hyphae situated at the longest distance of a Petri plate (i.e. 90 mm) is expected to occur within an approximate of 10 h, to which

should be added the time needed from a signal to be transmitted from infected shoot of the donor plantlets to hyphae in the RC and from hyphae in the HC to the uninfected shoot of the receiver plantlets. This should take less than 24 h and allow elicitation of defense mechanisms in the healthy receiver plants (Johnson and Gilbert, 2015; Gilbert and Johnson, 2017).

The highest response in the receiver plantlets was generally recorded between 48 and 100 hpi of the pathogen and decreased thereafter (Gilbert and Johnson, 2017), while in our experiment it was within 24 hpi. However, the current experiment was conducted in 90 mm Petri plate as compared with pot experiments where plant were separated by a distance of 15 cm (Song et al., 2010) or 20 cm (Babikova et al., 2013b). The smaller distance between roots in our system may have shortened the time necessary for defense activation and may have contributed to decrease the time required before defense response return to basal level. Indeed, even if the duration over which the signals are effective is still unknown (Gilbert and Johnson, 2017), the receiver plant response seems to be transient. If the receiver plant is not challenged by a pathogen/attacker, then the gene expression returns to basal level and the short distance between plantlets in the *in vitro* system may explain the absence of gene expression 48 and 100 hpi. Babikova et al. (2014) have evaluated the speed of electrical signal such as calcium signal at 40 m s^{-1} , therefore short distances between plants (i.e. around 10 cm) should not have impacted the time for gene defense elicitation in the receiver plantlets. Therefore, it could be hypothesized, but still should be confirmed experimentally, that electric signal is not involved (Johnson and Gilbert, 2015).

The first report of warning signals transmission between plants via CMN was made by Song et al. (2010). These authors demonstrated the activation of plant defense genes of both SA and JA pathways (*PR-1*, *PR-2*, *PR-3*, *PAL*, *LOX*, *AOC*). In another study, Song et al. (2014) used tomato mutants defective in JA biosynthesis and observed that they were unable to induce defense response, strongly suggesting that the JA signaling pathway was required. Our results seem to indicate similar results. Indeed, JA dependent gene (*LOX*) was up regulated as well as *PR-1b*, *PAL* and *ERF3* (JA/ET pathway). Furthermore, no activation of SA dependent genes was measured in our study.

Our results suggested a priming of the four genes (i.e. *PR-1b*, *PAL*, *ERF3*, *LOX*) in the ($\text{D}^{-\text{c+Pinf}}$ R) $^{+\text{M}}$ treatment. Priming state involve

notably histones modification (i.e. methylation or acetylation) facilitating transcript factor such as WRKY or ERF and MPK signalization cascade activation (Balmer et al., 2015; Conrath et al., 2015). *ERF3* has been shown to play a role in the activation of plant systemic defense system in potato plants (Velivelli et al., 2015) and against leaf infection by *P. infestans* (Gallou et al., 2011). The basic *PR-1b* protein (i.e. JA/ET dependent) is involved in innate immunity resistance of potato against *P. infestans* (Vleeshouwers et al., 2000) and was reported in AMF-colonized potato plants challenged by *P. infestans* (Gallou et al., 2011). Priming of potato plants also induced the activation of *LOX* which is involved in systemic signaling (Halim et al., 2009). *PAL* was also used as a JA/ET dependent gene involved in innate immunity against pathogens (Song et al., 2010) and was shown in AMF-colonized potato plants challenged by *P. infestans* (Gallou et al., 2011). The over expression of these four genes (*PR-1b*, *PAL*, *ERF3* and *LOX*) clearly suggested the elicitation of the ET/JA dependent defense gene in uninfected potato plants connected to infected potato plants. In conclusion, we have clearly demonstrated the transitory induction of JA/ET (i.e. *PAL*, *PR-1b* and *ERF3*) and JA (*LOX*) dependent genes in uninfected receiver plantlets connected via a CMN to a diseased plantlet. These findings reveal the direct involvement of hyphae in the transmission of the warning signals and their indubitable role in providing a route for activating defense responses in uninfected plants. The results further raise crucial questions about the specificity of signal transfer and nature of the signaling processes involved.

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Competing interests

The author declares no competing interests.

Author contributions

P-L.A, S.C. and S.D. conceived the experiments, P-L.A. and F.N. conducted the experiments, P-L.A. analyzed the results, P-L.A. wrote the manuscript. P-L.A, S.C. and S.D. reviewed the manuscript.

Data availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

GENERAL DISCUSSION

Since its appearance in Europe and its worldwide expansion, the potato has become one of the main food commodities for human consumption (Hardigan et al., 2017). This crop is threatened by several diseases amongst which late blight is the most important (Haas et al., 2009). Indeed, during its worldwide expansion, potato was followed by *Phytophthora infestans* (Dyer et al., 1993), the causal agent of late blight.

In conventional agriculture, the management of late blight relies mainly on the repeated application of chemical pesticides during the cropping season (Haverkort et al., 2009). However, those chemicals may have adverse effects on the environment and human health (Wilson and Tisdell, 2001; Beketov et al., 2013). Conversely, in organic agriculture, no synthetic pesticides are accepted, and control of the disease is mainly achieved by the use of copper. Though, the number of applications and long remanence have led to its accumulation in soils over the years, impacting soil microorganisms (Graham et al., 1986; Brun et al., 1998; Van-Zwieten et al., 2004) as well as the food chain (Vincevica-Gaile and Klavins, 2012). To limit the accumulation of copper in the environment, the EU has set a number of regulations and directives. In 1991 the Council Regulation (EEC) No 2092/91 restricted the use of copper to 6 kg/ha per year and in 2009, the EU directive 2009/128/CE established a framework for a sustainable use of pesticides by reducing the risks associated with their application, particularly on human health and environment. Therefore, integrated and alternative approaches to combat late blight are nowadays strongly promoted. Amongst those innovative strategies, the use of biological control agents (i.e. microorganisms) appears particularly promising (Olanya and Larkin, 2006).

Potato roots provide a unique environment for a diverse assemblage of microorganisms (e.g. bacteria, fungi), both beneficial and harmful, promoting or affecting the plant development. Among the beneficial microorganisms are arbuscular mycorrhizal fungi (AMF), *Trichoderma* sp. and various plant growth promoting rhizobacteria and fungi (PGPR and PGPF, respectively) (Dorn et al., 2007). These beneficial microorganisms have been used successfully in controlled environments (e.g. greenhouse) to decrease the disease severity of

various pests and diseases. Within the field, the results are more erratic with positive to no and even negative effects. This is partly due to the environmental context (e.g. edapho-climatic conditions, agricultural practices, competition with indigenous microflora) that may affect the survival and/or efficacy of the microbial inoculants. It is thus important to develop tracing tools allowing to link the observed outcomes to the applied inoculant (Guyer et al., 2015). The positive effects observed on plants may be attributed to various mechanisms from a better nutrition of the plant to the potentiation of plant defense genes. It is obvious, that different mechanisms can simultaneously be activated by one single beneficial microorganism.

Plant defense responses have been shown to be more efficient after potentiation by beneficial microorganisms (Conrath et al., 2006). This unique physiological state of the plant is called priming (Balmer et al., 2015; Mauch-Mani et al., 2017). Priming of plant defenses has been shown to impact hormonal signaling pathways and transcription factors (Cameron et al., 2013 ; Conrath et al., 2015; Yi et al., 2015). Salicylic acid (SA), Jasmonic acid (JA), Ethylene (ET) pathways are key elements in the control of pathogens in plants and more specifically in potato against late blight. Therefore, SA, JA and ET dependent genes and transcript factor are good indicator of plant defense priming state.

Plants are not isolated in their environment. They interact with numerous microorganisms and communicate with each other (Le Fevre and Schornack, 2016) via below-ground fungal connections called common mycorrhizal networks (CMNs) (Johnson and Gilbert, 2015). These CMNs are the rule in soil either with ectomycorrhizal (ECM) fungi linking trees or with AMF linking herbaceous plants or trees. The CMNs formed by ECM fungi have been shown to transport nutrients between plants (Selosse et al., 2006) and CMNs formed by AMF have been reported to transfer defense signals from infected to healthy plants (Song et al., 2010). Plants can therefore potentially anticipate pathogens outbreak and activate in advance their defense mechanisms. Increasing the knowledge on the signals transferred by CMNs during potato interaction with late blight may help in the development of innovative control strategies.

In this thesis we have used different approaches (from field to *in vitro*) to study the effects of AMF on potato protection against late blight and to investigate the potential mechanisms involved.

Traceability and detection of AMF inoculants within the field

One of the main issues of using AMF inoculants in the field is to be able to relate their effects on plants (e.g. growth stimulation, increased resistance to pests and diseases) to their presence in the roots. Therefore, the development of molecular tools allowing discriminating the AMF inoculants from the indigenous AMF belonging to different or to the same species is essential. Indeed, molecular marker using one-step RT-qPCR does not require a heavy equipment and can be used to evaluate the presence/absence of inoculant in the target plant and therefore can be used as a quality control for farmers. However, a prerequisite to evaluate the molecular barcoding tool developed is to ascertain that the AMF inoculant is well established in the root system of the target plant. Two parallel approaches were thus developed for potato. The first consisted in the development of an inoculation technique ensuring the presence of the inoculant in the potato roots. The second concerned the development of a molecular marker allowing to discriminate the inoculant versus any other AMF colonizing the potato roots.

During inoculation in the field, several factors may affect root colonization, among which the cultural practices, the edapho-climatic conditions and the competition with soil microflora among which the indigenous AMF. The inoculation technique developed was inspired from the *in vitro* plant donor system. It consisted in placing a sprouted potato tuber (i.e. the receiver plant) close to an AMF-colonized donor plant (i.e. *Medicago truncatula*) in biodegradable pots (Jiffy®). After a few days, the system was transferred to the field. *Medicago truncatula* is a highly mycotrophic plant that remain small and will thus not compete with the potato plants. The system allowed the fast and homogenous colonization of the sprouted potato tubers. Root colonization of Bintje and Nicola in the field were good with values close to 20% and 30%, respectively. Although the system is not usable as such in potato production, it solves the first problem that is obtaining mycorrhizal potato plants by the selected inoculant, thus allowing to test the molecular tools in the field. To ensure that the higher colonization of potato plants was indeed due to the added strain and not solely to the indigenous AMF community, specific molecular markers for the strain *Rhizophagus irregularis* MUCL 41833 (i.e. the inoculant) and for *Rhizophagus irregularis* species (i.e. potentially present in the indigenous AMF community) at large were developed. The mitochondrial genome (MtLSU) was used to distinguish the inoculant

R. irregularis MUCL 41833 from native AMF species and in particular the *R. irregularis* strains, following the recommendations of Börstler et al. (2008) and Badri et al. (2016). Indeed, the genomic markers used to date (ITS, rDNA SSU or LSU, AFLP) did not allow discrimination beyond the species level. The generally used ITS marker is unsuitable as species specific markers (Croll et al., 2008) but can be used to measure impact on family distribution induced by ecological shifts and interactions across the kingdom fungi (Lekberg et al., 2018). The partial Small Subunit (SSU) of the nuclear ribosomal genes (rDNA) has been used to discriminate up to the genus level. The LSU of the rDNA marker was also used to separate almost all morpho-species tested, with the exception of *Gigaspora* sp. (Stockinger et al., 2014). Amplified fragment length polymorphism (AFLP) was used to identify *G. intraradices* isolates from the field (Koch et al., 2004). Krüger et al. (2012) developed a phylogenetic reference data for systematics and taxonomy of AMF. The phylogeny was based on the nuclear ribosomal genes (rDNA) and more precisely on a sequence of 1500 bp formed by the partial small subunit rRNA gene (SSU), the partial large subunit rRNA gene (LSU), 5.8S rRNA gene (5.8S) and ITS. Unfortunately, the sequence was too long for most NGS technique and only around 760 bp long sequence could be used with 454 pyrosequencing (Senés-Guerrero and Schüßler, 2016). Schlaeppli et al. (2016) proposed a novel sequencing method to describe AMF communities: the single molecule real-time (SMRT) methodology. This technique offers long sequence reads of up to 40 kb and allows monitoring shift in community after specific strain inoculation. Nevertheless, this technique required heavy investment and did not allow to distinguish below species level. Phylogenetic analyses require the discrimination of taxa at the species level, but traceability in the field may require discrimination at the strain level. Therefore, other standardized molecular techniques are needed. Mating type loci are multi allelic and offer a great variability opening the perspective of discrimination between closely related strains (Halary et al., 2013; Ropars et al., 2016) but still need to be sequenced on different species. The use of mitochondrial genes avoids potential complications attached to the heterogeneous sequences with nuclear genes (i.e. ITS). Typical mitochondrial genomes comprise a single molecule of DNA, which is usually circular and contains all mitochondrial genes (Nadimi et al., 2016). The sequencing of the MtLSU of *R. irregularis* MUCL 41833 resulted in a 2000 bp sequence. This sequence was compared to an MtLSU database thus allowing to find species-specific and a strain-specific molecular marker. The

markers were tested on different AMF species and strains of *R. irregularis* coming from the GINCO (<http://www.mycorrhiza.be/ginco-bel/>) collection. The species-specific marker was only detected in strains of *R. irregularis*, confirming its level of specificity. The relatively short mitochondrial genome sequence used was identical for some closely related strains tested, thus forming a small group of strains sharing the same sequence on a specific genome area (e.g. a haplotype). The *R. irregularis* MUCL 41833 haplotype specific molecular marker was never detected on other AMF species and was detected in the *R. irregularis* MUCL 41833. The drawback of the technique is the limited number of available sequences, mainly *Rhizophagus* sp. and requires obtaining sequence of strain of interest before use. The mitochondrial genome is formed of 80000 bp and some regions could potentially offer high variability required for strains traceability but have to be identified. Inoculated strain diversity is very low and mostly relies on *Rhizophagus* sp. Furthermore, the introduction of exotic strain could result in deleterious effects on local soil environment.

To further evaluate the molecular marker, the MtLSU was quantified and linked to different stages of AMF development. It is well known from the literature that molecular detection is strongly impacted by specific AMF structures such as arbuscules and spores (Badri et al., 2016). These structures are good indicators of a successful colonization of the host as they are involved in nutrient exchange and life cycle of the AMF, respectively. Different approaches have been made to inoculate AMF strain on potato field: inoculation of cover crops using beads (Buysens et al., 2016) and coating have been used (Hijri, 2016). Coating seed with AMF appears as one promising technique since seeds are often coated with protective treatments before sowing. Coating with AMF could therefore be integrated to agricultural systems.

Before the field experiments, the molecular markers were tested on soil samples and only the *R. irregularis* species specific marker was detected. In both fields experiment the presence of *R. irregularis* in potato roots was identified by the species-specific marker. The haplotype specific marker was detected only on the potato plants inoculated as described above with *R. irregularis* MUCL 41833. Furthermore, during the second year of field experiment, molecular detection of *R. irregularis* was higher in pre-colonized potato plants as compared to the control plants (i.e. colonized only by the indigenous *R. irregularis* strains), indicating an increase of *R. irregularis* presence in AMF roots colonization. Inoculation of *R. irregularis* MUCL 41833

was successful over the two cropping seasons allowing the evaluation of its effect on late blight management.

Impact of AMF on late blight outbreak and symptoms

AMF are a key players in agroecosystems, increasing plant growth and resistance/tolerance to various biotic or abiotic stresses (Gianinazzi et al., 2010). However, most convincing results nowadays were obtained under controlled condition with reduced symptoms or elicitation of defense genes noticed *in vitro* (Gallou et al., 2011) and in the greenhouse (see Table 1 in section state of the art), while few or even no conclusive beneficial effects were observed within the field.

Two field experiments were conducted over two growing seasons with the inoculant *R. irregularis* MUCL41833 associated to potato plants (following the method developed in chapter I) before late blight outbreak. Copper treatment was used as a control. During the first cropping season, no symptoms were observed in the copper treatment, while during the second cropping season, copper only partially reduced late blight. Pressure by *P. infestans* was thus higher during the second year. This was paralleled with the climatic conditions, hot and dry during the first year and cold and humid during the second year, with late and early outbreak of the disease, respectively. Interestingly a marked decrease in symptoms was noticed in the AMF-colonized plants during the first cropping season, while no effect was noticed during the second cropping season. This clearly suggested the importance of pathogen pressure (and thus climatic conditions) on the potential efficacy of AMF. Indeed, cool and wet weather (Magarey et al., 2005) has a strong impact on the pathogen outbreak. Under low pathogen pressure *R. irregularis* MUCL 41833 reduced late blight but not under high pathogens pressure. Weather conditions over a growing season are unpredictable and thus the potential benefits of AMF inoculants as well. However, in the context of climate change, predicting a higher variability in rainfall in the coming decades and increased risk of water deficits during summers, AMF may become a more interesting option.

Numerous mechanisms have been suggested to explain the higher resistance/tolerance of AMF-colonized plants to diseases, from competition for space or nutrients to elicitation of defense genes. In the present study, no mechanism could be excluded, and it is even probable that several mechanisms were involved synergistically. Inoculation of AMF strains could potentially induce qualitative and quantitative

changes in microbial populations in the rhizosphere. Late blight management using AMF could therefore benefit from co-evolution or competition between beneficial microbial community and *P. infestans*. It would be interesting to analyze AMF strains and species effect on late blight, as it was shown for bacteria or fungi (see Table 3 in section state of the art). Most of the studies so far have focused on only two species (see Table 1 in section state of the art) and less than 10% of known species have been used in bio-protection experiment. Combination of different AMF strain and potato varieties could result in increased resistance of the plant against late blight.

Common mycorrhizal networks signaling during late blight outbreak

Hyphae of AMF have the capacity to interconnect numerous plants belonging to the same or different species (Simard et al., 1997; Rhodes, 2017; Simard, 2018). The functions of these CMNs range from the transport of minerals to the transfer of warning signals from diseased to healthy plants. For instance, Song et al. (2010) demonstrated that plants connected by CMNs could exchange information upon pathogen infection. Similar results were obtained with other pathosystems (Babikova et al., 2013a; Song et al., 2014; Gilbert and Johnson, 2017). However, the nature of the signals remains speculative. For instance, bacterial biofilms were hypothesized as one of the potential signal carriers (Barto et al., 2012) in parallel to chemical, volatile or electrical signals. So far, all the studies were conducted in pots, thus making it difficult to assess the exact contribution of the sole extra radical mycelium. The MDP *in vitro* culture system (Voets et al., 2009) represent a serious option to discriminate the role of hyphae from any other confounding effects. This system was used to demonstrate the transport of caesium between plants but never for the transfer of warning signals. For the first time an active mycorrhizal networks (CMNs) linking a donor plant to a receiver plant was used to investigate transfer of warning signals upon infestation of a donor plant by *P. infestans*. The defense genes were analyzed in the received plants at the time of inoculation of the pathogen and 24, 48 and 120 h post-infection by the pathogen.

Gene defense activation was noticed in the receiver plant 24 h post infection (hpi) of the donor plant only if the CMN was intact and the donor plant infected by *P. infestans*. The plant defense activation in the healthy receiver plant was transitory, as gene activation was not

measured 48 or 120 hpi. The system was free from any unwanted microorganism, indubitably demonstrating that hyphae were the unique pipeline for signal transport. The response was JA/ET dependent, as earlier reported by Song et al. (2014), with activation of *LOX/PAL/PR-1b/EFR3* genes. It would be interesting to evaluate if the signal is specific to the pathogen and to the host plant or is more general by using non-host receiver plants. The experimental setup did not directly allow to identify the nature of the signal. Nevertheless, VOCs could most probably been discarded, as potential accumulation of volatiles in the Petri plates did not lead to signaling between plants. Furthermore, involvement of electric signal seems to be unlikely. Indeed electric signal is so fast (Babikova et al., 2014) that the reduction of distance between the donor and receiver plant (10cm) in the present system comparing to pots systems should not have impacted the maximum genes defense response, contrarily to our observations. The results clearly suggested the direct involvement of hyphae in signal transport probably via cytoplasmic flux as suggested by (Whiteside et al., 2019). It could be interesting to evaluate the ecological impact of the CMN signaling at the field scale to further integrate AMF in late blight or any other disease management. Indeed, in field environments, CMNs need to connect the individual crop plant, to potentially affect disease development. Since deep ploughing and other forms of tilling destroy the fungal networks, this type of disease control could operate only in minimal till or no till systems, including perennials. Further studies are needed on the extent to which soil management helps or inhibits CMN formation and signal transfer. It would also be essential to understand which crop species can send or receive signals, how far signals can travel and through how many individual crops plants (Gilbert and Johnson, 2017). It appears critical to quantify the fitness costs/benefits to receiver crop plants involved in CMN signaling. It would be important to identify specificity of these signals in relation to plant species and pathogen and tests the hypothesis that AMF have a key role in shaping the patterns of plant to plant signaling in response to pest and disease infection, as demonstrated for nutrients exchange between host and fungi in CMN (Kiers et al., 2011; Whiteside et al., 2019).

CONCLUSIONS AND PERSPECTIVES

Potato is one of the most important crops cultivated worldwide for human consumption. However, this crop is threatened by numerous diseases among which late blight is the more problematic (Haas et al., 2009). The control of *P. infestans* under cool and wet climate conditions as in Belgium often requires the repeated applications of pesticides during the cropping season. This can lead to adverse effects on the environment and human health (Wilson and Tisdell, 2001; Beketov et al., 2013). In organic farming, the most widely used and efficient treatment to control late blight is copper. However, copper has a long remanence, may accumulate in soil over cropping seasons and has a detrimental impact on flora and fauna (Graham et al., 1986; Brun et al., 1998). Alternative methods must be evaluated, such as the application of beneficial microorganisms, in particular AMF. These organisms have been reported to protect plants against a number of pests and diseases, under controlled conditions for most studies, and in the field for only a few of them. The discrepancies between pots and field results may be related to competition with local flora, to agricultural practices and to edapho-climatic conditions. The use of AMF as well as any microbial inoculant into the field, therefore requires a thorough knowledge of the environmental factors that may affect them. Within this thesis, three major questions were addressed with the aim of better understanding the role of AMF in the field, and in particular in evaluating its potential for the control of late blight.

Are molecular markers based on MtLSU specific to an inoculated strain and can we link molecular quantification to root colonization in the greenhouse and field?

In chapter I, we have confirmed the efficiency of the MDP system to colonize potato tubers in the greenhouse. A good root colonization was obtained on all the inoculated sprouted tubers within short time. Key structures of AMF (i.e. hyphae, arbuscules, vesicles/spores) were identified in the roots. The MDP system allowed inoculating a great number of plants, therefore ensuring the presence of the AMF inoculant in the potato plants at transfer to the field, which is an essential step to evaluate the role of specific inoculants on potato growth and resistance to stresses. Although this system is time consuming and not adapted to the current plantation system. A number of products are proposed on the market such as coating of potato tubers with high density of isolated

spore solutions. Coating has been successfully used on large scale field experiments (Hijri, 2016) Tuber yield were significantly increased in most of the field, resulting in global increase of incomes for farmers (Hijri, 2016). However, so far, no data were provided demonstrating that the specific AMF inoculant was present in the roots, therefore that the effect on tuber yield was strictly linked to the inoculated AMF. Moreover, marketed mycorrhizal products are not systematically controlled under field condition, misleading the farmer when results are not consistent. It appears thus critical to implement traceability tools, to confirm the successful establishment of the AMF within the root system. Microscopic identification is inadequate and the classically-used genomic ribosomal sequence cannot separate strains belonging to the same species. Therefore, mitochondrial LSU of *R. irregularis* MUCL 41833 was sequenced and haplotype or species-specific primers were designed. The development of molecular markers requires extensive work and investment at first but allows the traceability of inoculated strain as noticed by Badri et al. (2016). Interestingly, the molecular quantification using RT- qPCR matched with the AMF stages development allowing to evaluate the dynamics of root colonization of the inoculant. The MtLSU marker was used to ascertain the successful establishment of *R. irregularis* MUCL 41833. The haplotype marker could discriminate between indigenous and inoculated *R. irregularis* during the two-field experiments conducted over two cropping seasons. Molecular marker design on 1500 bp sequence of the mitochondrial genome was specific to a haplotype (i.e. short number of strains sharing the same 1500 bp sequence).

- Specific attention should be devoted to microorganism communities. Nowadays, the Next Generation Sequencing (NGS) method allows to picture the microbial community at different steps of crop development but require heavy equipment and investment. It is of great interest to identify AMF and to have a better understanding of the dynamics of AMF communities. Indeed, AMF core species have been associated with potato and may take part of a sustainable cropping approach. This method mostly remains confidential to research purposes. On the other hand, introduction of a foreign strain can potentially disturb the established communities. Therefore, molecular markers have to be developed. They are extremely precise for detecting a specific inoculant strain, using only a one step RT-qPCR. The fast-diagnostic allow measuring the

inoculated strain during the cropping season and also during the following season. Therefore, should be part of quality requirement for marketed products.

Is *Rhizophagus irregularis* MUCL 41833 able to constrain late blight symptoms in the field and what are the environmental conditions more effective in disease control by the AMF?

Rhizophagus irregularis MUCL 41833 induced a reduction of late blight under low pathogen pressure (first cropping season under warm and dry conditions) but could not prevent completely the disease. Indeed, once the pathogen was installed, the growth rate of the disease was similar to the non AMF-inoculated plants. Therefore, the AMF was able to delay the emergence of symptoms for a couple of weeks without eradicating the disease. Under high pressure of the pathogen (second cropping season under cool and wet conditions), bio protection by AMF was not effective. No decrease in disease development was noticed on the sensitive Bintje or more resistant Nicola cultivars.

Low pathogen pressure was characterized by a warm and dry environment, whereas high pathogen pressure was measured under high humidity and cool temperatures. Those environmental data are already available to the farmer using risks prevision tools. Identification of those factors could lead to the use of AMF in integrated pest management (IPM) approaches. It would be interesting to test combined approaches. For instance, the above-ground application of reduced doses of copper with the below-ground application of AMF may be considered to evaluate the potential benefits of a BCA and reduced chemicals to control late blight.

- No cultivar dependence response was noticed, but synergistic interactions between AMF strains and cultivars against late blight is highly possible as it has been shown on plant nutrition aspect (Johnson and Pflieger, 1992). Therefore, special attention should be given to AMF dependency in breeding programs. Omics approach could be used to understand long-term response of mycorrhized potato against *P. infestans*. Identification of molecular responses and hormonal dependent pathways could help to also develop plant breeding programs against late blight for long term and stable resistance. This approach could also help to evaluate which part of the bioprotection can be attributed to the inoculated AMF. Indeed,

only global response can be evaluated for now including inoculated AMF effect but also indigenous microorganism effect.

Does extra radical hyphae connecting diseased to healthy plants transfer warning signals? What is the nature of the signals (chemical, electrical, volatiles) and which are the transcriptional changes observed in the healthy plants?

Since the past ten-year, the involvement of CMNs in the transfer of warning signals between diseased and healthy plants has been shown in various pathosystems, from necrotrophic pathogens to insects infestation (Song et al., 2010; Babikova et al., 2013a; Song et al., 2014). All the studies so far have been conducted in pots and the role of bacteria biofilms could not be completely overruled. The development of *in vitro* culture systems associating plants with AMF (Voets et al. (2009) allows to conduct studies under condition free from unwanted microorganisms. Gallou et al. (2011) used such system to demonstrate the elicitation of defense gene in AMF-colonized potato plants grown in presence of *P. infestans*. The signaling observed in our *in vitro* experiment using CMN allowed to exclude bacterial involvement since the growth environment is free of any unwanted microorganism.

Up-regulation of targeted defense gene expression was noticed 24 hpi by *P. infestans*. The drop of activity at 48 and 120 hpi seems to indicate that the pic of activation was earlier than the previously recorded: 48 to 100 h post infection/infestation of donor plant in the study of Gilbert and Johnson (2017). The Petri plates used in our experiment were 9 cm wide, reducing the distance between plants as compared to pots experiments (15 to 20 cm) (Song et al., 2010; Babikova et al., 2013a). This reduced distance seemed to induce a shortening of time required for gene activation, indicating that the signal was perceived earlier by the receiver plant. Pointing out, that time for signaling is distance-dependent, the involvement of electric signal could probably be excluded. Involvement of VOCs in aerial part or inside the Petri plate can also be excluded in accordance with Song et al. (2010). Speed measure of cytoplasmic flux using *in vitro* system from Whiteside et al. (2019) and our personal observation indicate that signal could follow the stream inside the hyphae and activate receiver plant response before 24 hpi of donor plant.

- The signal seemed to activate JA and JA/ET dependent gene, although it remains unclear whether the signal is specific or generic. It could be interesting to test if a non host (for late blight) would response or not to the signal from a *P. infestans* infected potato donor plant. The response of uninfested receiver plants connected via CMN to infested donors has to be evaluated at the community scale, allowing evaluation of different species or variety to a “stressor” signal. The plant diversity is a key factor but also the AMF diversity. Experiment should be conducted to evaluate potential dilution effect in a patchy network. The impact of bioprotection should also be evaluated to compare plant receiver response to pattern of gene activity measured and identified a potential priming effect of CMN signaling, as shown by Song et al. (2010). Long-term experiment should be conducted to evaluate the fitness cost/benefit of CMN signaling during cropping season. It could pave the way to understand a part of the ecological impact of CMN signals.

In conclusion, the AMF *Rhizophagus irregularis* can mitigate late blight in potato plants. The evaluation of the successful establishment of a mycorrhizal inoculant in the root system is a priority step to link it to the potential effects observed on plants. Using specific molecular markers, we could demonstrate potato colonization by the inoculant *R. irregularis* MUCL 41833 and evaluate its biocontrol potential against late blight. The effects of AMF on late blight appeared dependent of the environmental condition. It was transitorily efficient under warm and dry conditions characterized by a low pressure of the pathogen, while under cool and wet conditions no effect was observed. The CMN can play an important ecological role as they can warn neighbor plants from pathogen outbreak. Even if the nature of the signal remains unknown, a clear demonstration of hyphae involvement was made using the MDP *in vitro* culture system. Therefore, CMNs in the field can potentially warn uninfected potato plants from the presence of *P. infestans* and prepare those plants to better resist the disease.

OVERVIEW OF THE SCIENTIFIC ACHIEVEMENTS

Scientific publications

Research articles published

Buysens C., **Alaux P-L.**, César V., Huret S., Cranenbrouck S., Declerck S. (2017) **Tracing native and inoculated *Rhizophagus irregularis* in three potato cultivars (Charlotte, Nicola and Bintje) grown under field conditions.** *Applied Soil Ecology*, vol. 115, p. 1-9.

Alaux P-L., César V., Naveau F., Declerck S., Cranenbrouck S. (2018) **Impact of *Rhizophagus irregularis* MUCL 41833 on disease symptoms caused by *Phytophthora infestans* in potato grown under field conditions.** *Crop protection*, vol. 107, p. 26-33.

Research articles submitted

Alaux P-L., Naveau F., Declerck S., Cranenbrouck S. (2019) **Interplant signaling via common mycorrhizal networks might warn uninfected potato plants from late blight outbreak.** *Frontiers in plant science*.

Research articles in preparation

Alaux P-L., Naveau F., Dupré de Boulois H., Declerck S., (2019) **Selection of BCA against *Phytophthora infestans* on cultivars Bintje.** Biocontrol.

Conference participation

Alaux P-L., Sipel A., Dupré de Boulois H., Enaud E., Declerck S. (2012) **Screening of fungal candidates for the control of *Phytophthora infestans* on potato.** Poster at the 1st World Congress on the use of Biostimulants in Agriculture, Strasbourg, France, 26 to 29 November 2012.

Alaux P-L., Sipel A., Dupré de Boulois H., Declerck S. (2014) **"Contrôle du mildiou chez la pomme de terre par l'utilisation d'Agents de Bio-contrôle Microbiens".** Poster at the "10èmes

Rencontres de Phytopathologie - Mycologie de la Société Française de Phytopathologie". Aussois, France, 27 to 31 January 2014.

Alaux P-L., Sipel A., Dupré de Boulois H., Declerck S. (2014) Management of late blight of potato by the use of bio-effectors of fungal origin. Poster at the European Association for Potato Research (EAPR) at Bruxelles, Belgium, 6 to 11 July 2014.

Alaux P-L., Naveau F., César V., Cranenbrouck S., Declerck S. (2018) "Impact of *Rhizophagus irregularis* MUCL 41833 on the development of *Phytophthora infestans* in field potatoes". Oral presentation during Francophone day of mycorrhiza at Dunkerke, France, 27 to 29 Jun 2018.

Teaching and Student supervision

International training on *in vitro* culture of Arbuscular Mycorrhizal Fungi, organized by the CESAMM laboratory, UCL, Pr. Declerck: **Host preparation training.** Louvain la Neuve, Belgium (2013).

International training on *in vitro* culture of Arbuscular Mycorrhizal Fungi, organized by the CESAMM laboratory, UCL, Pr. Declerck: **Inoculation techniques** training. Louvain la Neuve, Belgium (2014-2017).

International training on *in vitro* culture of Arbuscular Mycorrhizal Fungi, organized by the CESAMM laboratory, UCL, Pr. Declerck: **Preparation and maintenance of *in vitro* HAM-P systems** training. Louvain la Neuve, Belgium (2018).

Supervision of one student (Isaure Lohest) from the "Bioingénieurs" faculty (UCL), who performed her master thesis at the laboratory of Prof. S. Declerck and Prof P. Bertin. ("Bioingénieurs" UCL, 2017-2018). **"Influence de la composition spécifique de couverts végétaux sur les paramètres physiques, chimiques, et biologiques du système sol-plante : étude des champignons mycorrhiziens à arbuscules".**

SUPPLEMENTARY DATA

Supplementary Table S1. *PCR primers and conditions used for the characterization of R. irregularis MUCL 41833 in the mitochondrial Large SubUnit DNA.*

Primer name(s)	Primer sequences (5' -> 3')	Applica tion	Primer conc. (µM)	Annea ling temp. (°C)	MgCl ₂ conc. (mM)
RNL28a/ RNL5	CCATGGCCA AGTGCTATTT A (Börstler et al. 2008) / GAGCTTCCTT TGCCATCCTA (Raab et al. 2005)	1st step of nested PCR	0.2 / 0.2	59	1.5
RNL29/ GImt4510 R	TAATAAGAC TGAACGGGT GT (Börstler et al. 2008)/ CATCCACGCT AGTGTTAGC (Krak et al. 2012)	2nd step of nested PCR	0.1 / 0.1	53	2
RNL27	CCAACTATGC AACCGTAGG	Sequenc ing			
RNL24	GAGCATACT AAGGCGTAG AG	Sequenc ing			

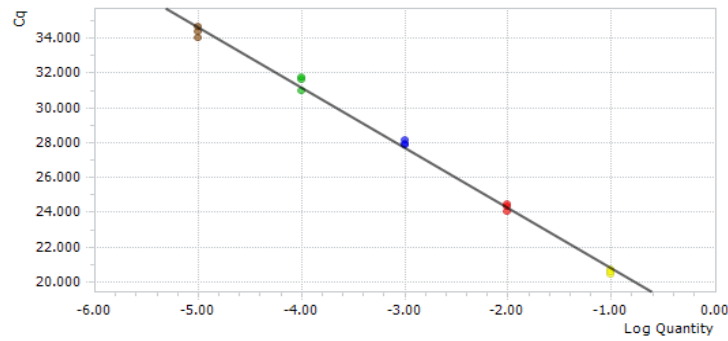
Supplementary Table S2. *Arbuscular mycorrhizal fungal strains and root samples (colonized or not by AMF) included in the test of specificity of primers.*

AMF isolates/ root samples				mitochondrial genome sequence		qPCR	
Species		Isolates		Origin			
Recent classification	Old classification	GINCO strain code	Other codes		accession number	BF7/BR5	BF8/BR6
<i>Rhizophagus irregularis</i>	<i>Glomus intraradices</i>	MUCL 41833 (spores/ mycelium in vitro)	/	Spain, Canary Islands	KY347874	+	+
<i>R. irregularis</i>	<i>G. intraradices</i>	MUCL 41833 (spores of in vitro culture)	/	Spain, Canary Islands	KY347874	+	+
<i>R. irregularis</i>	<i>G. intraradices</i>	MUCL 41833 (roots of maize colonised by strain in vitro)	/	Spain, Canary Islands	KY347874	+	+
<i>R. irregularis</i>	<i>G. intraradices</i>	MUCL 41833 (roots of potato colonised by strain in vitro)	/	Spain, Canary Islands	KY347874	+	+
<i>R. irregularis</i>	<i>Glomus irregularis</i>	MUCL 46241	DAOM 234180 / 4348YD	Canada, Quebec	JQ514225 (Formey et al. 2012) GQ204983 (Sokolaki et al. 2010)	+	-
<i>R. irregularis</i>	<i>G. irregulare</i>	MUCL 46239	DAOM 234181 / 4375YD	Canada, Quebec	JQ514223.2 (Formey et al. 2012) GQ204981.1 (Sokolaki et al. 2010)	+	-
<i>R. irregularis</i>	<i>G. irregulare</i>	MUCL 43194	DAOM 197198 / DAOM 181602	Canada, Quebec	KF848212.1 (Borriello et al. 2014) KI297408.1 KI274799.1 KI296156.1 ...	+	-
<i>R. irregularis</i>	<i>G. irregulare</i>	MUCL 46240	DAOM 234179 / 4341YD	Canada, Quebec	KC164354.1 (Beaudet et al. 2013)	+	-
<i>R. irregularis</i>	<i>G. irregulare</i>	MUCL 43195	DAOM 212349 / 3938 (YD)	Canada, Ontario	/	+	-
<i>R. irregularis</i>	<i>G. irregulare</i>	MUCL 43204	DAOM 229457 / CC-4	Canada, Ontario	JQ514224 (Formey et al. 2012) GQ204984.1 (Sokolaki et al. 2010) AM950206.1 AM950205.1 AM950204.1 (Borstler et al. 2008)	+	+
<i>Rhizophagus diaphanus</i>	<i>Glomus sp.</i>	MUCL 43196	DAOM 229456 / SD1 (SD)	Unknown	JX065416.1 (Beaudet et al. 2013) GQ204985.1 HE583398.1	+	+
<i>Rhizophagus clarus</i>	<i>Glomus clarus</i>	MUCL 46238	INCAM 12	Cuba, Pinar del Rio		-	-
<i>Rhizophagus intraradices</i>	<i>Glomus intraradices</i>	MUCL 49410	/	USA, Florida		-	-
<i>Rhizophagus</i>	<i>Glomus</i>	MUCL	/	Unknown		+	-
<i>facciculatus</i>	<i>facciculatus</i>	46100					
potato roots not colonized by AMF from in vitro and in vitro culture	/	/	/	/	/	-	-

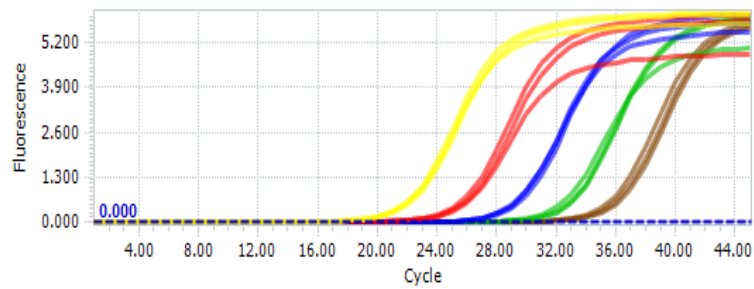
Supplementary Table S3. *Time line of the field trial and agricultural practices or treatments applied.*

Time	Agricultural practices
16 th December 2013	Ploughing
9 th April 2014	Herbicide Roundup (4 L ha ⁻¹)
16 th May 2014	Tillage
19 th May 2014	Tillage Plantation Mounding
26 th 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
28 th August 2014	Defoliant Reglone (2.5 L ha ⁻¹)
2 nd September 2014	Defoliant Reglone (2.5 L ha ⁻¹)
16 th September 2014	Mechanical defoliation
24 th September 2014	Harvest

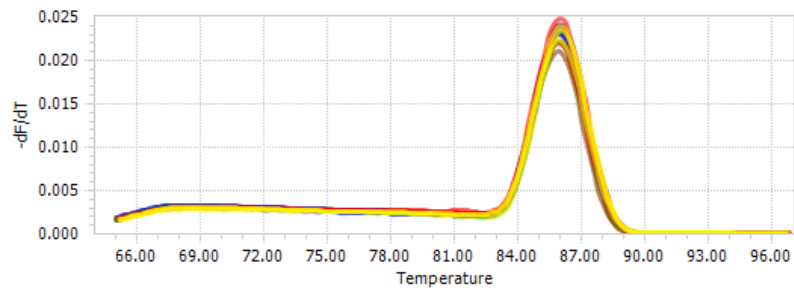
a



b

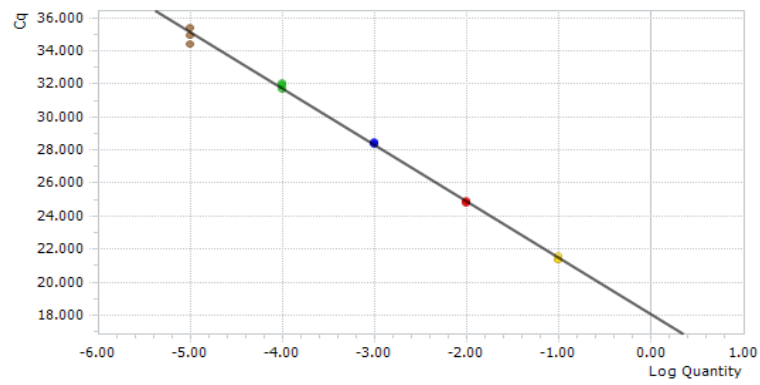


c

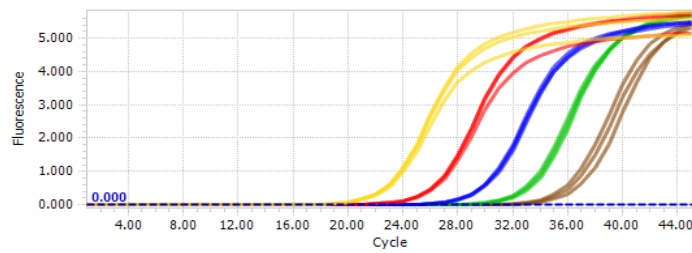


Supplementary Figure S1. Graphical view of standard curve (a), Real time qPCR amplifications (b) and melting peak (c) Using the *R. irregularis* MUCL 41833 haplotype-specific marker.

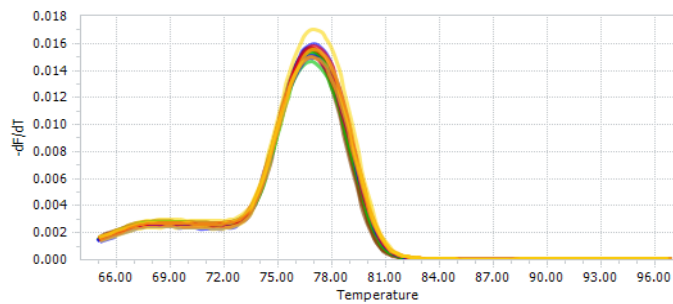
a



b



c



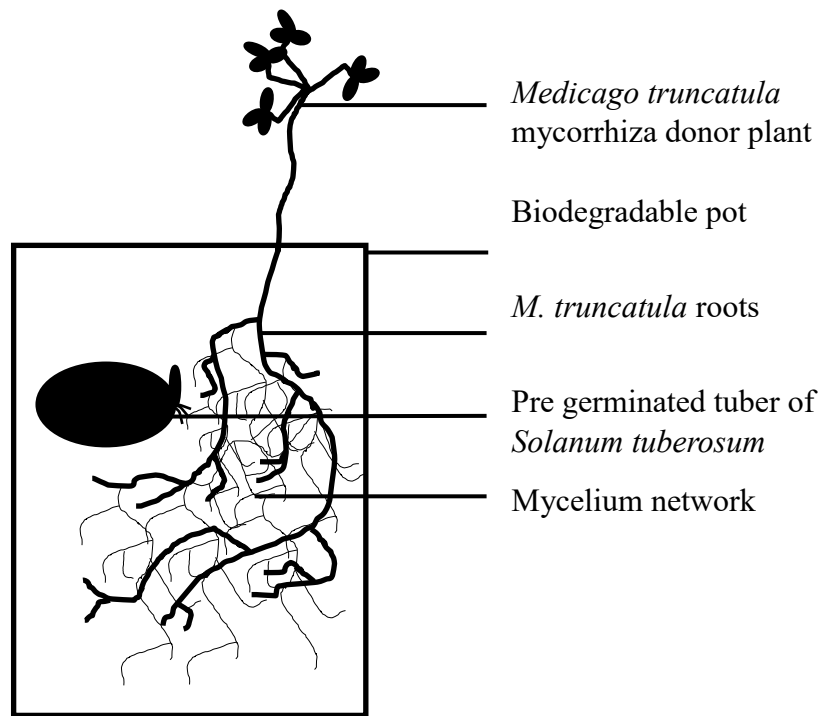
Supplementary Figure S2. Graphical view of standard curve (a), Real time qPCR amplifications (b) and melting peak (c) Using the *R. irregularis* species-specific marker.

Supplementary Table S4. Statistics of total (% RC), arbuscular (% A) and spore/vesicular (%V) colonization of the roots of potato at flowering stage, before defoliation 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). Main effects and interactions between the factor’s ‘cultivar’ and ‘inoculation treatment’ are presented. Tests that revealed significant differences at the 0.05 α level are indicated in bold. The Tukey’s test was used to compare means.

	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 x Inoculation treatment	0.5813	0.2645	0.6764	0.0466	0.0833	0.3833	0.9557	0.9499	0.9397
Tukey’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	0.1083	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

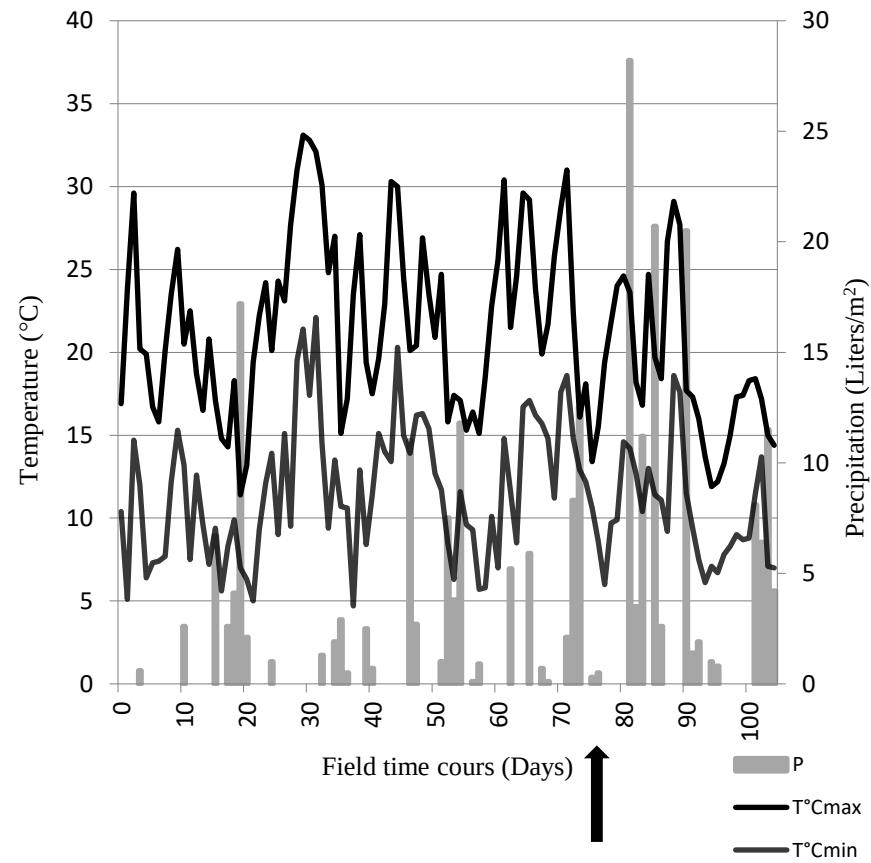
Supplementary Table S5. Quantification and statistics of the MtLSU specific to *R. irregularis* detected in the roots of potato at flowering stage, before defoliation and at harvest for following “inoculation treatments” (inoculation with beads containing *R. irregularis* MUCL 41833 (Ri41833) or beads without the AMF (Control) and of following “cultivars” (Bintje, Nicola and Charlotte). Data are means $\pm$ 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 Tukey’s test was used to compare means

MtLSU_<i>Rhizophagus irregularis</i> (10⁻⁶ ng/ ng DNA)			
	Flowering stage	Before defoliation	Harvest
Bintje/Ri41833	2.9 $\pm$ 0.5	39 $\pm$ 21	24 $\pm$ 5
Bintje/Control	2.8 $\pm$ 0.2	22 $\pm$ 10	18 $\pm$ 2
Nicola/Ri41833	22 $\pm$ 8	67 $\pm$ 12	190 $\pm$ 32
Nicola/Control	17 $\pm$ 9	94 $\pm$ 39	160 $\pm$ 21
Charlotte/Ri41833	7.3 $\pm$ 0.2	76 $\pm$ 25	49 $\pm$ 8
Charlotte/Control	6.2 $\pm$ 0.8	93 $\pm$ 40	69 $\pm$ 1
Effects (p-values)			
Cultivar	< 0.0001	0.0002	< 0.0001
Inoculation treatment	0.3767	0.5212	0.7615
Cultivar x Inoculation treatment	0.0569	0.8484	0.6638
Tukey’s test			
Bintje	2.9 $\pm$ 0.3	31 $\pm$ 11	21 $\pm$ 2.7
Nicola	20 $\pm$ 5.9	81 $\pm$ 20	180 $\pm$ 19
Charlotte	6.7 $\pm$ 1.1	84 $\pm$ 23	59 $\pm$ 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

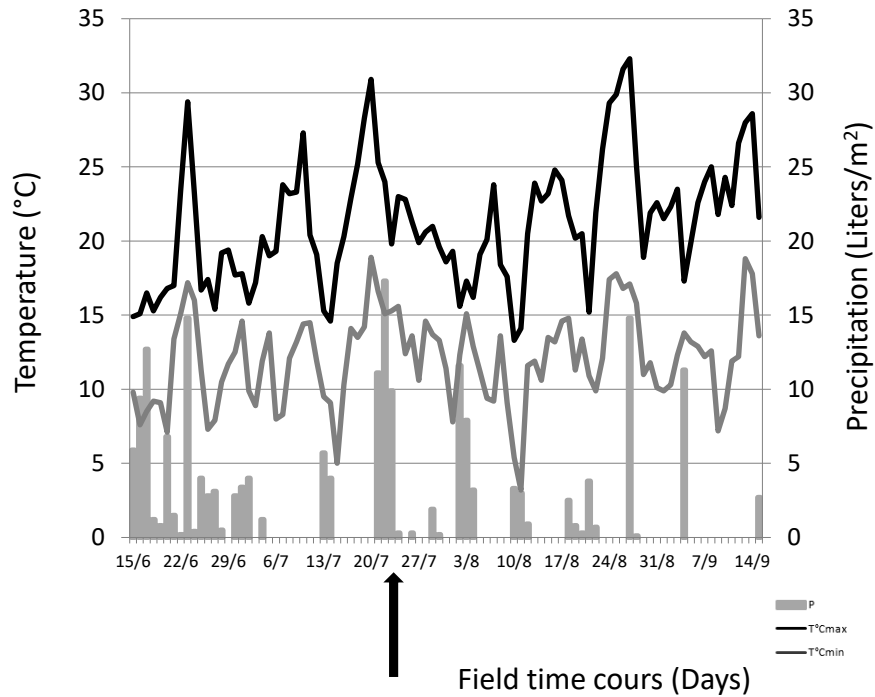


Supplementary Figure S3. Schematic representation of the “Donor” plant system (inspired from Voets et al. 2009). A rapid mycorrhization of potato plantlets was obtained in biodegradable pots using *M. truncatula* as mycorrhiza donor plant. Pre germinated tuber developed their roots in an active network of AMF.

2. a. Trial 1



2. b. Trial 2



Supplementary Figure S4. *Environmental conditions during the field experiment were recorded every day: rainfall and temperature in 2015 (Fig. 4.a) and in 2016 (Fig. 4. b).*

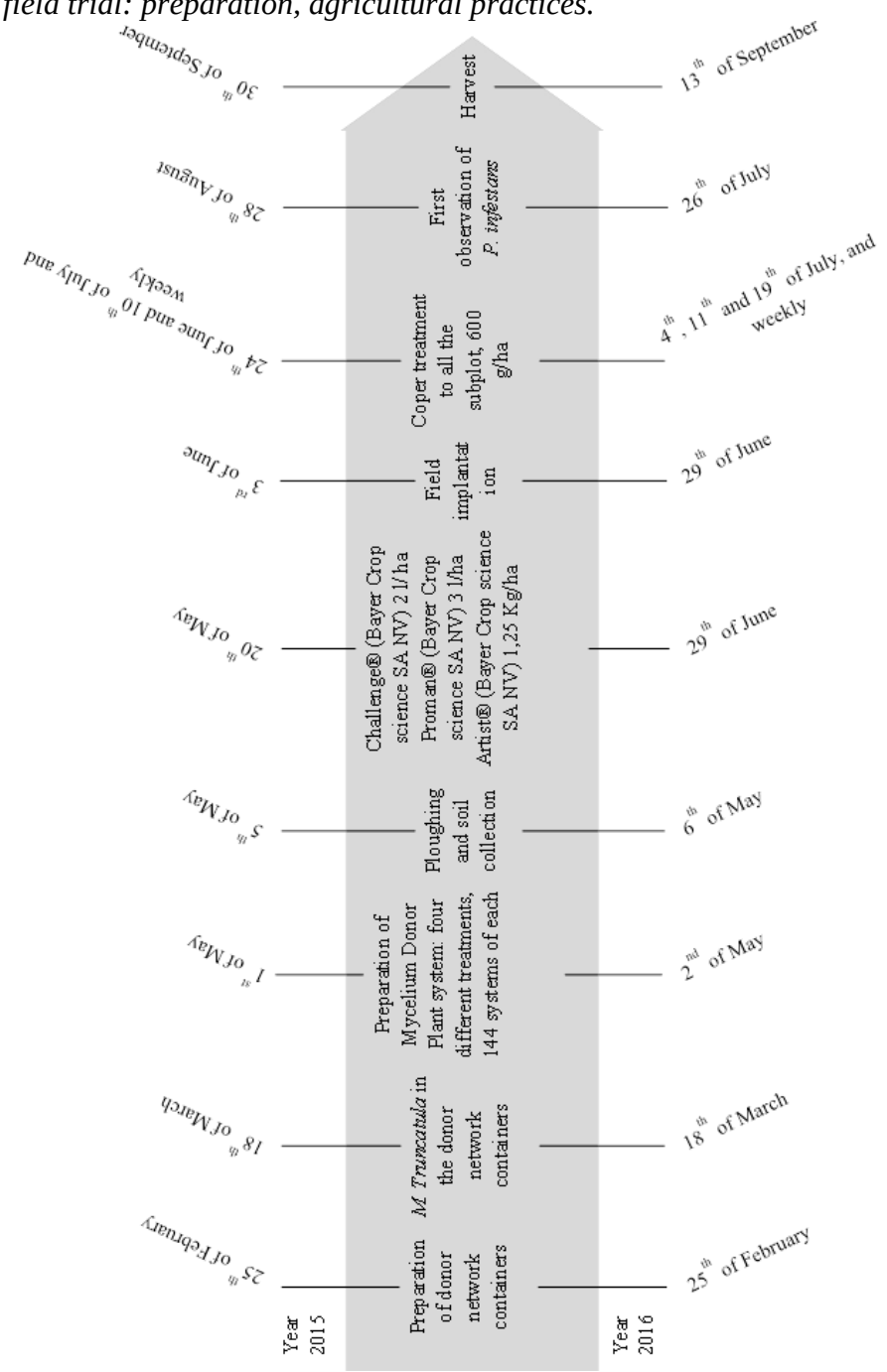
*Fig. 4.a. The dark arrow indicates the first observation of *P. infestans* symptoms in the field, after 83 days of field culture. Precipitation (p), lower temperature (T°C min) and higher temperature (T°C max) were beneficial for *P. infestans* after 83 days.*

*Fig. 4. B. The dark arrow indicates the first observation of *P. infestans* symptoms in the field, after 28 days of field culture. Precipitation (p), lower temperature (T°C min) and higher temperature (T°C max) were beneficial for *P. infestans* after 28 days.*

Supplementary Table S6. *Soil minerals content trial1 (2015) and trial 2 (2016).*

Soil minerals and content	Trial 1	Trial 2
P corresponded	2.2 mg/100g soil	2 mg/100g soil
K	20 mg/100g soil	20 mg/100g soil
Ca	152 mg/100g soil	100 mg/100g soil
Mg	11 mg /100g soil	10 mg/100g soil
Na	2 mg/100g soil	1 mg/100g soil
pH H ₂ O	6.4	6
pH KCl	5.3	4.9
Soil organic matter	3.4 %	3.4 %
C content	2 %	2 %

Supplementary Table S7. Time line of 2015 and 2016 main events for field trial: preparation, agricultural practices.

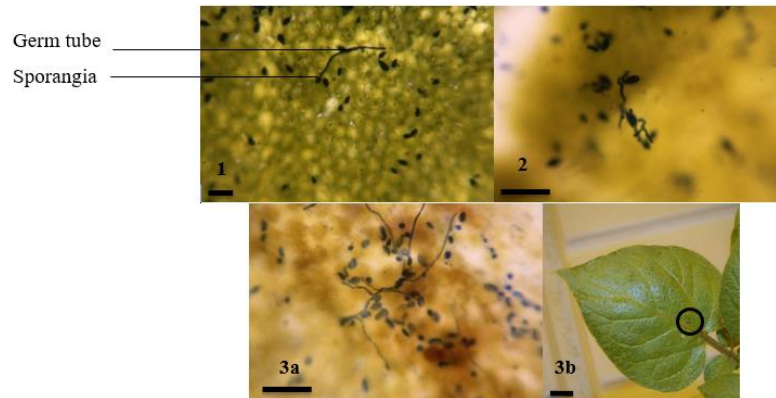


Supplementary Table S8. *Scale of assessment of Phytophthora infestans in field trial adapted from EUCABLIGHT by V. César (Walloon Agricultural Research Centre, Life Sciences Department, Breeding and Biodiversity Unit, Libramont, Belgium).*

Severity	Leaf infection index: scale description
0%	No disease
0,01%	1 to 2 leaflets affected / subplot
0,05%	3 à 5 leaflets affecteddb/ subplot
0,10%	Around 10 leaflets affected / subplot
0,50%	1 leaflets / plant
1,00%	4 leaflets / plant
2,50%	8 to 10 leaflets / plant
10%	1 leaflets affected / 10 leaflets
25%	Nearly every leaflet affected, field looks green
50%	50% of leaf area destroyed, field green flecked brown
75%	75% of leaf area destroyed, field both green and brown
95%	Only a few leaves left but stems green
99%	Only a few leaflets left and stems brown
100%	leaf area completely destroyed

Supplementary Table S9. *Phytophthora infestans identification.*

Date	Mating-type	Metalaxyl resistance	Virulence R gene from <i>Solanum demissum</i>	Genotype	Collector
28-August	A1	S	1-2-3-4-5-7-8-10-11	other	V. César, CRA-W
07-September	A2	R	1-2-3-4-5-6-7-8-9-10-11	13_A2	
07-September	A1	-	1-2-3-4-5-7-8-9-10-11	-	
31-August	A2	R	1-2-3-4-5-6-7-8-9-10-11	13_A2	
31-August	A2	R	1-2-3-4-5-6-7-8-9-10-11	13_A2	



Supplementary Figure S5. *Early stages of P. infestans infection on potato plants grown in vitro.* (1) Germinating sporangia 24 h post inoculation. (2) Hyphae branching and plant tissue infection 48 h post inoculation (3a) Spreading of hyphae on the leaf surface with the first symptom observed 120 h post inoculation. (3b) Round circle showing first necrosis. Scale bar = 0.25mm for Picture 1, 2, 3a and scale bar = 5mm for 3b.

Supplementary Table S10. Overview of the acid salicylic (SA), ethylene (ET) and jasmonic acid (JA) dependent genes, and their expression fold change in the Receiver plantlets after 24, 48 and 120h post inoculation (hpi) of the Donor plantlets by *P. infestans*.

Treatments	PR-1		PR-2		PR-3		PR-9		WRKY6		MAPK		GSTI		OSM		NPR1		PAL		PR-1b		ERF3		LOX		AOC		OPR3	
	means	standard error	means	standard error	means	standard error	means	standard error	means	standard error	means	standard error	means	standard error	means	standard error	means	standard error	means	standard error	means	standard error	means	standard error	means	standard error	means	standard error	means	standard error
24hpi																														
(D ⁺ WRKY6)	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
(D ⁺ PR1)	1.21	1.35	0.64	0.40	0.78	0.62	1.07	0.92	0.88	0.70	0.99	0.69	0.89	0.65	1.50	0.61	1.07	0.70	1.41	2.00	0.71	0.67	0.65	0.43	0.59	0.37	1.85	1.19	1.22	0.64
(D ⁺ PR2)	1.34	0.64	1.23	0.60	0.78	0.56	0.71	0.33	0.73	0.22	0.81	0.24	1.06	0.40	0.73	0.14	0.85	0.27	0.65	0.30	1.57	0.81	1.08	0.41	1.20	0.73	0.96	0.25	1.07	0.36
(D ⁺ PR3)	0.62	0.32	1.36	1.23	0.43	0.33	0.47	0.08	0.39	0.15	0.65	0.31	0.49	0.33	0.81	0.49	0.64	0.21	1.02	1.05	1.29	0.73	0.81	0.29	0.99	0.93	1.18	0.34	0.93	0.36
(D ⁺ PR9)	0.98	0.99	2.21	2.34	0.45	0.27	0.62	0.40	0.84	0.30	0.58	0.19	0.52	0.22	0.79	0.12	0.81	0.38	1.24	1.44	1.25	1.22	0.65	1.44	0.91	0.64	0.22	0.69	0.41	
(D ⁺ WRKY6)	0.62	0.46	1.30	1.43	0.48	0.47	0.92	0.42	0.52	0.33	0.43	0.17	0.41	0.30	0.71	0.39	1.30	0.99	0.64	0.38	1.68	1.09	0.95	0.64	1.43	0.65	0.96	0.35	0.95	0.53
(D ⁺ PR1)	0.87	0.69	1.27	1.01	0.28	0.16	0.58	0.40	0.43	0.13	0.77	0.49	0.48	0.34	0.81	0.33	1.12	0.45	1.17	0.83	1.17	0.94	1.09	0.40	1.80	1.66	1.28	0.49	0.98	0.42
(D ⁺ PR2)	1.38	1.11	4.20	2.67	0.51	0.39	0.61	0.34	0.77	0.35	1.06	0.48	0.72	0.29	1.04	0.86	1.58	0.33	26.54	33.82	3.91	3.39	4.05	2.43	9.77	10.98	1.10	0.38	0.89	0.44
48hpi																														
(D ⁺ WRKY6)	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
(D ⁺ PR1)	1.63	0.76	4.10	4.76	1.96	1.67	1.61	0.75	0.82	0.27	2.48	2.07	1.45	0.71	1.77	1.78	1.73	1.67	0.33	0.77	0.26	0.74	0.47	1.96	1.47	1.76	1.43	1.67	1.13	1.13
(D ⁺ PR2)	1.07	0.57	3.39	1.99	1.87	0.72	1.51	0.84	1.00	0.56	2.19	1.23	1.42	0.75	1.73	0.73	1.97	1.78	0.77	0.35	0.95	0.75	0.98	0.44	1.67	1.19	1.69	1.12	1.86	0.85
(D ⁺ PR3)	0.89	0.79	2.95	1.31	1.74	0.50	1.34	0.88	0.87	0.26	1.76	0.82	1.47	0.64	1.12	0.49	1.49	1.43	0.57	0.29	1.00	0.54	0.86	0.41	1.40	0.47	1.69	1.01	1.24	0.37
(D ⁺ PR9)	2.11	2.52	4.31	3.84	1.87	1.15	1.57	0.64	1.75	0.79	2.37	1.90	1.32	0.55	2.13	1.69	2.47	2.68	1.42	0.85	1.55	1.15	1.35	0.98	1.53	1.29	1.15	0.77	2.07	1.16
(D ⁺ WRKY6)	1.07	0.99	7.11	14.35	1.88	2.54	2.30	2.13	1.23	0.97	4.60	5.61	1.23	0.69	1.11	1.22	3.62	5.76	2.18	2.46	0.81	0.44	1.44	2.06	1.62	1.93	1.67	2.14	4.23	6.00
(D ⁺ PR1)	0.97	0.76	3.50	3.52	1.18	0.70	1.29	0.53	0.96	0.61	2.29	1.61	1.39	0.44	0.85	0.61	1.94	2.53	1.20	1.01	0.68	0.44	0.99	0.88	1.29	1.00	1.06	0.94	1.37	1.02
(D ⁺ PR2)	1.63	0.80	2.96	2.83	1.49	0.76	1.74	1.04	1.12	0.73	3.09	2.59	1.43	0.71	1.33	0.93	2.25	2.37	6.02	9.28	0.87	0.58	3.78	5.79	0.90	1.17	0.96	1.79	0.82	0.82
120hpi																														
(D ⁺ WRKY6)	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
(D ⁺ PR1)	0.67	0.19	1.06	0.45	0.89	0.36	1.02	0.16	0.78	0.62	0.78	0.32	0.81	0.23	0.24	0.04	0.52	0.14	1.61	0.92	0.67	0.49	0.67	0.43	0.84	0.33	1.12	0.16	2.33	2.77
(D ⁺ PR2)	1.36	1.72	0.63	0.42	0.38	0.17	0.52	0.22	0.14	0.08	0.79	0.52	0.79	0.45	0.21	0.12	0.44	0.22	0.60	0.49	0.60	0.23	0.36	0.20	0.52	0.32	0.62	0.43	0.52	0.29
(D ⁺ PR3)	1.37	0.20	3.29	2.06	1.35	1.18	1.52	0.22	0.41	0.05	2.45	0.44	1.83	0.38	0.39	0.14	1.33	0.22	0.91	0.70	2.25	0.92	0.85	0.30	2.01	1.38	2.45	0.53	2.13	0.81
(D ⁺ PR9)	1.82	1.50	3.52	3.02	1.20	0.48	1.78	0.42	0.75	0.22	2.30	0.83	1.46	0.45	0.80	0.55	1.57	0.24	2.71	1.69	1.67	0.20	1.30	0.52	1.40	0.32	2.33	0.53	2.14	1.20
(D ⁺ WRKY6)	2.16	1.08	1.05	0.67	0.91	0.16	0.81	0.12	0.27	0.04	1.29	0.39	1.36	0.52	0.31	0.05	0.88	0.18	0.96	0.82	1.21	0.41	1.07	0.22	1.02	0.41	1.00	0.32	1.19	0.21
(D ⁺ PR1)	0.95	0.27	1.26	0.74	0.61	0.24	0.92	0.11	0.23	0.02	1.14	0.25	0.87	0.15	0.20	0.05	0.68	0.12	0.94	0.66	0.93	0.35	0.23	0.43	0.77	1.28	0.27	1.21	0.19	0.19
(D ⁺ PR2)	0.83	0.23	0.98	0.64	0.66	0.26	0.93	0.18	0.30	0.05	0.91	0.17	0.64	0.15	0.21	0.06	0.61	0.09	0.34	0.13	0.98	0.23	0.46	0.37	0.62	0.14	1.17	0.14	1.38	0.41

Genes significantly up- or down regulated (in boldface). Genes were considered as differentially regulated if p -values ≤ 0.05 and the values of fold change compared with the controls was $\geq \pm 2.5$. Linear mixed model, where “Treatment” and “Time” were regarded as fixed factors and “Experiment” as random factor to account for potential differences between experiments, Differences between means were tested by Tukey’s test, significant values were set at $P < 0.05$. Quantitative real time RT-PCR was used to detect the transcripts of 15 target genes. Seven Salicylic acid (SA) dependent genes; pathogenesis related 1 (PR-1), pathogenesis related 2 (PR-2), pathogenesis related 3 (PR-3), pathogenesis related 9 (PR9), WRKY transcription factor 6 gene (WRKY6), mitogen-activated protein kinase (MAPK), osmotine like (OSM, glutathione-S-transferase 1 (GST1), Putative regulatory protein Non-expressor of PR genes 1 (NPR1). Two Jasmonic acid (JA) and Ethylen (ET) dependent genes; phenylalanine ammonia lyase (PAL), pathogenesis related 1 basic (PR-1b). One ET dependent gene: ethylene response factor 3 (ERF3). Three JA dependent genes: lipoxygenase (LOX), allene oxide cyclase (AOC), OPDA reductase 3 (OPR3). Every means are composed of eight replicates at 24 and 48 hpi and four replicates at 120 hpi.

Supplementary Table S11. Analysis of variance using statistical model.

Test variance between experiments	PR-1	PR-2	PR-3	PR-9	WRKY6	MAPK	GST1	OSM	NPR1	PAL	PR-1b	ERF3	LOX	AOC	OPR3
Factor "Experiment" p-value	0.5144	0.3631	0.3555	0.3908	0.3375	0.4151	0.3140	0.8666	0.7229	0.8326	0.2142	0.9952	0.2839	0.9035	0.5476

Analysis of variance using statistical model (Fit Least square) without fixed factor for both experiment at 24 and 48hpi. Significant values were set at $P < 0.05$. Seven Salicylic acid (SA) dependent genes; pathogenesis related 1 (PR-1), pathogenesis related 2 (PR-2), pathogenesis related 3 (PR-3), pathogenesis related 9 (PR9), WRKY transcription factor 6 gene (WRKY6), mitogen-activated protein kinase (MAPK), osmotine like (OSM, glutathione-S-transferase 1 (GST1), Putative regulatory protein Non-expressor of PR genes 1 (NPR1). Two Jasmonic acid (JA) and Ethylen (ET) dependent genes; phenylalanine ammonia lyase (PAL), pathogenesis related 1 basic (PR-1b). One ET dependent gene: ethylene response factor 3 (ERF3). Three JA dependent genes: lipoxygenase (LOX), allene oxide cyclase (AOC), OPDA reductase 3 (OPR3).

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Annex

Selection of BCA against *Phytophthora* *infestans* on cultivars Bintje

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Preface

In the different chapters of the thesis our attention was focused on arbuscular mycorrhizal fungi (AMF). The objectives were to evaluate their bioprotective effect against potato late blight and investigate some of the potential mechanisms involved. In the present annex, the attention was focused on selecting potential fungal biological control agents (BCA) from the BCCM/MUCL fungal collection. This approach was part of a DGO3 project entitled copper-free late blight control in potato production using natural agents and innovative delivery systems. This project aims to pursue research for developing alternative BCAs compatible with organic and low input farming.

The selection of BCAs is a long process that comprises several steps. Applying BCAs often starts with an *in vitro* screening (e.g. antagonisms test) to select a number of potential candidates. However, this approach only represents a measurement of the metabolite potential of the strains grown on rather rich laboratory media and does not guarantee that the strains that are active *in vitro* will also be efficient *in planta* (Guyer et al., 2015). Additional experiments must be conducted before transfer to the field. In the present study, we conducted dual tests *in vitro* and assays on potato plants grown *in vitro* and within the greenhouse, as a pipeline to select a number of potential BCA candidates able to reduce the symptoms caused by *P. infestans* either via the induced systemic plant defense activation or direct interaction with the pathogen. Experiments were conducted in growth chambers (for the *in vitro* tests) and in the greenhouse (for the *in vivo* tests) to ensure consistency of performance.

Abstract

Phytophthora infestans, the causal agent of late blight, is a major threat to potato production in conventional as well as organic production systems. In organic cultures, this pathogen is mainly controlled by copper fungicides. However, this compound may cause environmental problems by accumulation in the soil. The European Commission has set up directives to decrease its use drastically with the objective to ban its utilization in the medium-term. Therefore, alternative approaches are urgently needed to control *P. infestans*. Among the innovative approaches is the use of biocontrol microorganisms. From the literature, it is well known that microorganisms may directly or via the production of metabolites control, at least partly, root and shoot pathogens. Here we aimed to screen a collection of filamentous fungi and yeasts for their biological control effects against *P. infestans*.

Based on a literature survey, 77 fungal candidates were selected from the Mycothèque de l'Université catholique de Louvain (MUCL). Their effects against *P. infestans* were first tested *in vitro* with dual cultures. Seventeen of them induced a growth decrease of *P. infestans* superior to 50% and 4 had an inhibition zone. Within a second step, these microorganisms were tested *in vitro* on potato plants var Bintje and the twelve more efficient were selected for a final assay in the greenhouse on the same potato cultivar. One fungus (*Chaetomium cupreum* MUCL 49932) induced a strong decrease (50%) of *P. infestans* development, representing a promising BCA against this major potato pathogen.

Keywords

Phytophthora infestans; Biological control agent; potato late blight management; stepwise selection

Introduction

Phytophthora infestans, the causal agent of late blight, is the most devastating potato pathogen worldwide (Haas et al., 2009). Its control is mostly achieved via the repeated applications (10 to 16 per growing season in the most severe conditions) of fungicides, especially in cool and wet climatic conditions as in Belgium (Haverkort et al., 2009). This may have serious side-effects on the environment and human health (Wilson and Tisdell, 2001; Beketov et al., 2013). Moreover, the damages caused by this pathogen as well as the measures to control the disease accounts for more than 1 billion € per year in Europe (Haverkort et al., 2008; Vos and Kazan, 2016). It is therefore necessary to develop alternatives to chemicals in order to reduce costs and promote a sustainable approach to potato cropping.

In 2009, The European Union (EU) directive 2009/128/CE – (directive, 2009), promoting the use of integrated pest management (IPM) practices, entered into force and became compulsory in 2014. Integrated pest management is “*a holistic ‘approach’ or ‘strategy’ to combat plant pests and diseases using all available methods, while minimizing applications of chemical pesticides*” (Stenberg, 2017). It comprises the responsible use of pesticides and plant biotechnology solutions, including among others the use of biological control agents (BCAs) belonging for most to bacteria or fungi. Direct competition with the pathogen, elicitation of plant defenses (e.g. priming or induced systemic response (ISR)) (Ab Rahman et al., 2018) and/or production of bioactive antimicrobial metabolites (de Vries et al., 2018) have been suggested as potential mechanisms (Velivelli et al., 2014). Different microorganisms (e.g. *Trichoderma* sp., *Chaetomium* sp., arbuscular mycorrhizal fungi) and compounds (e.g. chitosan) compatible with organic farming have been tested on late blight (Soytong and Ratanacherdchai, 2005; Shanthiyaa et al., 2013; Nechwatal and Zellner, 2015; Yao et al., 2016; Alaux et al., 2018). Fungi in the genus *Trichoderma* and *Chaetomium* were reported to decrease symptoms caused by *P. infestans* and other *Phytophthora* species under laboratory and greenhouse conditions (Dorn et al., 2007; Zegeye et al., 2011; Shanthiyaa et al., 2013; Yao et al., 2016). A few results were also reported in the field. For instance, *C. globosum* isolate Cg-6 was shown to produce an antibiotic called ‘chaetomin’ and had a great exo- and endo-glucanase enzyme activity, resulting in a decrease by 30% of the infection index (Shanthiyaa et al., 2013). The *Trichoderma* isolate

HNA14 was the most efficient against the pathogen out of all the *Trichoderma* strains tested and reduced by almost 50% the disease severity compared to the control (Yao et al., 2016). Controlling an aggressive pathogen such as *P. infestans* remains, however, a major challenge, as evidenced by the number of biocontrol treatments that have been tested but did not, or only partially, meet the required level of efficacy (De Vrieze et al., 2018). One factor, among others, explaining this low level of success might be that *P. infestans* can infect its host plants by different means, e.g., through direct germination of sporangia or release of motile zoospores, both leading to host tissue penetration and mycelial development (Fry, 2008). An efficient BCA should therefore either induce plant resistance (Ab Rahman et al., 2018) or, when acting directly on the pathogen, inhibit both types of infection routes (De Vrieze et al., 2018). It is obvious that the environmental conditions (e.g. °T; humidity...) and agricultural practices (e.g. application of fungicides...) or cultivars used may also impact the efficacy of the BCAs (Ke-qiang and Forrer, 2001; Shanthiyaa et al., 2013; Sylla et al., 2013; Cray et al., 2016). Selecting efficient BCAs often starts with the screening of many microbial candidates. Screening is mostly conducted *in vitro* via dual tests (Badalyan et al., 2002; Yao et al., 2016; de Vries et al., 2018). However, this approach only represent a metabolite potential of the strains grown on rather rich laboratory media and do not guaranty that the strains that are active *in vitro* will also be efficient *in planta* (Guyer et al., 2015). A screening test on plants may thus be considered based on a restricted number of candidates having shown promising results in dual culture (Köhl et al., 2011). For instance, *T. harzianum* MUCL 29707 was successfully tested as BCA candidate against *P. infestans* (Gallou et al., 2009) using the *in vitro* plant system developed by Voets et al. (2009) for potato. Greenhouse trials using soil substrate under various conditions (e.g. °T, humidity...) may further help in selecting the final BCA candidates to be evaluated under real productive conditions in an appropriate number of testing sites. In the present study, 77 fungal BCA candidates were selected from the literature and tested against *P. infestans*. A thorough screening was conducted *in vitro* in dual culture and on potato plants c.v. Bintje, and the more efficient candidates were tested in the greenhouse on the same potato cv. Growth inhibition of the pathogen was measured in dual tests and development of symptoms was followed on potato plants grown *in vitro* and within the greenhouse.

Material and methods

Phytophthora infestans

Phytophthora infestans (mont.) de Bary MUCL 54981 – mating type A1 – was provided by the “Mycothèque de l’Université catholique de Louvain” (MUCL - <http://bccm.belspo.be/about-us/bccm-mucl>). It was isolated in July 2012 by the “Centre Wallon de Recherches agronomiques” (CRAW, Libramont, Belgium) from a potato field in Temploux (Belgium). The strain was sub-cultured every two weeks on Petri plates (90 mm diam.) containing 20 ml of potato dextrose agar (PDA, Oxoid: CM0139) medium. Leaves from *in vitro* micropropagated potato plants were placed on the surface of an actively growing *P. infestans* colony in Petri plates (90 mm diam.) to stimulate the production of sporangia. The Petri plates were sealed with cellophane, wrapped in aluminum foil and placed in the dark in a culture chamber set at 20°C.

Fungal candidates

Fungal candidates were selected from the collection of the “Mycothèque de l’Université catholique de Louvain” (MUCL - <http://bccm.belspo.be/about/mucl.php>) (see Table 1). The selection was made on species or genus known from literature to have bio-control or to produce active molecules against pathogens (they will be termed biological control agents – BCA – in the text). The strains were sub-cultured every three weeks on Petri plates (90 mm diam.) containing 20 ml of PDA medium. The Petri plates were sealed and placed in the dark in a culture chamber set at 27°C.

Solanum tuberosum

Solanum tuberosum L. var. Bintje was provided by the “Station de Haute Belgique”, Libramont (Belgium). The plants were micropropagated every 4 weeks in culture microboxes, sealed with breathing filters in the lid (ref: 0118/120+OD118, Combines, Belgium), containing Murashige and Skoog medium (MS) (Declerck et al., 1998) medium (Duchefa, The Netherlands) solidified with 4.2 g L⁻¹ Gelrite™ and supplemented with 20 g L⁻¹ sucrose. The microboxes were placed in a growth chamber (Snijders Scientific B. V, Netherlands), under a relative humidity (RH) of 75 %, a photoperiod of 16 h/8 h (day/night),

a photosynthetic photon flux (PPF) of $24.5 \mu\text{mol sec}^{-1} \text{m}^{-2}$ and a temperature of 20 °C/18 °C. Tubers (size 28-35mm) were also provided by the “Station de Haute Belgique”, Libramont (Belgium) for the greenhouse experiment. The tubers were placed in a growth chamber set à 20°C for 15 days to break the dormancy.

Experiment 1: Impact of BCA candidates on the growth of *Phytophthora infestans* MUCL 54981 evaluated in dual cultures.

The effect of the potential BCAs (see Table 1) on *P. infestans* growth was evaluated using dual cultures on Petri plate (90 mm diam.) following Badalyan et al. (2002) with some modifications. Briefly, a 5-mm-diam disc of *P. infestans* isolated from a 15-day old PDA culture was placed at one corner (1.5 cm from the edge) of the Petri plate. After 12 day, the outline of the culture was delimited with a black marker and a 5-mm-diam disc of the BCA candidates (from a 12-day old culture grown on PDA) was placed at the opposite corner (1.5 cm from the edge) of the Petri plate. Petri plates without BCA were used as control. The Petri plates were incubated at 25 °C in the dark. The assay was stopped when the mycelium of *P. infestans* in the control reached the opposite corner of the Petri plate. The growth of the pathogen in each Petri plate containing the BCA was then delimited with a black marker. Eight replicates were considered per BCA and the assay was repeated three times independently.

The growth surface of *P. infestans* was expressed as the difference between areas of growth in presence and absence of the BCA. The surface of growth was measured using ImageJ software (Schneider et al., 2012). The growth inhibition of *P. infestans* was further calculated using the following formula: Growth inhibition (%) = $((\text{GS} - \text{GSI}) \times 100) / \text{GS}$. Where, GS = growth surface of *P. infestans* in absence of the BCA (i.e. the control). GSI = growth surface of *P. infestans* in presence of the BCA.

Experiment 2: Impact of BCA candidates on *Phytophthora infestans* MUCL 54981 evaluated on *in vitro* cultures of potato plants.

The BCAs showing the highest inhibition of *P. infestans* MUCL 54981 in experiment 1(see Table 1) were selected and cultured on potato plants grown in the half closed arbuscular mycorrhizal-plant (HAM-P) *in vitro* culture system (Voets et al. 2009) with slight

modifications. Briefly, *in vitro* produced potato plants (20 day old) were transferred to mono-compartmented Petri plates (90 mm diam.) filled with 40 ml of Modified Strullu-Romand (MSR) medium (Declerck et al., 1998). The roots of the plants were in contact with the MSR medium and the shoot extended outside the Petri plate via a hole cautiously plastered with silicon grease (VWR International, Belgium) to avoid contaminations. The Petri plates were covered with aluminum foil to maintain roots in the dark. Starting from the second week of culture, 10 ml MSR medium was added weekly. The plants systems were placed during four weeks in a growth chamber, under 75 % RH, a photoperiod 16 h /8 h (day /night), a PPF of, $24.5 \mu\text{mol sec}^{-1} \text{m}^{-2}$ and a °T of 20 °C /18°C.

The 21 most active potential BCAs (see Table 1) from experiment 1 were selected for inoculation of the plants. Ten ml of sterilized (121 °C for 15min) deionized water was added in the Petri plates of each BCA. Conidia of BCA were harvested in the Petri Plates maintained in a slope at 40°. The conidia accumulating at the bottom of the Petri plates were harvested with a micropipette and placed in a small Petri plate (45 mm diam.) Concentration of each BCA was adjusted to 1×10^6 conidia /ml using Thoma cell. Fifteen μl of each solution was inoculated using micropipette (Sartorius Stedim Biotec, France) on four different spots on the surface of the MSR medium. The Petri plates were covered with aluminum foil to maintain roots in the dark. The systems were then placed in the growth chamber under the same conditions as above for one week.

Spores of *P. infestans* were collected from 10 days-old detached leaves of potato plants inoculated with a 0.2 mm diameter plug from *P. infestans*. Sporangia were delicately washed-off the leaves with deionized sterilized (121 °C for 15 min) water and their concentration adjusted to 100 per μl^{-1} . Sterilized cellophane disks of 0.5 mm diameter were placed on the adaxial part of leaves of the donor potato plants, preventing evaporation and drop fall. Then 20 μl of the sporangia solution was disposed between the cellophane and the leaf using a micropipette (Sartorius Stedim Biotec, France). The three oldest leaves on each plant were infected. The plant systems were placed for 20 h in the dark in a growth chamber, under 95 % RH and a °T of 18 °C /16°C. Photoperiod was adjusted to 16 h /8 h (day /night) with a PPF of $24.5 \mu\text{mol sec}^{-1} \text{m}^{-2}$.

Leaf infection by *P. infestans* was monitored every day after 4 days. Disease development was assessed according to James (1971) and EUCABLIGHT (www.eucablight.org) guidelines and translated into relative area under the disease progress curve (rAUDPC) as described in EUCABLIGHT guidelines. The AUDPC was calculated according to the equation of Madden and Campbell (1990): $AUDPC = \sum^{n-1} (y + y_{+1})/2 \times (t_{+1} - t)$, where n = number of observations, y = the leaf infection index and t = the day post emergence. Relative AUDPC (rAUDPC) was calculated by dividing the AUDPC of each treatment by the number of days of effective *P. infestans* infection.

Ten replicates were considered per BCA. The experiment was repeated two times independently.

Experiment 3: Impact of BCA candidates on *Phytophthora infestans* MUCL 54981 evaluated on potato plants var. Bintje under greenhouse conditions.

A greenhouse experiment was set up to validate the efficacy of the selected BCAs on the development of *P. infestans* MUCL 54981 in potato plants. Pre-germinated potato tubers were cultured for two months in pots of 8 dm³ filled with sand and vermiculite (1v/1v). They received once 1.5g L⁻¹ substrate granular slow release fertilizer of osmocote®. The plants were placed during four weeks in the greenhouse, under 75 % RH, a photoperiod of 16 h /8 h (day /night) under natural light conditions and a °T of 24 °C /18°C.

Solutions of BCAs were made as described previously in experiment 2. Concentrations were adjusted at 1 x 10⁶ conidia /ml using Thoma cell. Two hundred µl of the solution was inoculated using a micropipette (Sartorius Stedim Biotec, France) on 4 different areas (total volume 800 µl) on the substrate. The plants were placed during 10 days in greenhouse under the same condition as above.

Inoculation of *P. infestans* was made as described in experiment 2. The pots were placed randomly, for 16h in the dark in greenhouse, under 95 % relative humidity (RH) and 15 °C. Photoperiod was then adjusted to 16 h /8 h (day /night) natural light conditions, 20°C /15°C and 90% RH. Ten replicates were considered per BCA.

Statistical analysis

Data analyses were performed with JMP[®] Pro statistical software version 14.0.0 (SAS Inc., Canada). All data were evaluated by one-way factorial analysis of variance (ANOVA) with treatment differences among means tested by post-hoc by Tukey's test for confrontation test and Dunnett test (comparing each treatment with a single control) for *in planta* essay, significant values were set at $P < 0.05$.

Table 1. *Biological control agent evaluated for efficacy against late blight, Phytophthora infestans: in vitro dual culture, in vitro plant system in growth chamber, and greenhouse.*

Nom	Collection number (MUCL)	Dual Test	In vitro test	Greenhouse test
Hypocrea pachybasioides	8860			
Chaetomium cupreum	10178			
Verticillium psalliotae	18310			
Trichoderma harzianum	29707			
Penicillium striatisporum	31202			
Torulaspora globosa	31277			
Hypomyces chrysospermus	38942			
Chaetomium globosum	39889			
Trichoderma atroviride	44894			
Chaetomium cupreum	49932			
Emericella rugulosa	49935			
Hypomyces odoratus	166			
Gliocladium album	4127			
Gliocladium catenulatum	8002			
Rhizopus oryzae	16179			
Trichoderma virens	18139			
Sporothrix insectorum	19334			
Melanospora argentinensis	31657			
Emericella nidulans	35515			
Ascoidea hylecoeti	44855			
Trametes versicolor	44889			
Cladosporium cladosporioides	689			
Cladosporium fulvum	722			
Beauveria bassiana	2947			
Hansfordia pulvinata	3153			
Phoma glomerata	3648			

Nom	Collection number MUCL	Dual Test	In vitro test	Greenhouse test
Metarhizium anisopliae	6859			
Sporothrix pallida	8061			
Pyricularia oryzae	9191			
Metarhizium anisopliae	9644			
Hyalodendron lignicola	9672			
Cladosporium sphaerospermum	11858			
Rhodotorula mucilaginosa	11862			
Pestalotiopsis disseminata	14123			
Dipodascus capitatus	14473			
Galactomyces geotrichum	15162			
Nectria ochroleuca	18031			
Paecilomyces farinosus	18885			
Beauveria felina	20193			
Chaetomium globosum	20345			
Aureobasidium pullulans	22377			
Schizoblastosporion starkeyi	27844			
Pichia membranifaciens	28481			
Aphanocladium dimorphum	28814			
Sporidiobolus pararoseus	29432			
Bulleromyces albus	29433			
Candida sake	29900			
Kluyveromyces marxianus	29917			
Candida saitoana	29934			
Filobasidium capsuligenum	30295			
Rhodosporidium diobovatum	30329			
Cryptococcus magnus	30399			
Cryptococcus albidus	30595			
Cryptococcus laurenti	30609			
Taphrina populina	30958			

Nom	Collection number MUCL	Dual Test	<i>In vitro</i> test	Greenhouse test
Laetisaria fuciformis	31092			
Candida saitoana	31230			
Fellomyces sp.	31787			
Phlebiopsis spissa	32711			
Debraryomyces hansenii	34575			
Dacrymyces stillatus	34795			
Beauveria brongniartii	34853			
Cephaloascus fragrans	35227			
Pichia guilliermondii	38119			
Sporidesmium goidanichii	39230			
Phytophthora parasitica	41913			
Rhodotorula dairenensis	43165			
Trichosporiella flavificans	43376			
Saccharomyces cerevisiae	45812			
Pichia guilliermondii	46637			
Candida oleophila	46717			
Aspergillus ustus	47331			
Hypholoma fasciculare	47611			
Clavisporea reshetovae	52545			
Pichia anomala	52882			
Dioszegia hungarica	53958			

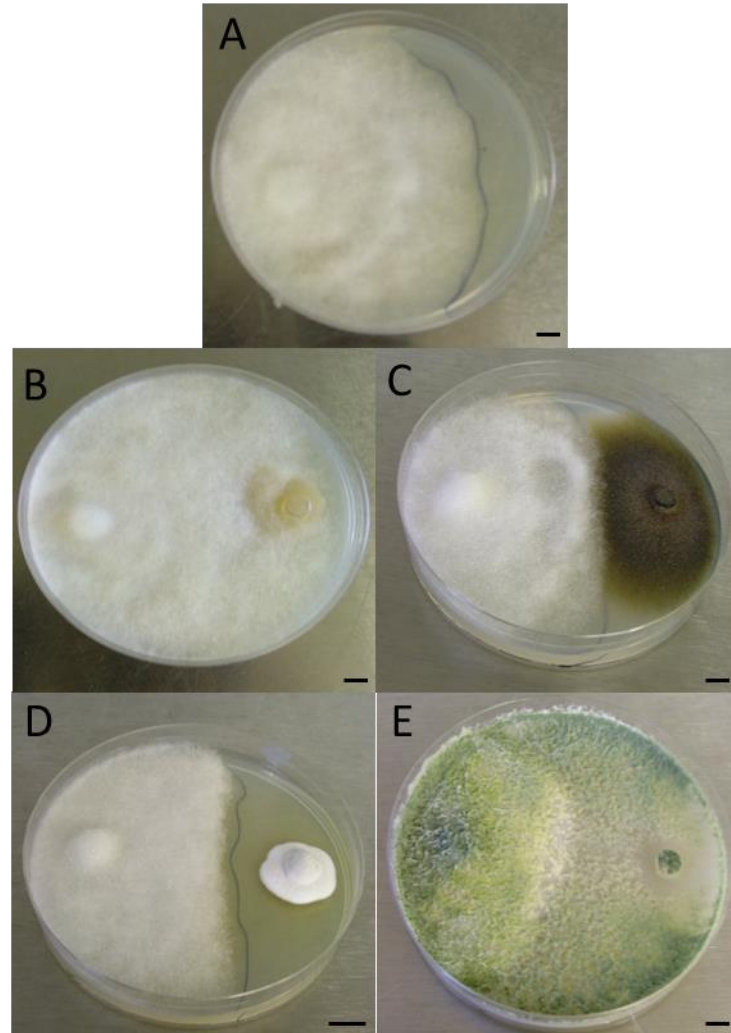


Figure 1. Dual test assays combining *P. infestans* MUCL 54981 with various potentials BCAs. Experiment was stopped when *P. infestans* in the control treatment (A) reached the opposite corner (1.5 cm from the edge). Growth of *P. infestans* could be stimulated by the potential BCA (B) or inhibited below 50% (C). Potential BCA could develop an inhibition zone around them (D) or completely cover the pathogen (E).

Results and discussion

It is nowadays widely accepted that the rhizobiome fulfills many functions for plants in the same way as the intestinal microbiome in humans. The control of pests and diseases is one of them and the exploitation of microbes involved in this function represent an innovative and alternative option to pesticides for the control of plant pathogens. However, the use of biological control agents (e.g. fungi) requires first to evaluate their potential against the target disease under controlled conditions (i.e. *in vitro* and in the greenhouse) before trialing in real productive conditions, in an appropriate number of testing sites. Here, 77 fungi, selected from the literature, were evaluated against *P. infestans*, the causal agent of late blight, under *in vitro* (in dual culture and on potato plants) and greenhouse conditions. Several fungi appeared effective throughout the testing procedure and one of them, i.e. *Chaetomium cupreum* MUCL 49932, reduced the symptoms of the disease, under greenhouse conditions, by 50%. This fungus should be tested further in the field, before any possible homologation, a process that is rather long and costly.

Experiment 1: Impact of BCA candidates on the growth of *Phytophthora infestans* MUCL 54981 evaluated in dual cultures.

A number of BCA candidates (Fig 1) were tested against *P. infestans* MUCL 54981 in dual cultures. Responses ranged from absence of effects on *P. infestans* to growth stimulation in a few cases or inhibition in most cases (Fig 2) as earlier noticed by (Heydari and Pessarakli, 2010). Out of the 76 BCA candidates tested, 60 induced a growth inhibition below 50%, while three BCA candidates slightly stimulated the growth of the pathogen (i.e. *Sporidesmium goidanichii* MUCL 39230, *Schizoblastosporion starkeyi* MUCL 27844, *Dioszegia hungarica* MUCL 53958). Finally, 17 BCA candidates induced a growth inhibition higher than 50% and four (i.e. *Hyalodendron lignicola* MUCL 9672, *Cladosporium sphaerospermum* MUCL11858, *Torulaspora globosa* MUCL 31277, *Hypholoma fasciculare* MUCL 47611) produced a clear inhibition area.

The most effective BCA candidates in dual cultures were fast growing fungi (i.e. *Trichoderma atroviride* 44894, *Trichoderma harzianum* MUCL 29707), with 88, 81% of growth inhibition respectively, possibly overgrowing *P. infestans* as reported in other studies (Zegeye et al., 2011; Yao et al., 2016). *In vitro* bioassays against

P. infestans are generally conducted with bacteria and only a few studies concerned fungal candidates (Daayf et al., 2003; Guyer et al., 2015; Hunziker et al., 2015; Cray et al., 2016; De Vrieze et al., 2018). *Trichoderma* sp. and *Chaetomium* sp. were shown effective against late blight (Soytong and Ratanacherdchai, 2005; Zegeye et al., 2011; Yao et al., 2016). The mechanism proposed was the production of bioactive antimicrobial metabolites released by the fungi in the medium or mycoparasitism (Heydari and Pessarakli, 2010). *Trichoderma viride*-ES1 as shown to reduce the radial growth of *P. infestans* *in vitro* by 36.7% after 3 days and a complete overgrowth of the pathogen was observed after 9 days (Zegeye et al., 2011). *In vitro*, the *Trichoderma* isolate HNQ11 showed the highest inhibition of mycelium growth (60.4 %) of *P. infestans*, followed by HNA14 with a percentage of reduction of 56.8 % (Yao et al., 2016). It is obvious that our experiment in dual culture favor fast growing strain like *Trichoderma* sp. and strain producing antimicrobial metabolite like *Torulospora* sp. Antagonistic relationship between microbes and pathogens mostly leads to significant disease control, where the established populations of metabolically active beneficial microbes initiate protection either by direct antagonistic activity of pathogens, by outcompeting pathogens or by stimulation of host plant defenses (priming) (Shoda, 2000). It also involves antibiosis which is the secretion of diffusible antibiotics, volatile organic compounds, and toxins, as well as the development of extracellular cell wall degrading enzymes such as chitinase, β -1,3-glucanase, beta-xylosidase, pectin methylesterase (Ab Rahman et al., 2018). It is therefore important to test on plant the potential of those BCA strains.

Experiment 2: Impact of BCA candidates on *Phytophthora infestans* MUCL 54981 evaluated on *in vitro* cultures of potato plants.

The ability of BCAs to induce a systemic protection on plants was tested on potato grown under *in vitro* conditions. Twenty-one BCA candidates were selected because they either induced a reduction of growth of *P. infestans* superior to 50% or formed a clear inhibition area during the dual culture bioassays (see above).

Late blight development on potato plants *in vitro* ranged from stimulation to no effect or even decrease of the symptoms of the disease as compared to the control treatment. Seventeen of the tested BCA candidates had no effect on late blight development, one (i.e. *Hypocrea pachybasioides* MUCL8860) increased the symptoms by 58% and three

decreased the rAUDPC measured by around three times (*Sporothrix insectorum* MUCL19934, *Chaetomium cupreum* MUCL 49932, *Torulaspora globosa* MUCL 31277) and one by around four times (i.e. *Hypomyces chrysospermus* MUCL 38942) as compared to the control treatment.

This experiment did not allow to directly investigate the mechanisms involved in the decrease of symptoms caused by *P. infestans*. But potentization of plant defense has been shown to affect leaf pathogens. Indeed, induced long distance (e.g. systemic) plant defense has been reported with beneficial rhizobacteria: induced systemic resistance (ISR) (Van Loon et al., 1998). Since this first study, ISR have been describe with fungal BCAs (Pieterse et al., 2014b). It was notably reported with AMF, *Trichoderma* sp., *Chaetomium* sp. (Soytong and Ratanacherdchai, 2005; Gallou et al., 2009; Gallou et al., 2011) against late blight. *T. viride* reduced the disease severity by 75% respectively as compared to the control (Zegeye et al., 2011). *In planta*, the lowest disease index (10.0) was obtained with *Trichoderma* isolate HNA12, followed by isolate HNA14 with a disease index of 11.5 compared to the control 42.5 (Yao et al., 2016). Stepwise screening of BCA notably proposed by Köhl et al. (2011) highlight notably low cost selective step such has *in vitro* dual test and *in planta* experiment. Both test allow to evaluate important criteria for BCA potential commercialization: spores production on low cost medium and compatibility with target crop (Köhl et al., 2011). Our experiment in dual test have shown that BCA tested produced numerous spores per plate and *in planta* experiment has shown that most of the BCA were compatible with potato plants, only one of them had a negative impact on plant fitness during the assay. From the literature, experiments on BCA *in planta* have also shown production of antimicrobial compounds (chitinase, glucanases and detoxifications enzymes) and induce systemic resistance (ISR) (Ab Rahman et al., 2018). Plant roots offer an ecological habitat for the growth of soil microorganisms that thrive on root exudates and lysates as nutrients. The mutualistic interaction between plants and beneficial microbes that commonly occurs in nature often leads to either an improvement in plant nutrition and/or enhancement of the plant's ability to prevail over biotic and abiotic stress. In all cases, this provides a competitive advantage leading to improved growth and plant proliferation (Haney et al., 2015). To test further the mutualistic interaction between potato host plant and the BCA greenhouse experiment come closer to natural environment.

Experiment 3: Impact of BCA candidates on *Phytophthora infestans* MUCL 54981 evaluated on potato plants var. Bintje under greenhouse conditions.

The capacity to induce a systemic protection on plant was further investigated on plants, in a greenhouse experiment. Late blight development was measured on plant associated with twelve BCA candidates selected from the above experiments. Eleven candidates did not affect the disease development. The association with *C. cupreum* MUCL 49932 induced a decrease of late blight by 50% as compared to the control. So far, *Chaetomium* have been used to extract antagonist compound (Mao et al., 2010; Sibounnavong et al., 2011; Sibounnavong et al., 2012) but not on plant essays. *Chaetomium* have not been shown to produce mycotoxin and therefore do not present potential toxicity for the environment and human health, which are prerequisite for BCA marketing (Köhl et al., 2011).

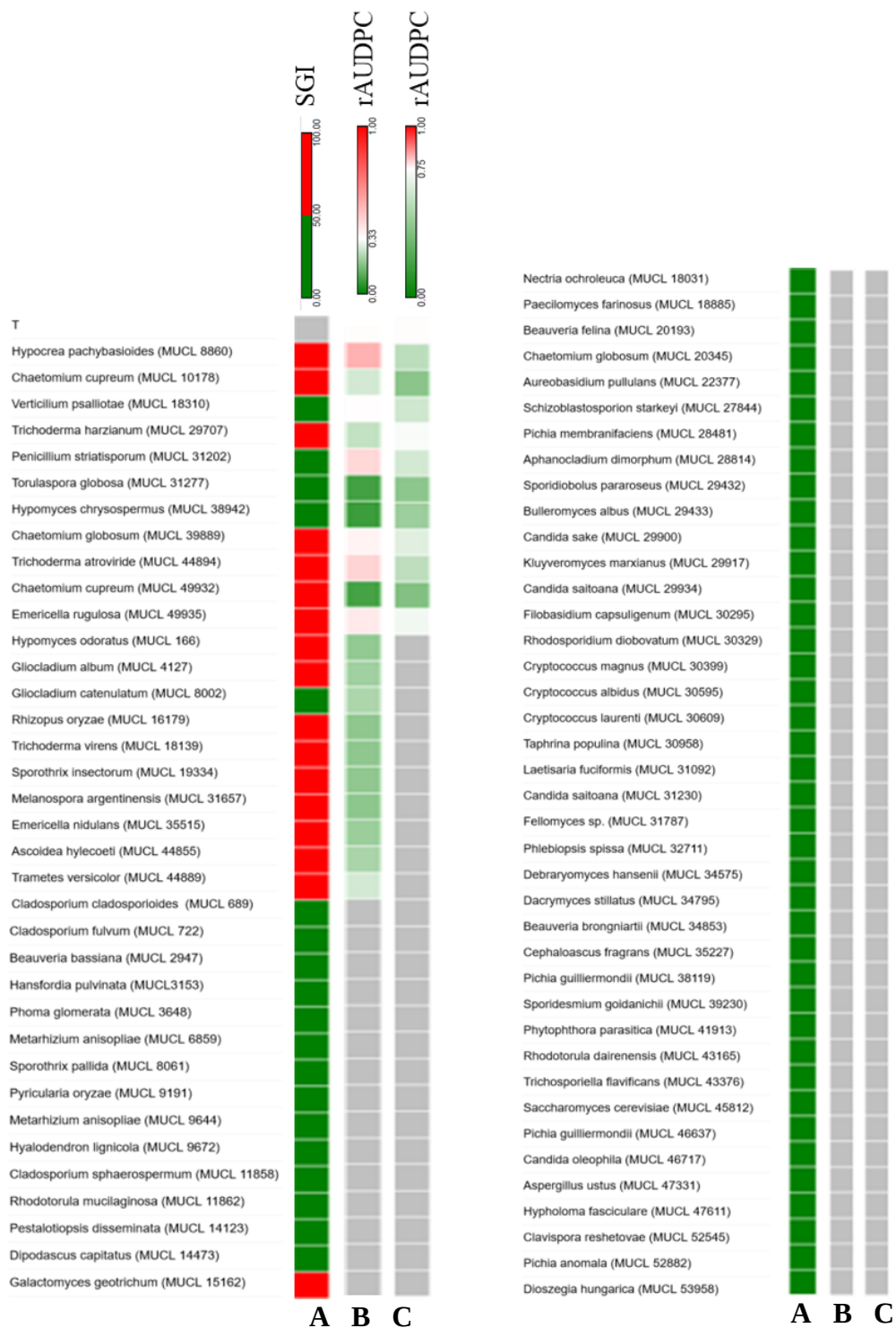


Figure 2. Heat map presenting results of dual test bioassays (A), *in vitro* plant (B) and greenhouse (C) assays. Surface growth inhibition (SGI) of *P. infestans* with limit of selection set a 50% of inhibition. Essay on late blight development on *in vitro* or greenhouse experiment, with control rAUDPC 0.33 and 0.75 respectively.

Conclusion

The results of our study demonstrated that some fungal BCAs have the potential to control *P. infestans* under controlled *in vitro* and greenhouse conditions. Indeed, *Solanum tuberosum* was found to tolerate different BCAs. Various effects on the growth rate of *P. infestans* and late blight development have been measured. Further studies should be carried out to investigate the mechanisms by which *C. cupreum* MUCL 49932 help the plant to fight against late blight (i.e. induced systemic resistance, plant nutrition). The strain developed well, with numerous conidia (10^9 spores per gram of substrate) under PDA medium, which is a prerequisite for mass production (Köhl et al., 2011). Pursuing investigation on this strain under field conditions should also be done in order to establish its real potential for biological control over late blight during the cropping season.

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