Strategies to improve mycorrhizal inoculum quality for field application

Dissertation

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Declaration of Authorship

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Abstract

Agriculture represents the biggest interface between mankind and environment. It ensures the provision of food together with several other ecosystem services. However, modern agriculture, based on a wide use of chemical products, turned out to be not sustainable in the long run. Therefore, new production strategies are needed in order to maintain high crop production with a reduced use of chemical inputs. Among the strategies proposed, use of beneficial microorganism, like arbuscular mycorrhizal fungi, has been proved to be one of the most promising technics.

Arbuscular mycorrhizal fungi are well-known class of microorganism able to colonize the majority of plant species. These fungi provide a series of benefits to the host plant, such as: improvement of mineral nutrition and reduction of biotic and abiotic stress. For these reasons, implementation of their use in agriculture have been suggested as a valid strategy to reduce the need for chemicals in the field. However, their application in practical condition is still limited by a series of factors, among them, the high concentration of phosphate in soil deriving from fertilization. High concentration of this nutrient reduces the colonization capacity of the fungus and the functionality of the symbiosis. In order to exploit the benefits that arbuscular mycorrhizal fungi may provide to crop plants, the development of a newly formulated inocula, adapted to the environment present in the field, are needed and represent the subject of the present work.

One of the main challenges in facing the "phosphate problem" is the comprehension of its mode of action. Giving the obligate nature of the symbiosis, it is extremely complex to differentiate the effect of this nutrient on the two partners. Therefore, different strategies have been proposed and tested for the development of suitable inocula, one directed to the fungus and one to the plant. In the first approach, the ability of the fungi to adjust to hostile environment has been exploited. Along this line, the fungal strain of interest was acclimatised *in vitro* for several generations and tested in greenhouse for its ability to promote plant growth and to maintain normal levels of root colonization. The fifth generation of acclimatised strain was associated with positive effect on plant growth, and higher frequency of root infection at high phosphate availability. The comprehension of genetic pathways involved in acclimatisation have been investigated via formulation of different hypotheses, but gene expression analysis did not allow to confirm any of them. The acclimatisation process has been tested even *in vivo*, in commercial production system set-up, and tested in the field. Field application was not associated with plant growth promotion effects, even though the colonized strain was associated with higher colonization capacity in presence of phosphate fertilization.

The second strategy assumed that plant physiology is the main driver of mycorrhizal symbiosis. Here, use of inducer molecules, able to modulate plant metabolism, have been proposed and tested, *in vitro*, and *in vivo*, both in greenhouse test and in the field. Selected inducer molecules confirmed to act as expected in the activation of different plant metabolic responses. In greenhouse, these responses have been associated with modulation of colonization rate but not with growth promotion of plants. Field tests confirmed the absence of growth response and also showed the absence of mycorrhizal root colonization promotion.

The results indicated acclimatisation as a promising technique to cope with phosphate stress in practical conditions. However, the tests underline that phosphate cannot be considered as the only limiting factor occurring in the field that limit the possibility to exploit the symbiosis. Therefore, in order to develop suitable inocula, the attention has to be directed also to other elements of inhibition of the symbiosis in the field. Based on this consideration, strategies are proposed to develop new inocula for the future.

Abbreviation

ABA	Abscisic acid
AM	Arbuscular mycorrhizal
AM-	Non-acclimatised AM strain
AM+	Acclimatised AM strain
AOX	Alternative oxidase
Aze	Azelaic acid
BR	Brassinosteroid
COX	Cytochrome oxidase
CytC	Cytochrome C
ERM	Extra-radical mycelium
ET	Ethylene
G3P	Glycerol 3-phosphate
GA	Gibberellin
Glu	Glucose
GSE	Germinative spore exudate
IAA	Indol-3-acetic acid
IRM	Intraradical mycelium
ISR	Induced systemic resistance
JA	Jasmonic acid
NADPH ox.	NADPH oxydase
Pi	Phosphate
[Pi]	Phosphate molar concentration
Pip	Pipecolic acid
PR protein	Pathogenesis related protein
RDW	Root dry weight
RFW	Root fresh weight
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SDW	Shoot dry weight
SFW	Shoot fresh weight
TCA	Tricarboxylic acid cycle (Krebs cycle)
Xyl	Xylose

Table of contents

I. General introduction

Agriculture has been defined as the mother of all inventions (Rosenberg, 1990). Hunter-gatherers lifestyle could support 4 million of humans worldwide (Cohen, 1995), while agriculture laid the foundations for the development of mankind as we know it today, supporting more than 7 billion of people (www.fao.org/faostat/en). However, modern agriculture, is even responsible for several environmental issues that are highlighted below and in the coming years, strategies need to be implemented to make agriculture sustainable in long run.

Despite that the definition of "modern agriculture" is vague, it is possible to recognize its starting point during the so called "green revolution". Since the beginning of the XX century, mechanization and the first studies on plant nutrition helped global crop yield to increase. However, the application of the knowledge and diffusion of modern practices were patch-worked. It was only from the middle of the last century that the a series of practice, like: optimized irrigation, use of pesticides for disease controls, use of chemical fertilizers and development of new plant varieties permitted to increase steadily the crop production (Tilman et al., 2002) were adopted worldwide as standard. The positive effects of green revolution were impressive in terms of yield, for example, cereals production worldwide doubled the yield per hectare in less than 40 years (Tilman et al., 2002). However, the green revolution was based on large inputs with a long series of negative side effects. Today, agriculture is responsible for nearly 30% of greenhouse gases emission (Tubiello et al., 2014). Large area of the planet are affected by eutrophication problems due to nitrogen and phosphorus leaching, consequence of excess of fertilization in the field (Conley et al., 2009). Moreover, intensive agriculture, based on monoculture causes problems of deforestation, habitat fragmentation and biodiversity loss (Ramankutty & Foley, 1999), with negative impacting the resilience of ecosystems (Hooper et al., 2012).

The concern for sustainability of all human practices, included agriculture, reached authorities (Paris Agreement, COP 21, https://ec.europa.eu/clima/policies/international/negotiations/paris_en) and public opinion, as witnessed by the recent "global strike for future", started in Sweden and developed worldwide. Agriculture should urgently shift from "foe" of environment, to "friend", ensuring, in the same time, crop production and environment protection (Foley et al., 2011; Rockström et al., 2017). Wide range of sustainable practices have been proposed in the last decades as solutions to maintain or increase the production while reducing the inputs; and they share the same objective: the sustainable intensification of agriculture.

I.1 Sustainable intensification of agriculture

The sustainable intensification of agriculture is described as that series of practices that allow "yield increase without adverse environmental impacts and without the cultivation of more land" (Royal Society, 2009). Several action have been shown to be effective to reach this scope, both a scientific and practical level (Scoones, 2009). However, considering the differences in agriculture systems worldwide, in terms of climatic conditions, soil properties and agronomic management, it is wrong to consider that a single practice alone may allow the sustainable intensification of agriculture for the future. Rather a mix of farming systems, ranging from conservative agriculture to conventional may be adopted according to the various situations (Davis et al., 2012; Reganold & Wachter, 2016). Nevertheless, among the plethora of possible solutions that can be adopted, increasing attention have been direct to beneficial soil microorganisms. Soil represents one

of the richest ecosystems in terms of species, and to date, only a small fraction of its microbial biodiversity is known, due to the difficulties in isolate and cultivate the microorganism in controlled conditions. Only in recent years, scientists started to disclose soil microbial communities thanks to high throughput sequencing studies, that permitted identification of microorganisms without the necessity for cultivation (Thompson et al., 2017). Discovery and exploitation of new microorganisms will probably represent one of the corner stone of future cultivations. However, some of the beneficial microorganisms are known since long time for their positive effects on plants, and they have been already implemented in some agriculture practices. As examples, the bacterium *Bacillus thuringiensis* is widely used for insect control (Nexter et al., 2002), and the fungus *Trichoderma harzianum* is one of the most promising biological fungicide, active against many fungal species (Elad, 2000). The mode of action of the beneficial microorganisms is different, and not all of them are used for plant protection. Many of them, in fact, can help plant nutrition, like the nitrogen fixing bacteria (Burns & Hardy, 2012) or the phosphate (Pi) solubilizing bacteria, that make insoluble Pi available for plant nutrition (Khan et al., 2009). Other microorganisms have more general effects, like mycorrhizal fungi, that are known to be able to support plant nutrition, enhance plant resistance and tolerance against biotic and abiotic stresses, improve quality of plant products and increase soil quality (Rouphael et al., 2015).

I.2 Mycorrhizal fungi

Mycorrhiza is a Greek word formed by two components: *mýkēs* (fungus) and *rhiza* (root), and indicates a symbiotic relationship between a fungus and root of plants. Nowadays, the definition of mycorrhiza results quite vague, and many fungal species could be described as “mycorrhiza”. Originally, this term is referred to the fungi that were observed and firstly described by Albert Bernhard Frank in 1877 (Frank, 1877a, 1877b; Trappe, 2005), classified today as arbuscular mycorrhizal fungi, member of *Glomeromycotina* (Spatafora et al., 2016). Curiously, the interest for these fungi remained quite low for many decades and only in the second half of the last century in-depth study on this association took place. Today, the term mycorrhizal is referred to fungi which form particular structures during their interaction with the plant (Peterson & Massicotte, 2004). From this point of view, it is important to notice that fungi falling in the definition of “mycorrhiza” have in common the characteristic of forming peculiar and specific structures during the establishing of the symbiosis, while they differ among them for other aspects, like in the ecology, host range and phylogeny. These characteristics are also those that differentiate mycorrhizal fungi from endophytic fungi. In fact, although some authors assimilate mycorrhizal fungi with endophytes (which literally means being in the plant), endophytes are generally described as those organisms whose "infections are inconspicuous" (Stone et al., 2000). It is precisely this inconspicuous, or lack of easily recognizable structures, that differentiates mycorrhizae from other endophytic fungi. Based on these structures, mycorrhizal fungi are distinguished as ectomycorrhizal, ecto-endomycorrhizal and endomycorrhizal.

Ectomycorrhizal fungi are characterized by their mode of colonization, that never involves penetration of the host cell wall, and the interface between the host is limited to intercellular spaces. They produce a thick mycelial cover surrounding root tips, called mantle (Figure I.1). From the mantle, hyphae proliferate outwards, for foraging activities, and inwards, colonizing the host root as Hartig net (Smith and Read, 2008). These fungi belong mainly to the phyla *Basidiomycot*a and *Ascomycota*. They form the symbiosis with roughly 2% of plant species and almost all of them are arboreal, like the genera *Fagus*, *Pinus*, *Larix*, *Picea*, *Quercus* (Tedersoo et al., 2010).

Ecto-endomycorrhizal fungi share similarities with ectomycorrhizal fungi, with the difference that they penetrate host cell walls. This behaviour has been observed on plants of *Pinus* and *Larix* genera. These mycorrhizal species belong mainly to *Basidiomycota* phylum (Smith and Read, 2008).

Endomycorrhizal fungi are characterized by hyphal penetration of host cells. They do not form mantle and during the colonization process host cells are invaded both at symplastic and apoplastic level. These fungi form specific hyphal structures, like arbuscule or coils, inside the apoplast of host roots. These structures are the main site for nutrient exchange between the host and the guest and they are a clear sign of the successful symbiosis (Smith and Read, 2008). This mycorrhizal type is divided in three main groups: ericoid, orchid and arbuscular mycorrhizal.

Ericoid mycorrhizal fungi belong to *Ascomycota* phylum and form symbiosis with plants of *Ericaceae* family, usually growing in acid, wet and poor of nutrient soils (Cairney & Meharg, 2003). Anatomically, ericoid mycorrhizal fungi are characterized by the formation of hyphal coils developing inside host cells. These coils penetrate cell walls, but they never enter the cytoplasm. The contact zone between coils and cytoplasm represent the location where nutrients exchange take place (Smith and Read, 2008).

Orchid mycorrhiza involves all orchid species. In fact, the plants belonging to this family, have some phase of their life cycle were the nutrition depends completely on the fungus, especially during seed germination (McCormick et al., 2012). Orchid mycorrhizal fungi form specific coil structures called "pelotons", that represent the site where nutrient are transferred from fungus to plant (Smith and Read, 2008).

The last group of mycorrhizal are the arbuscular mycorrhizal (AM) fungi, they are the subject of this work, and they are be described in depth in the next section.

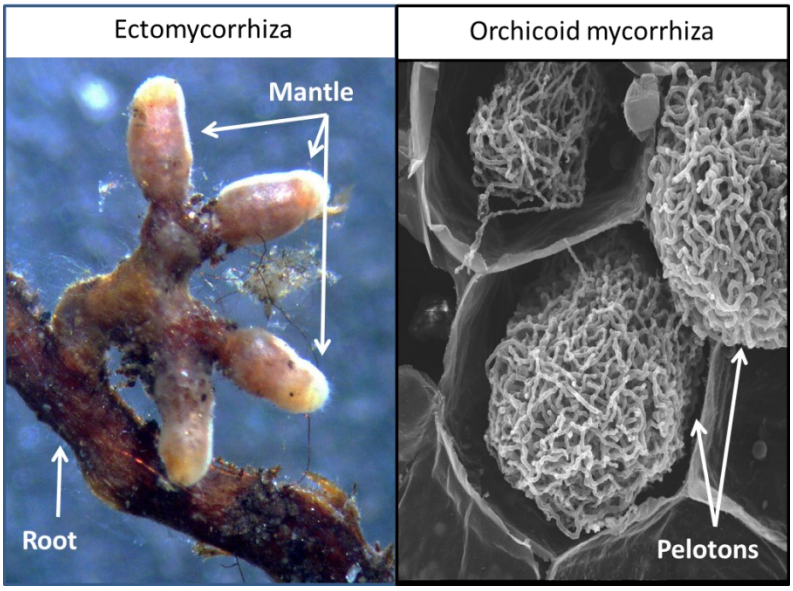

Figure I.1 Typical structures of ectomycorrhiza and orchid mycorrhiza. Pictures adapted from (https://public-media.smithsonianmag.com) and (Nedelin, 2014).

I.3 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi belong to the phylum *Glomeromycotina* among the Mucoromycota (Spatafora et al., 2016). AM fungi form the most widespread kind of mycorrhiza occurring worldwide (Smith and Read, 2008), in fact they form a mutualistic symbiosis with the majority of plant families (74%; van der Heijden et al., 2015). They are considered as key element of colonization of lands by plants in prehistoric time, and first fossils appeared together with the first land plants during the Ordovician, 460 million years ago (Heckman et al., 2001; Redecker et al., 2000; Remy et al., 1994; Simon et al., 1993). For these reasons, they are considered as one of the most important symbiosis in terrestrial ecosystems (Francis & Read, 1994). AM fungi are obligate biotrophs, and they cannot complete their life cycle without the host plants (Smith and Read, 2008). The name comes from the characteristic branched structure that they form within the cortical cells of roots, called arbuscule (Figure I.2; Smith and Read, 2008). It represents the primary site of nutrient exchange between the partners. AM fungi, provide mineral nutrients to plant in exchange for hexoses synthesized during photosynthesis (Helber et al., 2011; van der Heijden et al., 2015). The obligatory symbiotic nature of AM fungi, however, was shown to depend on lipids which seemed to be only available during the symbiotic phase of their life cycle (Bago et al., 2000; Trepanier et al., 2005). The plant origin was then assumed by the finding that AM fungi lack the enzymatic tools for *de novo* fatty acid synthesis (Tisserant et al., 2013; Wewer et al., 2014). That they were indeed derived from the plant could be confirmed by Luginbuehl et al. (2017) and Jiang et al. (2017). Beneficial effects of the symbiosis are, however, not only limited to the nutrient exchange. AM fungi, in fact, ensure enhanced water availability and use (Bitterlich, et al., 2018; Quiroga et al., 2019), and increase plant resistance against pathogens and tolerance to several abiotic stresses like salinity, cold, heat, drought and heavy metals (Aroca et al., 2007; Pozo et al., 2002; Smith and Read, 2008).

I.3.1 Life cycle and root colonization

Life cycle of AM fungi starts from a source of inoculum. It can be made by spores, colonized root fragments or mycelium. Particularly, spores are relatively big, they can reach 500 µm of diameter, and contain several nuclei, ranging from 800 to 35000 in the different fungal species (Hosny et al., 1998). Every spore contains huge numbers of lipid bodies and some carbohydrates delimited by a thick and strong wall containing chitin (Smith and Read, 2008). Thanks to the thick wall, spores maintain an high vitality and they can colonize new areas, thanks to wind transport and passing through gut system of soil arthropoda, annelida, birds and mammals (Allen, 1987; Reddell et al., 1997; Warner et al., 1987). Colonized root fragments are another common source of inoculum. The growth of hyphae from root fragments has been observed several times, even in this case, the presence of a thick wall around the hyphae allows the long term availability of the inoculum (Hepper & Jakobsen, 1983; Powell, 1976). Finally, the most important source of inoculum in natural environments with perennial vegetation, is represented by the existing mycelium from already colonized plants, that can colonize the new plants. Spores germinate in soil in presence of favourable conditions, in terms of pH, temperature, humidity, and mineral content (Clark, 1997; Daniels & Trappe, 1980; Green et al., 1976). When the asymbiotic germ tubes that developed from the spores recognize signals coming from the roots, they switch to presymbiosis and show intensive mycelium branching, and this phenomenon increases the chance for random contact between the partners (Giovannetti et al., 1993). Strigolactones have been identified as the essential signal in the root exudates acting on the respiratory pathway of the fungus (Akiyama et al., 2005; Besserer et al., 2006). At the same time, fungus communicates with the roots via production of short-chain chitin oligomers, that trigger calcium spiking response in plant (Genre et al., 2013). When the contact takes place, the fungus enters the symbiotic phase of its life cycle.

Hyphae adhere to the root via formation of hyphopodia and after 2-3 days, the fungus starts to penetrate the root epidermis (Brundrett et al., 1985; Cox & Sanders, 1974; Gianinazzi-Pearson et al., 1981; Giovannetti et al., 1993; Holley & Peterson, 1979; Kinden & Brown, 1975a, 1975b; Rosewarne et al., 1997).

During the symbiotic phase, colonization can follow two main morphological types: the Arum- or the Paris-type. Arum colonization is characterized by apoplastic invasion of cortex cells with development of highly branched structure called arbuscule in the host cells. Paris-type colonization, instead, follows a symplastic development in exodermal cell layers (Smith and Read, 2008). Development of one or another colonization pattern seems under genetic control of both host plant (Bedini et al., 2000; Brundrett & Kendrick, 1990a, 1990b; Gerdemann, 1965; Jacquelinet-Jeanmougin & Gianinazzi-Pearson, 1983) and fungus (Smith & Smith, 1997) even though some authors suggest that nutritional status of roots may play a role as well (Mercy et al., 2017). Arum-type development, moreover, is characterized by the presence of large cell-filling arbuscules, while in Paris-type development, the arbuscule are substitute by hyphal coils with small intercalated arbuscules. The plant plasma membrane surrounding the arbuscule is the periarbuscular membrane, and together with the membrane of the fungal arbuscule and the matrix between the two membranes, it represents the interface where the majority of exchanges between the symbionts take place. Many studies highlights the presence of several nutrient transporters, aquaporins and enzymatic activities at this site (Aroca et al., 2007; Gianinazzi-Pearson et al., 2000; Harrison, 2002; Porcel et al., 2006; Rausch et al., 2001). In contrast to hyphae, arbuscules have generally a short life, in fact, they need 2-3 days to be formed, and they are active for 4-5 days before collapsing (Brundrett et al., 1985). Several AM fungal species develop vesicles in intercellular space of roots (Abbott and Robson, 1982). Vesicles contain lipids and nuclei, they have different shapes, a thick wall and many nuclei representing the most important storage organ of AM fungi (Smith and Read, 2008).

Once that the fungus is established in the plant, hyphae start to grow outside the roots forming the extra-radical mycelium. The high branch number and the tiny diameter of the mycelium significantly increase the volume that can be explored in the soil for the nutrients and water uptake; and especially in the case of non-mobile nutrient, like P, the presence of the mycelium allows the plant to overcome the depletion zone usually formed around the root system (Smith and Read, 2008). The extra-radical mycelium from one colonized plant can fuse itself with mycelia of AM fungi colonizing other plants. The junction point, where the mycelia of different fungi meet each other, is called anastomosis. Anastomosis result in cytoplasmic continuum and nuclei migration between different AM fungi (Giovannetti et al., 2004; Giovannetti et al., 1999) and exchange of nutrients among different plants via the AM fungi, in a system that was described as wood wide web (Simard et al., 1997) and nowadays as common mycorrhizal networks (Weremijewicz et al., 2018). Furthermore, extra-radical mycelium secrete a protein called glomalin that improves soil aggregation, water holding capacity with consequently decreased soil erosion (Bedini et al., 2009; Rillig, 2004; Rillig & Steinberg, 2002).

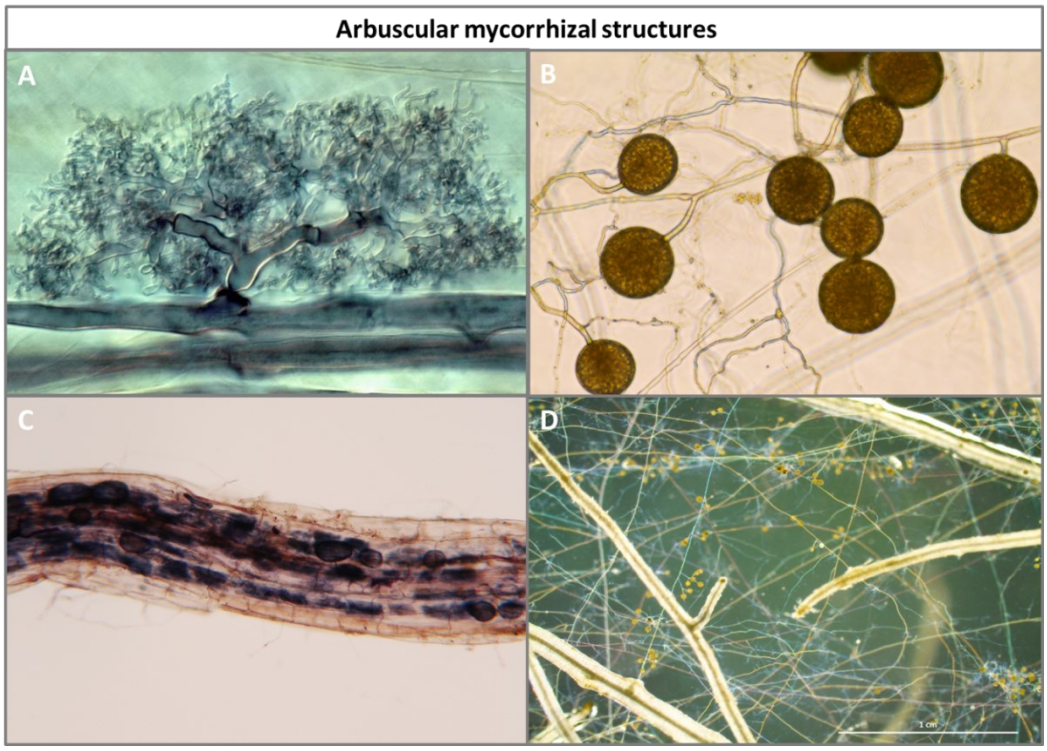

Figure I.2 Arbuscular mycorrhizal structures. (A) Arbuscule (B) spores (C) colonized roots, with vesicules and arbuscule after staining (D) hyphal network *in vitro*, with transformed carrot roots. Photo (A) from https://namyco.org/mycorrhizae_explained.php. Photo B,C,D taken by the author.

I.3.2 Genetic organization

The genetic organization of AM fungi is not well understood, despite several steps forward have been made in the last years. AM fungal hyphae harbour thousands of nuclei that can move freely within the cytoplasm of the coenocytic mycelium (Giovannetti et al., 1999). There are no observations reporting the presence of mono- or di-nucleated stages in these organisms, while the smallest propagating units, represented by the spore, contain hundreds of nuclei (Sanders & Croll, 2010). The absence of observed sexual stages induced many researchers to see AM fungi as an "evolutionary scandal" (Judson & Normark, 1996), considering them as ancient clones, unable to delete deleterious mutation via sexual reproduction (Rosendahl, 2008; Sanders, 1999; Sanders, 2002). It has been proposed that, in absence of sexual stages, the presence of many nuclei in the same spore or hyphae can complement each other via coexisting functional alleles (Sanders & Croll, 2010). This hypothesis implies that hyphae are heterokaryotic, harbouring genetically different nuclei. However, this unique system seems not to be stable in long term and this suggest that cryptic sexual processes may occur to offset the incremental addition of deleterious alleles (Corradi & Brachmann, 2017). Heterokaryotic nature of AM fungi was considered true during the first decade of the century, and it was confirmed by fluorescent *in situ* hybridization showing that rDNA variants locate on different coexisting nuclei (Kuhn et al., 2001). Other clues suggesting the heterokaryotic hypothesis came from genes analysis showing high divergence and variances within the same isolate (Kuhn et al., 2001; Sanders, 2002; 1999). Nevertheless, in the last decade, with the development of high-throughput sequencing techniques, the heterokaryotic theory has been confuted. In fact, the genome size was too small to be heterokaryotic (Bianciotto and Bonfante, 1992; Hijri and Sanders, 2005; Hosny et al., 1998) and further studies showed that AM fungal hyphae contain low level of diversity, comparable to other not-heterokaryotic fungi (Martin et al., 2010; Martin et al., 2008; Payen et al., 2015). However, recent data suggest that the two hypothesis of homokaryosis and heterokaryosis may both be correct (Corradi & Brachmann, 2017). In fact, it

was shown that some isolates of *Rhizophagus irregulare* were slightly heterokaryotic, while others were not (Ropars et al., 2016) showing that AM fungal genetics can be either homokaryotic-like, made up nuclei with one dominant genotype; or dikaryote-like harbouring nuclei of two dominant genotypes at similar frequencies (Corradi & Brachmann, 2017). Interestingly, AM fungi own in their genome the set of meiosis-related genes (Halary et al., 2011; Tang et al., 2016), and it is possible to speculate that the observed dikaryote-like isolates represent the initial stage of sexual events in AM fungi, originated from anastomosis between compatible strains (Corradi & Brachmann, 2017).

I.3.3 AM fungi as biostimulants in agriculture.

Despite the genetic basis of these fungi being still not clear (Kokkoris et al., 2020; Yildirir et al., 2020), the numerous reports of beneficial effects induced by AM fungi stimulated the interest for their use in agriculture as biostimulants (Berruti et al., 2016; Rouphael et al., 2015; Vosátka et al., 2008). Coupled with the need to find strategies to maintain the crop productivity while reducing the use of chemicals promoted by politics (European Directive 2009/128/EC), the last years created a positive scenario for the implementation and use of beneficial microorganisms like AM fungi for crop production.

The use of AM fungi in agriculture as biofertilizer has increased in the last decade, due to the several benefits that they can provide to plants (Berruti et al., 2016). AM fungi have been shown to be important in plant nutrition, enhancing the uptake of low-mobile phosphate (Pi) ions in soil (Fitter et al., 2011; Tinker and Nye, 2000), but even for nitrogen (Gomez et al., 2009; Guether et al., 2009; Kobae et al., 2010; Koegel et al., 2013), sulfur (Allen & Shachar-Hill, 2009; Sieh et al., 2013) and micronutrients, like: copper, iron, manganese and zinc (reviewed by Lehmann et al., 2014; Lehmann and Rillig, 2015). Improved uptake of these elements has been associated with positive effects in terms of yield increase (Berruti et al., 2016; Hijri, 2016; Wall et al., 2013) and quality parameters of crops, like: higher content of important secondary metabolites with nutraceutical potential (Giovannetti et al., 2012; Larose et al., 2002; Sbrana et al., 2014; Schliemann et al., 2008). AM fungi play a role in the protection of plants from different kind of pathogens, for example, they reduce the negative effect of nematodes (Anene et al. 2013; Koffi et al. 2013; Li et al. 2006; Peña et al. 2006), and they provide protection against several soil-borne disease caused by fungi, like: *Rhizoctonia*, *Fusarium* and *Verticillium*, or by oomycetes such as: *Phytophthora*, *Pythium* and *Aphanomyces* (Reviewed by Whipps, 2004). AM fungi, furthermore, can strengthen the defence system of shoots, via activation of defence mechanisms similar to induced systemic resistance (ISR, Pozo et al., 2002). Finally, AM fungi are well known to be able to alleviate almost all the abiotic stress occurring in the field, like: drought, heat, salinity and presence of heavy metals (Al-Karaki, 2006; Aroca et al., 2007; Bui and Franken, 2018; Pozo et al., 2002; Sharifi et al., 2007; Smith and Read, 2008). For this reason, they have been described as “health insurance” for plants (Gianinazzi & Gianinazzi-Pearson, 1988). Recently, two reviews on the use of AM fungi in field have been published, and they confirmed that, generally, AM fungal inoculation results in beneficial effect on plants growth (Berruti et al., 2016; Bitterlich et al., 2020; Hijri, 2016), and inoculation can be even economically profitable, in comparison to conventional fertilization (Baar, 2010). However, as for any other biological agent implemented in agriculture, it has to be noted that the positive effects depends on: (i) AM fungal isolate; (ii) plant genotype; (iii) environmental conditions and (iv) agronomic management of field (Azcón-Aguilar and Bare,a 1997; Pozo and Azcón-Aguilar, 2007; Whipps, 2004). Many agriculture practices occurring in field are known to be detrimental to AM fungi, deleting the positive effects associated to their inoculation (Gosling et al., 2006). Use of biocides for pathogens control, for example, may have negative effect on AM fungi too (Carrenho et al., 2000; Li et al., 2010; Miller and Jackson, 1998). Cropping with non-host plant may be detrimental (Njeru et al., 2015), as

well as period of bare fallows and deep ploughing of the soil (Avio et al., 2013; Jansa et al., 2002; Njeru et al., 2015). However, one of the most important factor impairing AM fungal symbiosis is the fertilizer application, especially with respect of Pi (Avio et al., 2013; Daniell et al., 2001; Lehmann et al., 2014), often occurring in crops for ensuring high yield.

I.4 The problem of phosphate.

Phosphorus (P) is one of the most important macronutrient for plant growth, it represent 0.2 % of total plant dry weight, playing fundamental role in major cellular processes like: photosynthesis, respiration, nucleic acid synthesis, energy storage and transfer and many other vital processes (Marschner, 2011; Schachtman et al., 1998). In soil, P can be generally abundant, but only a small portion is available for plant nutrition, due to several immobilization events involving fixation and precipitation (Fitter et al., 2011; Tinker and Nye, 2000). Plants assimilate P in form of orthophosphate (Pi; Sharpley et al., 2000; Sharpley and Rekolainen, 1997). The assimilation can follow two paths, the first one is the so-called direct pathway, that is the uptake of Pi from the soil solution by roots. The second one involves AM fungi, and their extra-radical hyphae that actively take up Pi from soil areas beyond root depletion zones.

Pi absorption represents one of the major limiting factors in plant growth in the field and application of Pi in soil as fertilizer has been shown to reliability promote plant growth and yield increase (Eltelib et al., 2006; Wang and Li, 2004). However, Pi application is associated with several problems. As Pi is a non-renewable resource, deposits worldwide are limited and the peak of extraction is likely to occur in the next decades (reviewed by Cordell and White, 2011). The excessive use of Pi fertilizer causes eutrophication problems by contaminating surface water (Conley et al., 2009). Moreover, phosphate rock, from which Pi is obtained, are often contaminated with low amount of heavy metals like cadmium, uranium and lead. Years of application in the fields, results in accumulation of these harmful elements in soil, leading to potential problems for plants, humans and animals (López Carnelo et al., 1997; Mendes et al., 2006). Finally, high soil concentration of available Pi, due to high application of fertilizer, inhibit the indirect Pi uptake pathway and concomitantly the mycorrhizal symbiosis (Tóth et al., 2014). In fact, while the cause of AM fungal symbiosis inhibition is not fully understood, it is known that Pi reduces spore germination and mycelium development (Olsson et al., 2002), reduces the colonization of roots (Balzergue et al., 2011; Breuillin et al., 2010; Thomson et al., 1986) and symbiosis functionality. In other words, Pi impairs the ability of the fungus to provide benefits to its host (Smith & Smith, 2011).

I.5 Strategies to overcome Pi inhibition.

AM fungi represent a potential element of sustainable intensification of agriculture. Their application in field, however, can be limited by a series of constraints. One of the most important, as described in the previous paragraph, is represented by elevated [Pi] caused by fertilization. Despite Pi inhibition of AM fungi being known for a long time, the causes are not known, and the obligate nature of the relationship between plant and fungus makes the understanding of the process complex. The mode of action of Pi can be: (i) direct, therefore acting on the fungus, limiting its developing capacity, resulting in symbiosis failure; or (ii) indirect, that is affecting plant physiology, which, in consequence limits the development of the fungus in the roots. In this work, we explored both scenarios, testing strategies to overcome Pi inhibition with the aim to obtain performing inocula for practical use.

I.5.1 Acclimatisation as tool for improving AM fungal inoculum

The obligate nature of the symbiosis makes it impossible to study the fungus without its host, thus making the understanding of the phenomena rather challenging. However, if the action of phosphorus acts directly on the fungus, it could be considered as an abiotic stress element for mycorrhiza. In this scenario, AM fungi has shown several times the ability to acclimatize themselves to hostile environments (Sochacki et al., 2013) and different sources of stress like: salinity (Sharifi et al., 2007), heavy metals (Bui & Franken, 2018) or freezing (Addy et al., 1998). Acclimatisation is the process that involves changes in the content of protein, catalysts and/or enzymes as a consequence of the activation of genes that was previously silent or inactivated (Giordano, 2013; Raven & Geider, 2003), caused by a shift in the environmental conditions. In general, acclimatisation occurs with the establishment of a new equilibrium inside the cells, bringing to a new homeostatic condition in presence of the variable object of the acclimatisation (Cram, 1976; Giordano, 2013). Acclimatisation is distinguished by other processes like regulation and adaptation. In fact, regulation is described as post-translational modification occurring to enzymes or proteins via e.g. phosphorylation–dephosphorylation or other similar process (Giordano, 2013). These changes occur in very short time, ranging from seconds to minutes (Raven & Geider, 2003). Adaptation, instead, is a quite long process and requires several generations (Lohbeck et al. 2012; Collins and Bell 2004). It involves changes in the genome, with modification occurring to proteins that are expressed and in which conditions they are expressed (Giordano, 2013; Raven & Geider, 2003).

No observations on Pi acclimatisation have been reported in literature until now. However, given the importance of this element for crop development, and the effects on AM fungi, it is of primary importance to test whether AM fungi can be acclimatised to high Pi condition, allowing a wide use of AM inocula in field, ensuring the set of benefits to crops associated with these fungi. Despite being different, Pi shows similarities with Zn that plays the double role of essential nutrient (Zenk 1996; Vamerali et al., 2010) and potential toxic element, at least for the fungus (Sharma et al., 2007; Malviya and Chaudhary, 2006). However, Pi acclimatisation shows unique characteristics, therefore the understanding of possible mode of action requires specific sets of research questions to be answered, and these will be explored in this work.

I.5.2 Regulator as modulator of AM fungal inoculum performance

In the previous paragraph, the scenario was described in which Pi inhibition acts by targeting the AM fungal site of the symbiosis. However, other studies have highlighted that Pi could act indirectly inducing metabolic modifications in plants, resulting in a hostile root cell environment for AM fungi. Information available in literature suggests that the AM fungi behaviour within roots is driven by the metabolic interplay initially set in the plant. This interplay involves the regulation of plant hormones, plant energetic pathways, plant defence and AM development and growth. The following paragraph is an excerpt from a review including three figures published by Bedini et al. (2018).

Hormone signalling is tightly linked with the defence pathway activation *in planta* (Bonneau et al., 2013). Contact with pathogens, beneficial microorganisms, natural and synthetic compounds or the presence of abiotic stress triggers at physiological, transcriptional, metabolic and epigenetic levels an unique plant state called “priming,” resulting in the establishment of induced defence mechanisms (Conrath et al., 2006; Mauch-Mani et al., 2017). Usually, but non-exclusively, two main antagonistic induced responses are engaged in plants, depending on the priming signal (named elicitor): systemic acquired resistance (SAR) and induced systemic resistance (ISR). The SAR response is induced by biotrophic pathogens (Thakur & Sohal,

2013; Ton et al., 2009) and involves SA accumulation, which mediates the activation of pathogenesis-related (PR) genes (Durrant & Dong, 2004). PR proteins are known especially for their antifungal activity based mainly on the hydrolytic capacity toward fungal cell wall components (Edreva, 2005). The ISR response, instead, is induced by necrotrophs or plant growth-promoting rhizobacteria (PGPRs) and involves JA and ET signalling without modification of defence gene expression (Pieterse et al., 1996; Pieterse et al., 2002). Specifically, ISR is based more on enhanced sensitivity to these plant hormones rather than to an increase in their production (De Vleesschauwer et al., 2006; Pieterse et al., 1998; Pieterse & Van Loon, 2004). Finally, many studies showed that almost all the plant hormones could participate to different extent in induced plant resistance (Pieterse et al., 2012). For example, additionally to abiotic stresses, ABA has a role in plant pathogen interactions (Cao et al., 2011; Fan et al., 2009). Emerging evidences state importance of ABA in plant defence system, with suppression of SAR induction and involvement in SA-SAR-mediated signalling (Kusajima et al., 2010; Yasuda et al., 2008) but its potential role in ISR establishment is less clear as it can also counteract JA/ET defence related pathways (Cao et al., 2011).

Although the knowledge of plant pathogen interactions made important progress in the last years, classification of many important hormones involved as part of either the ISR or SAR system remains incomplete (Pieterse et al., 2012). Moreover, interactions between plants and beneficial microorganisms partially exploit the same defence related pathways. Firstly, as shown by Güimil et al., (2005), there is a 40% overlap between genes responding to AM fungi and pathogen agents in rice. Although these responses are temporally and spatially limited in mycorrhizal symbiosis compared to phytopathosystems, this suggests that the plant defence system may play a role in the establishment and control of the endomycorrhizal symbiosis (Dumas-Gaudot et al., 1996; García-Garrido & Ocampo, 2002). Secondly, several authors suggested that AM fungi implement ISR in the plant, during the first colonization stages (Hause et al., 2007; Hause & Fester, 2005; Kapoor et al., 2008; Pieterse et al., 2014; Pozo et al., 2002; Pozo & Azcón-Aguilar, 2007) but also that PGPRs, known to elicit ISR, can increase the mycorrhizal development (Alizadeh et al., 2013). By contrast, the SAR system seems to generate a non-favourable metabolic context for AM fungi, since the use of SAR elicitors can lead to inhibition of mycorrhizal development (de Román et al., 2011; Faessel et al., 2010) sharing therefore similarities with biotrophic pathogens (Delaney et al., 1994).

According to the proposed model (Figure I.4, Figure I.5), AM fungi development *in planta* seems to be promoted by the occurrence of ISR and its related signalling, prior to AM fungal contact. The induction of the ISR or SAR system can be primed by application of specific elicitors for one or the other system. The use of specific molecules able to generate a favourable metabolic context to promote an effective colonization can therefore be proposed to master mycorrhizal inoculum applications under practical field condition. In this view and among those stimulatory molecules, potential affordable strategies exist from the application at low doses (seen as signal) of oligosaccharides on plants. Interestingly, oligosaccharides were shown since some decades to act as elicitors and therefore implement specific plant defence responses against biotic but also abiotic stress (Trouvelot et al., 2014). Oligosaccharides possess several advantages, such as being cheap and available, non-toxic, biodegradable, easy to use and not classified as phytohormones (whose field application is highly restricted in Europe). Linking plant respiration and plant priming, the idea consists to induce a specific transient plant stress, by targeting the alternative oxidase (AOX) pathway and its related metabolism, as it was shown to play a crucial role in arbuscule formation and positive mycorrhizal response (Mercy et al., 2017). Sugar signalling can promote AOX pathway directly (Li et al., 2006) or indirectly via the ABA signalling. In this last case, sugar recognition by the hexokinase 1 (Ramon et al., 2008), present on the outer mitochondrial membrane, initiates ABA synthesis (Cheng et al., 2002) and then stimulates the AOX gene expression *via* transcription factors (Finkelstein et al., 1998; Giraud et al., 2009; Millar et al., 2011; Rook et al., 2006). Although this signalling scheme (Figure I.3) remains hypothetical, first trials using

application (soil or on leaves) of low dose of oligosaccharides (such as glucose, fructose, and xylose) show possibilities to improve mycorrhizal development and responses under various [Pi] and in several plant and AM fungal species (Lucic & Mercy, 2016; Mercy et al., 2017) Since the same compounds were termed initially as elicitors, related to the implementation of plant defence upon pathosystems but can also promote mycorrhizal performances, the term "inducer", has been used in this work. This term which defines signalling molecules that are intended to act specifically as stimulants in endomycorrhizal systems.

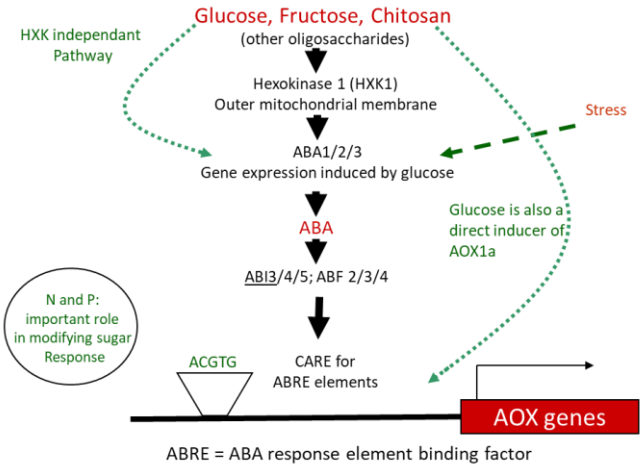

Figure I.3 Theoretical scheme of the oligosaccharides signalling on alternative oxidase pathway, via ABA-dependant and independent regulation. From (Bedini et al., 2018)**.**

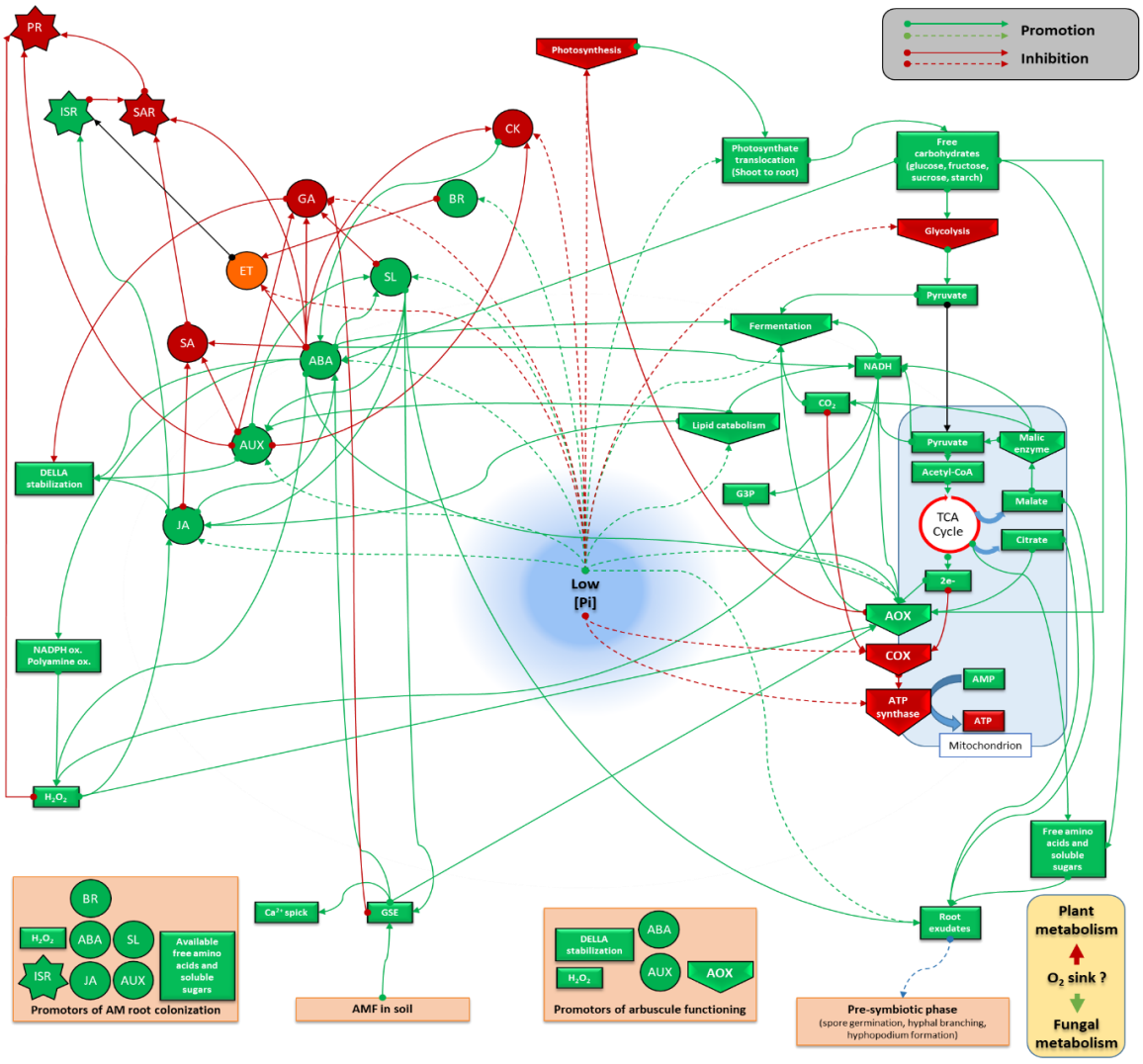

Figure I.4 Plant metabolic orientation of hormone interplay, carbon partitioning and responses on mycorrhizal development under low available P. Box and arrow colour indicate repression (red), or promotion (green). Orange boxes and black arrows are used for uncertain conditions. Based on the literature survey, mycorrhizal colonization is enhanced under low available P which goes together with the action of AM-promoting hormones (such as ABA, SL, and JA). This hormonal interplay is connected to a favourable metabolic frame which involves lower photosynthetic activity, higher translocation of photosynthates from shoots to roots, accumulation of sugars (reduced glycolysis flow and enhanced lipid oxidation), enhanced plant fermentation activity, cytosolic reductive potential (elevated NADH pool), electron partitioning, which is orientated toward the alternative oxidase pathway, reduced ATP formation and ISR implementation. Root exudation of several sugars, amino acids, some carboxylic acids and hormones (such as SL) participate to the molecular dialog with mycorrhizal fungi present in the rhizosphere. This can support physical contact with the root by stimulating hyphal branching and to induce plant responses by promoting Myc factor release from germinative spore exudates. It is questioned if this metabolic flux is accompanied by lower oxygen consumption by plant cells, which may become more available for the fungus (as aerobic organism) under low P. ABA, abscisic acid; JA, jasmonate; GA, gibberellins; SA, salicylic acid; SL, strigolactones; ET, ethylene; CK, cytokinins; IAA, auxins; BR, brassinosteroids; PR, pathogenesis related protein; ISR, induced systemic response; SAR, systemic acquired resistance; AOX, alternative oxidase; COX, cytochrome oxidase; CytC, cytochrome C; TCA, Krebs cycle; NADPH ox., NADPH oxidase; polyamine ox., polyamine oxidase; GSE, germinative spore exudate; G3P, Glycerol 3-phosphate. From (Bedini et al., 2018).

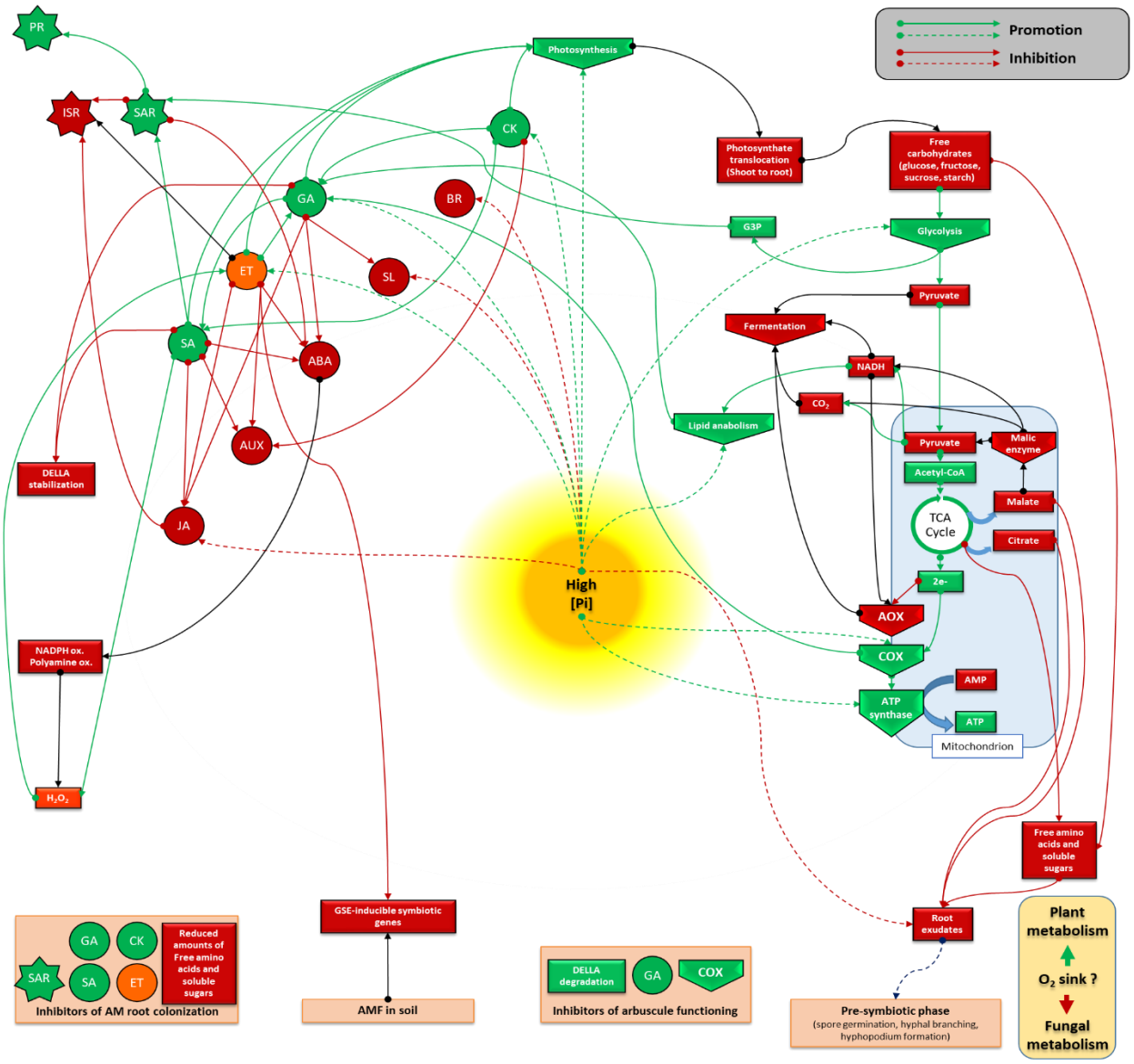

Figure I.5 Plant metabolic orientation of hormone interplay, carbon partitioning and responses on mycorrhizal development under high available P. Box and arrow colour indicate repression (red), or promotion (green). Orange boxes and black arrows are used for uncertain conditions. Based on the literature survey, high available P affects mycorrhizal performances mainly due to the activity of mycorrhiza-inhibiting hormones (such as GA, SA, and ET), and in which SAR is potentialized. This goes together with a non-favourable metabolic frame which seems connected to an enhanced photosynthesis activity, lower translocation of photosynthates from shoots to roots, a continuous flow of sugars processed via glycolysis, lipogenesis, and TCA, but also reduced fermentation activity and higher oxidative potential (reduced $NADH^+H^+$ cytosolic pool). In this system, lower free amounts of compounds (sugars, amino acids, SL, and carboxylate acids) are released in the root exudate, thus reducing possible molecular dialog between AM fungi and plant root. In addition, high P favours electron partitioning toward the plant COX pathway, thus participating to ATP formation. It is questioned if this metabolic flux is accompanied by higher oxygen consumption by plant cells, which may become less available for the fungus (as aerobic organism) under high P. ABA, abscisic acid; JA, jasmonate; GA, gibberellins; SA, salicylic acid; SL, strigolactones; ET, ethylene; CK, cytokinins; IAA, auxins; BR, brassinosteroids; PR, pathogenesis related protein; ISR, induced systemic response; SAR, systemic acquired resistance; AOX, alternative oxidase; COX, cytochrome oxidase; CytC, cytochrome C; TCA, Krebs cycle; NADPH ox., NADPH oxidase; polyamine ox., polyamine oxidase; GSE, germinative spore exudate; G3P, Glycerol 3-phosphate. From (Bedini et al., 2018).

I.6 Aim of the thesis: strategies to improve AM fungal inocula for high [Pi]

AM fungi represent a potential element of sustainable intensification of agriculture. Their application in field, however, can be limited by a series of constraints. One of the most important is represented by elevated [Pi] caused by fertilization. The aim of the thesis work was to propose and test two strategies to develop improved AM fungal inocula for field application, with stronger tolerance for high [Pi] in soil. The main idea behind this work states that: "an inoculum able to generate successful mycorrhizal symbiosis in presence of inhibiting [Pi], can help the plants to grow and better face environmental stress, reducing therefore the use of chemicals". In frame of the experimental work for the current thesis, two main hypotheses deduced in the previous paragraphs, were tested:

1. Acclimatisation as tool for improving AM fungal inoculum.
2. Regulator as modulator of AM fungal inoculum performance.

These two hypotheses were divided into a series of secondary hypotheses which are described in the following. Experiments have been performed with the model fungus *Rhizoglomus irregulare*, which genome has been recently sequenced, using the INOQ strain QS81. The model plant for the experiments was *Solanum tuberosum* cv. Jasia. It was chosen due to its importance in agriculture, especially in Germany, where experiments were performed. For the *in vitro* tests, *R. irregulare* was associated with *Daucus carota* root organ cultures.

I.6.1 Approaches for testing the "acclimatisation" hypothesis

As first step, the asymbiotic and the presymbiotic phases have been investigated, concerning the relationship between spore germination, mycelium development and Pi. Action of Pi inhibition on AM fungi is not fully understood, it can act directly on spores, reducing their ability to produce and develop a branched mycelium or, indirectly, acting on plant signal perception, required for stimulating the switch from asymbiotic to presymbiotic growth. Therefore, in frame of the two phases, the following hypotheses were tested:

(i) Acclimatisation is based on different response of acclimatised propagules to a direct Pi inhibition. Therefore, an acclimatised fungus, in presence of inhibiting [Pi], shows higher spore germination rate and improved mycelium development (length and branching) compared to a non-acclimatised fungus.

(ii) Acclimatisation is based on better response of acclimatised propagules to signals coming from plant roots in presence of inhibiting [Pi]. An acclimatised fungus, in presence of inhibiting [Pi], shows improved response to strigolactone as essential root signal, linked with improved presymbiotic mycelium development (growth and branching) compared to a non-acclimatised fungus.

The second step involved the investigation of the symbiotic phase in root organ culture (ROC) *in vitro* system. Therefore, acclimatised and non-acclimatised fungal strains have been investigated for their extra-radical mycelium development patterns. The tested hypothesis was the following:

(iii) An acclimatised strain shows higher sporulation and hyphal density compared to a non-acclimatised strain in presence of inhibiting [Pi].

The investigations of the result of acclimatisation continued from *in vitro* to *in vivo*, and spores from an acclimatised and a non-acclimatised strain were used as inoculum for plants grown in greenhouse. The hypothesis leading the experiment was the following:

(iv) Acclimatised fungi are better able to perform in terms of root colonization and plant growth promotion in presence of high [Pi] in greenhouse growing conditions.

The positive effects of an acclimatised inoculum in terms of root colonization and plant growth promotion observed in greenhouse, stimulated more questions, especially on which process the observed phenomena are based on. Therefore, gene expression analyses were performed to unveil the molecular pathways involved in the acclimatisation. Four hypotheses, based on previous studies on [Pi] and AM fungi, were tested:

(v) During the symbiotic phase, high levels of Pi inhibit the expression of genes related to cell cycle, among them: DNA polymerase delta subunit 4 (*RiDPD4*), proliferating cell nuclear antigen (*RiPCNA*), ribonucleutide reductase (*RiRNR*), myosin II heavy chain (*RiMhC*) and Major facilitator superfamily (*RiMFS*) (Sugimura & Saito, 2017). We hypothesise that an acclimatised strain shows normal expression of these genes at high-Pi conditions, comparable to a non-acclimatised fungus at low-Pi conditions.

(vi) Acclimatisation of *R. irregulare* for Zn is based on different expression of oxidative stress-related genes: superoxide dismutase (*RiSOD*) and glutathione S-transferase (*RiGST*) (Bui & Franken, 2018). Here, we tested the hypothesis that Pi and Zn acclimatisation shows similarity and therefore Pi acclimatisation is reflected by different regulation of *RiGST* and *RiSOD*.

(vii) Different respiration pathways can be at the basis of adaptation to specific conditions, like high [Pi]. Therefore, we tested the impact of acclimatisation on genes involved in fungal respiration pathways, specifically, encoding the alternative oxidase (*RiAOX*) and cytochrome oxidase (*RiCOX*) (Mercy et al., 2017). The hypothesis states that acclimatisation is accompanied by modulation of respiration related genes *RiAOX* and *RiCOX* in the acclimatised strain in presence of high [Pi].

(viii) Inoculation with acclimatised strain, in presence of high [Pi], resulted in higher plant biomass and growth. The hypothesis states that the improved growth is due to a more active symbiosis (in terms of exchange between partners), reflected by activation of the sugar transporter gene *RiMST2*, and a higher expression of the phosphate transporter genes *RiPT1, RiPT3, RiPT5, RiPT6, RiPT7* (Fiorilli et al., 2013; Helber et al., 2011; Mercy et al., 2017).

As the last step, it was tested whether the acclimatisation process can be implemented even in classical mass production set-up. Therefore, the inoculum was produced in presence of increased [Pi], in mass production system, in greenhouse. The inoculum was then used for inoculation of potato plant in “half field condition”, to test the hypothesis:

(ix) Acclimatised inoculum is able to better perform in terms of plant growth and root colonization in presence of high [Pi] in the field.

I.6.2 Approaches for testing the "regulation" hypothesis

Four candidates were selected as regulators, which are supposed mycorrhiza regulators: D-glucose (Glu) and D-xylose (Xyl), known to stimulate ISR responses (Ramon et al., 2008; Rolland et al., 2006; Trouvelot et al., 2014) and the supposed mycorrhiza inhibitors: pipecolic acid (Pip) and azelaic acid (Aze) known to stimulate SAR response (Aranega-Bou et al., 2014; Navarova et al., 2012; Jung et al., 2009; Shah, 2009).

Validation of this hypothesis started from *in vitro* studies of compatibility between the selected regulators and our strain. Specifically:

(i) Selected mycorrhiza regulators are compatible with AM fungi in terms of spore germination and presymbiotic mycelium development.

Once the compatibility was defined at the selected concentrations, the regulators have been tested in greenhouse with different application methods and in presence or not of high [Pi]. The experiment was performed to test the hypothesis:

(ii) Selected regulators are able to modulate AM symbiosis.

The best molecules in terms of phenotypic colonization response were analysed at transcript levels in order to test the hypothesis:

(iii) The regulators activate the expected defence pathways.

As last, the selected regulators, where applied in the field with the aim to test the main hypothesis.

(iv) The physiological state of the plant determines the outcome of the AM symbiosis. Therefore, regulators, which are able to modulate the plant hormone interplay, can be used to promote or inhibit AM symbiotic functioning.

The genes selected for hypothesis (iii) were selected from previous studies on activation of defence pathways in potato were they have been shown to be regulated by the SA-pathway (*StPR-1*, *StPR-3*, and *StGST*) (Genzel et al., 2018) or by the JA-pathway (*StPR-2*, *StPR-6*; Genzel et al., 2018; Lehtonen et al., 2008).

II. Material and Methods

II.1 *In vitro* experiments

R. irregulare was maintained in root organ culture (ROC) system with *Agrobacterium rhizogenes*-transformed carrot roots (*Daucus carota* L.) according to (Bécard & Fortin, 1988), in bi-compartment Petri dishes (Ø 9 cm), added or not with sugars and vitamins, according to (St-Arnaud et al., 1996). Briefly, the proximal compartment of Petri dishes was filled with Modified Strullu and Romand (MSR) medium and was used for ROC growth. The distal compartment was filled with medium deprived of sucrose and vitamins (Figure II.1). Absence of sucrose prevents root growth, stimulating at the same time spore formation. A fragment of three-weeks-old, transformed carrot roots, containing at least a couple of secondary roots, were placed in proximal compartment. After three weeks of growth in dark at 26 ºC, roots were inoculated with a gel plug (approx. 0.5 cm x 0.5 cm) from the distal compartment of fully colonized Petri dishes containing roughly 500 mature spores. After inoculation, cultures were placed in inverted position, in the dark, for twelve weeks at 26 ºC. MSR medium was prepared as described in (Declerck et al., 1998) and solidified using Gelrite™ (Duchefa, Haarlem, The Netherlands) at concentration of 3 g/L. The value of pH was set at 5.6 before autoclaving. The chemicals for the medium were supplied by Carl Roth GmbH, Karlsruhe, Germany. Stock solution of KH_2PO_4 (Carl Roth GmbH) 1 M was prepared and the pH was adjusted to 5.6. The required amount of solution was added to MSR-medium after autoclaving via filter sterilization (pore size 0.22 μm) according to the experimental setup.

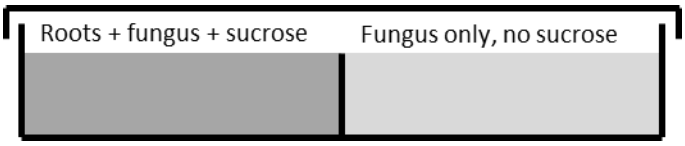

Figure II.1 Scheme of ROC *in vitro* propagation of AM fungi in 9 cm Petri plates. Figure modified from St-Arnaud et al., 1996.

II.1.1 Acclimatisation process

In order to stimulate the acclimatisation of the fungus to Pi, 3.23 mM of KH_2PO_4 were added to the MSR medium in both compartments. Spores, generated during the cultivation period, were used to inoculate new Petri dishes containing fresh ROC. The process was repeated five times and the sub-strains obtained from the first and last cultures were named “acclimatised” (AM+ with additional Pi) or “non-acclimatised” (AM- without additional Pi) and used for further experiments, as described in Figure II.2

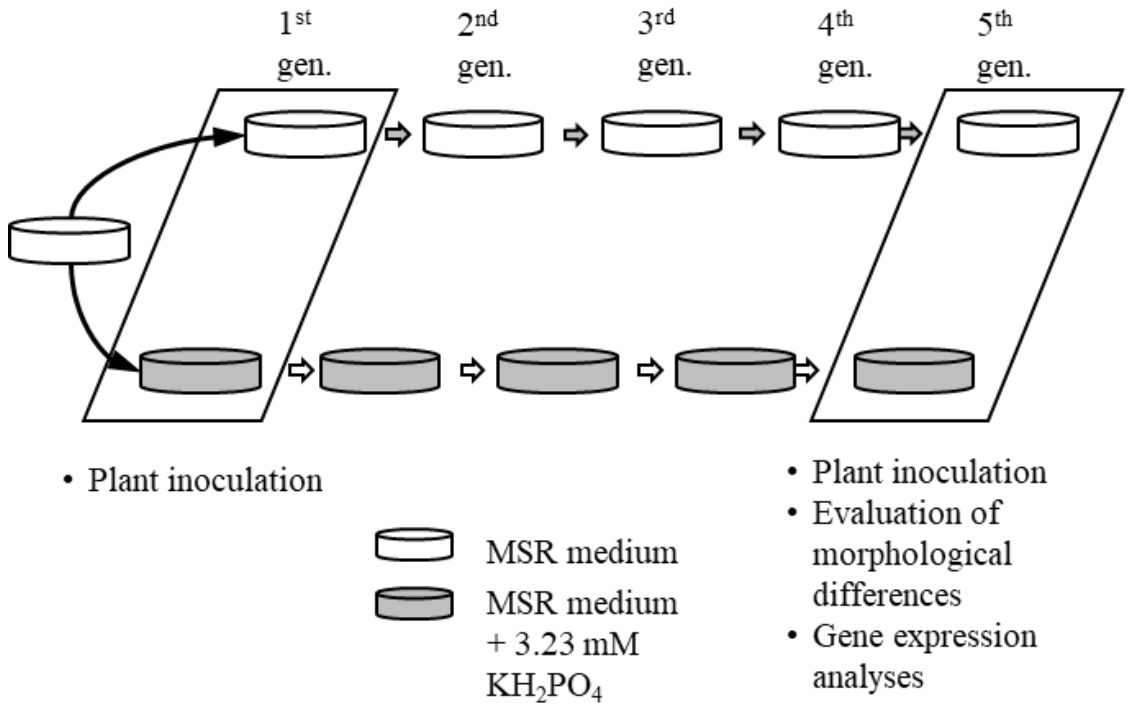

Figure II.2 Schematic representation of acclimatisation process *in vitro*. Below the first and fifth generation, the conducted analyses have been mentioned.

II.1.2 Evaluation of mycelium development

Spores of AM+ and AM- strains were collected from distal compartments and harvested after gel solubilisation in citrate buffer (0.1 M, pH 6). Collected spores were placed in Petri dishes (Ø 6 cm) in 10 ml of distilled water (pH 5.5) for stimulating germination. After two days, spores were added with the following solutions accordingly to the trial setup:

- Solution of KH_2PO_4 (pH 5.5) to a final concentration of 2 mM.
- Solution containing the synthetic strigolactone GR24 (StrigoLab, Turin, Italy) to a final concentration of 10^{-8} M.
- Mock solution for GR24, containing water and acetone.

GR24 solution was prepared by dissolving it in a few drops of acetone and then mixing it with sterile distilled water. Petri dishes containing spores were sealed and placed in dark in incubator at 26 ºC. After four days, evaluation of presymbiotic mycelium development was assessed. Five germinated spores were carefully collected with the help of micropipette and placed on microscope glass slide. Picture of single spores were taken with Zeiss SteREO Discovery V.20 (Carl Zeiss, Jena, Germany), equipped with lens Zeiss Plans 1.0x FWD 81 mm (Carl Zeiss) after 150x digital magnification. Numbers of branches and mycelium development were assessed with the help of digital grid line method as described in Figure II.3.

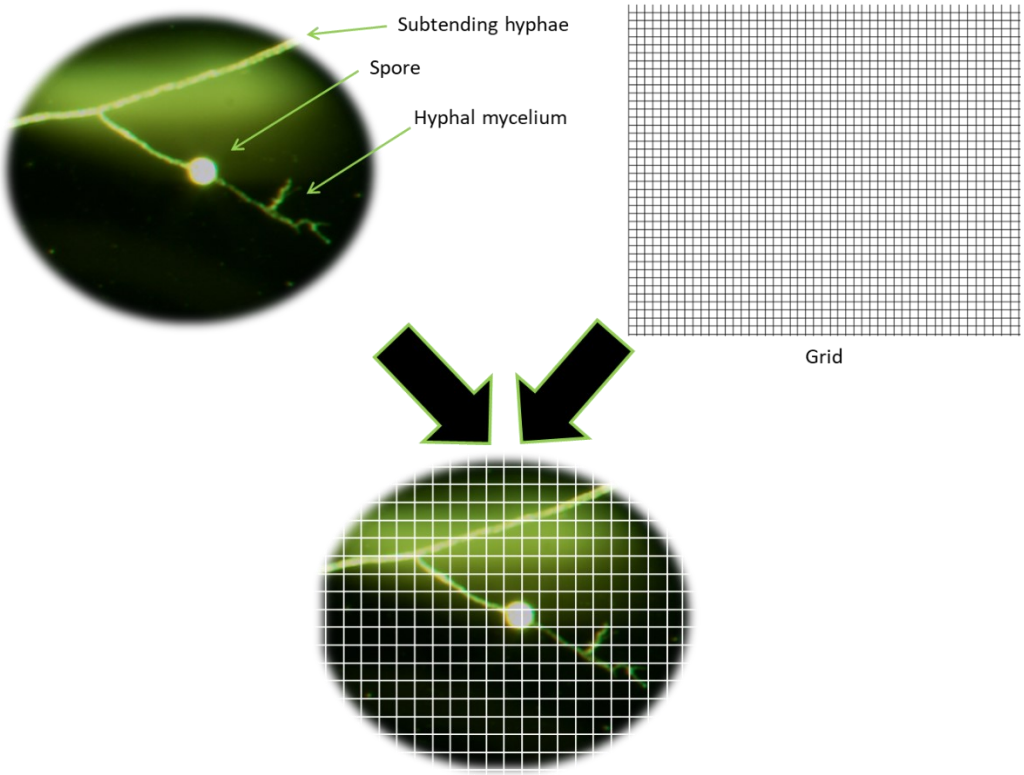

Figure II.3 Schematic representation of evaluation of hyphal development via the help of digital grid on the pictures.

II.1.3 Quantification of spores and hyphal density of acclimatised strain

Bi-compartment Petri dishes (Ø 9 cm) containing 25 mL MSR medium enriched or not with 3.23 mM of KH_2PO_4 were used for the analysis of acclimatised and non-acclimatised strains. Root organ pieces were inoculated with spores of AM+ or AM- strains as described above. After 7 weeks of cultivation, the number of spores and density of hyphae were quantified in the distal compartment. Each treatment consists of five biological replicates. Total number of spores and hyphal density were assessed under a dissecting microscope. Three different areas of 1 cm^2 were selected in each Petri dish in order to cope with the patchy development of the fungus. The average of the values obtained in the three observations was considered as one biological replicate. Spore number was calculated as the total number of spores in the selected area, while the mycelium density was assessed via the help of grid line method (Giovannetti & Mosse, 1980; Marsh, 1971).

II.1.4 Evaluation of compatibility between fungal propagules and selected regulators

Mature spores of the AM fungus *R. irregulare*, grown *in vitro* with transformed carrot roots were collected under dissecting microscope with a syringe needle and placed in Petri dishes (Ø 9 cm) containing 0.6% agarose (agarose standard, Carl Roth, Germany). Each Petri plate contained four spores displayed at the vertices of a square of 3 cm side. The four regulators known to be able to elicit the ISR/SAR responses in plants were tested. Solutions of pipecolic acid (Sigma Aldrich, Saint Louis, USA), azelaic acid (Sigma Aldrich), xylose (D-(+)-xylose, Carl Roth) and glucose (D-(+)-glucose, Carl Roth) were dissolved and diluted with H_2O to a working solution of 0.055 mM and 0.55 mM. The solutions obtained were incorporated in the autoclaved medium via filter sterilization.

At 24 day after inoculation, hyphal development patterns were observed under dissection microscope. Total number of branches was counted directly, while length of the longest hyphae was calculated with the measuring tool of microscope camera DinoCapture V 2.0 (Dunwell, California, USA). Germination rate was calculated as number of spores germinated to total number of spores, while spore viability was assessed by (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride salt staining (INT, BioChemica, Sauerlach, Germany), through counting spores showing red formazan colour (Walley & Germida, 1995). INT reduction (2 electrons and 2 protons convert INT to formazan) is connected to the electron chain transport (Berridge et al., 2005) as a marker of mitochondrial activity (Mukerji, 2011; Walley & Germida, 1995).

II.2 Greenhouse experiments

II.2.1 Growing conditions and inoculation

Solanum tuberosum cultivar selected for the experiments was "Jasia" developed by Dr. Niehoff (Bütow, Germany). It is a starch potato variety with medium to late development showing high yield and good drought tolerance and a very good storage behaviour. The tubers are round oval. Disease susceptibilities are described in Table II.1. *In vitro* plantlets of *S. tuberosum* cv. Jasia were propagated in 50 mL of modified Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) deprived of hormones and supplemented with 20g/L of sucrose and 3.5 g/L of Gelrite™ in 350 mL glass jars (Weck, Wehr, Germany). Twenty cuttings of potato, containing one node each, were placed in every jar and were grown for 12 days [20ºC/17ºC (day/night), 16 h d^{-1} photoperiod (55 µmol $m^{-2}s^{-1}$ photon flux density) and 70% relative humidity].

At the end of this phase, the twelve-days-old potato plantlets were transplanted in 1 L pots containing 700 ml of fine sand (0.2–1 mm; Euroquarz, Ottendorf-Okrilla, Germany) sterilized (baked twice in a dry oven, at 120 ºC for 6 h) in greenhouse [Loitze, Germany; 52.905286, 10.832907; 32°C/25°C (day/night), natural light and day] for 8 weeks, as indicated in Table II.1. Two layers of expanded clay were added to the bottom of the pot to help with drainage and to the top to limit evaporation of water. Hardening of plantlets was performed keeping them under transparent plastic foil during the first week after transplanting.

Table II.1. Date of experiments performed in greenhouse.

Experiment	Starting date	Harvesting day
Regulation	11.04.2017	20.05.2017
Trained I	24.05.2017	26.07.2017
Trained II	16.03.2018	07.05.2018

Two concentrations of Pi were mixed directly in the substrate in form of KH_2PO_4 (10 and 100 mg of P per kg of dry sand). The first concentration was in the range of mycorrhizal inoculum production under greenhouse and was set as reference concentration for proper colonization. The second concentration was set to mimic conventional field soil condition, usually inhibiting AM fungal development. Fertilization was performed once a week with 70 mL of modified Hoagland´s solution without Pi (Hoagland & Arnon, 1950). Watering was performed when needed, precautions were taken to avoid the contact with plant aerial parts.

For inoculation of plants in greenhouse, two different inocula were used. For the experiment with regulators, plants were inoculated (M) or not (NM) with 10 mg/L of commercial root powder based mycorrhizal inoculum, INOQ advanced (INOQ GmbH, Schnega, Germany) to a final concentration of

80,000 propagules/L of *R. irregulare* (INOQ strain QS81) mixed directly with the substrate. For the other experiments, AM fungal spores were collected from Petri dishes with the help of 40 µm stainless steel sieve after solubilizing the solid medium with citrate buffer (Doner & Bécard, 1991). Harvested spores were washed three times with distilled sterilized water. Aliquots of 1 mL of water, containing 100 ± 5 spores were used for inoculation, placing it at the vicinity of the roots at transplanting time, with the help of a pipet. Water from last washing was collected and use for mock inoculation.

For testing the regulators, glucose, xylose, pipecolic acid and azelaic acid were added in the substrate before transplanting at two concentrations in the soil water (0.05 and 0.55 mM), while leaves application was made by spraying the plant after acclimatisation period with 10 mL of regulators solution at two concentration (0.05 and 0.55 mM). During the spraying, precaution was taken to avoid spray drift and dropping of the solution on the soil.

Table II.1 Description of potato cv. Jasia. Data from breeder website (https://www.saatzucht-niehoff.de/en/kartoffel/jasia-2-2/).

Use	Starch potato
Maturity	Medium late
Resistance to potato wart disease *Synchytrium endobioticum*	Pathotype D1
Susceptibility PLRV	Very low
Susceptibility to PVA	-
Susceptibility to PVY	Very low
Susceptibility to *Rhizoctonia solani*	Low
Susceptibility to black leg	Low
Susceptibility to foliage blight	Low
Susceptibility to tuber blight	-
Susceptibility to internal rust spots	-
Susceptibility to common scab	-
Nematode resistance	Pathotypes Ro 1 + 4 *Globodera rostochiensis*

II.2.2 Analysis of plant growth and root colonization

Plants were harvested 8 weeks after transplanting. Approximately 1 g of leaves and roots were immediately frozen in liquid nitrogen for further analysis. Shoot and root fresh weight (SFW and RFW respectively) was assessed, material was dried at 65°C for 48 hours and dry weight (SDW and RDW respectively) was also assessed. A subsample of roots was collected for assessing the mycorrhization of the roots.

Root colonization was assessed after trypan blue staining (Phillips and Hayman, 1970) or by the ink vinegar method (Vierheilig et al., 1998). Roots were first cleared in 10% (w/v) KOH for 15 minutes at 90°C in a water bath. Cleared roots were carefully washed with tap water using a sieve, in order to get rid of any traces of KOH. After rinsing, roots were acidified submerging them in 2% HCl solution for 30 minutes. HCl was removed and replaced by 0.05% (w/v) trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water) for 30 min. at 90 °C in water bath, or in China ink solution (5% ink, 8% acetate). Excess of staining was removed by placing the roots in 50% (v/v) glycerol solution overnight at room temperature. 30 stained root fragments of 1 cm were placed on microscope glass slide and frequency of mycorrhization (F%), intensity of

mycorrhization in the root system (M%), intensity of mycorrhization in the root fragment (m%), arbuscule abundance in the root fragment (a%) and arbuscule abundance in the root system (A%) were assessed with the INOQ calculator Advanced (https://www.researchgate.net/publication/320869026_INOQ_Calculator_Advanced_Evaluate_the_mycorrhizal_rate_according_to_a_modified_Trouvelot_method) based on the Trouvelot et al. (1986) method (Figure II.4).

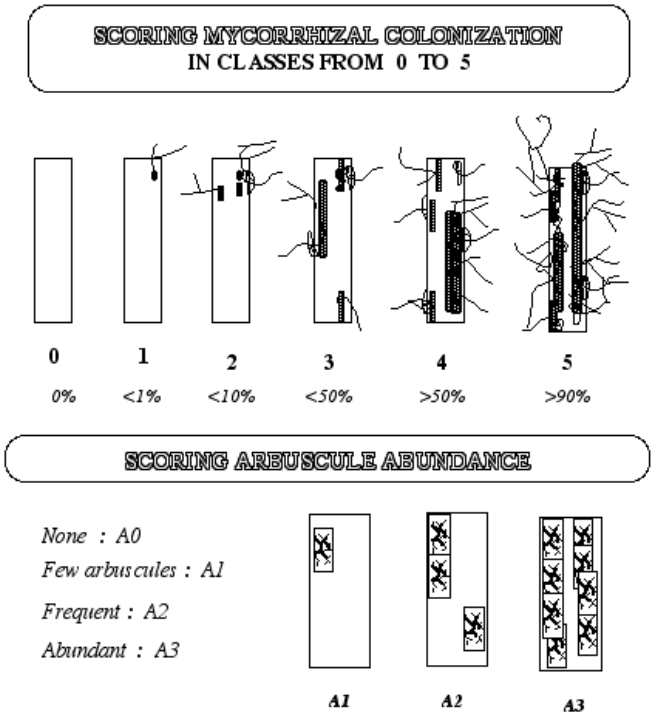

Figure II.4 Classification of mycorrhizal colonization and arbuscule abundance according to Trouvelot et al., 1986

II.2.3 Nutrient analysis of plant tissues

Dried shoot samples were finely grinded in the mixer mill MM200 (Retsch, Haan, Germany), and 200 mg of each sample were used for the analysis of total P content. To determine the P content, flow injection analysis was performed by means of colorimetric detection following ISO/EN/DIN 15681-1. At first, samples were digested in a solution with 5 mL of HNO_3 65% and 3 mL of H_2O_2 in a microwave (15 min. 2000°C; CEM Corporation, Kamp-Lintfort, Germany). Digested solution was brought up to 50 mL with double distilled water and filtered. The ortho-phosphate formed during digestion, was reduced in phosphomolybdate and then in molybdenum blue by zinc-(II)-chloride/hydroxylamine after treatment with molybdate in acidic environment. The intensity of the dye was proportional to P content and was detected via measurement of absorbance using the EPOS 5060 analyser (Eppendorf, Hamburg, Germany).

II.3 Molecular investigations

II.3.1 RNA extraction

RNA was extracted from frozen roots using innuPREP Plant RNA Kit (Analitik Jena, Jena, Germany) according to the instructions of the manufacturer. In brief: 50 mg of frozen roots were grinded in the extraction buffer using the mixer mill MM200 (Retsch, Haan, Germany). Samples were placed in columns,

and subjected to digestion with DNase, to remove genomic DNA contamination. After washing, samples were diluted in RNase free water. Quality and quantity of extracted RNA was measured with spectrophotometer NanoDrop® ND1000 (NanoDrop Technologies, Wilmington, USA). Ratio of absorbance at 260 and 280 nm (260/280 ratio) was used to assess RNA purity. Values ~2 of 260/280 ratio were accepted as pure for RNA. Moreover, the ratio of absorbance at 260 and 230 nm (260/230 ratio) was used to assess possible co-purified contaminants (e.g. phenolic compounds). RNA solution was stored at -80°C until further use.

II.3.2 cDNA synthesis

M-MLV Reverse Transcriptase kit was used to reversley transcribe 750 ng of extracted RNA into cDNA (Promega GmbH, Mannheim, Germany) in a 25 µL volume following the supplier's instruction. 100x diluted cDNA was used as template for each qRT-PCR reaction.

II.3.3 Primer design and validation

Primer pairs were designed using the NCBI primer design tool based on *R. irregulare* genome and transcriptome information (https://www.ncbi.nlm.nih.gov/tools/primer-blast/; Tisserant et al., 2012, 2013). Length of amplification products was below 200 bp and annealing temperature of 60 °C was set for all primer pairs, allowing simultaneous analysis of different genes in one single plate. All primer pairs had exponential increasing amplification curves. Specificity of different amplicons was tested with melting curve analysis at the end of each run (Ririe et al., 1997).

II. 3.4 Quantitative real-time RT-PCR

Target genes (Table II.2 List of genes used in this study. were quantified by qRT-PCR with 7500 fast real-time PCR system (Applied Biosystems, Foster city, USA) using SYBR green as fluorescent dye. Each reaction (10 µL) contained 5 µL SYBR green mix (SYBR green Low-ROX 2x Sensimix, Bioline, Luckenwalde, Germany), 200 nmol/L of each forward and reverse primers and 1 µL of diluted cDNA template. Amplification program was set as follow: 95 °C for 10 min, 40 cycles (95 °C for 15 s, 60 °C for 1 min). Three independent biological replicates and three technical replicates were used to analyse RNA accumulation in each treatment. For plant analysis, the reference genes of potato: β-tubulin (*Stβ-tub*), elongation factor (*StEF1*) and ubiquitin (*StUbc*) were tested as reference, and after evaluation of expression stability, the gene *Stβ-tub* was selected as reference. For fungal analysis, the translation elongation factor EF1-alpha (*RiTEF1a*) was selected as reference. Evaluation of expression stability was performed using Biogazelle qBase+ version 3.0 (Biogazelle, Zwijnaarde, Belgium). PCR efficiency calculation was calculated from a fourfold dilution series of cDNA and used to adjust the calculation of RNA accumulation as $E^{\Delta Ct}$ (ΔCt = Ct reference gene – Ct target genes) accordingly to Pfaffl (2001).

Table II.2 List of genes used in this study.

Target Gene	Putative gene product	Forward (5'-3') Reverse (3'-5')	Reference for primer sequences
StGST	Glutathione S-transferase	For: GTCGTGGCAGAGAACGAAG Rev: GGCCTAGCATCGAACAAGC	Genzel et al., 2018
StPR-3	Acidic class II chitinase ChtA2	For: ATGGCTGCCTTTTTCGGTCA Rev: TACCTTGTCCAGCTCGTTCG	Genzel et al., 2018
Stβ-tub	β-tubulin	For: ATGTTCAGGCGCAAGGCTT Rev: TCTGCAACCGGGTCATTCAT	Gallou et al., 2009
StUbc	Ubiquitin	For: TGATGGTTACCCATTTGAGCC Rev: ACTGGTCCTTCAGGATGTC	Gallou et al., 2009
StEF1	Elongation factor	For: ATTGGAAACGGATATGCTCCA Rev: TCCTTACCTGAACGCCTGTCA	Gallou et al., 2009
StPR-1	Pathogenesis related protein 1	For: GGTGCAGGAGAGAACCTT Rev: GGTACCATAGTTGTAGTTTGGCT	Genzel et al., 2018
StPR-2	Basic glucan endo-1,3-betaglucanase	For: CACATTGCTTCTGGGATGGA Rev: TTAACATCTGGCCAGAAATCTTTAA	Lehtonen et al., 2008
StPR-6	Proteinase-inhibitor II PI2	For: TGCCCACGTTCAGAAGGAAG Rev: TGGGTCAGATTCTCCTTCGC	Genzel et al., 2018
RiTEF1a	Translation elongation factor EF1-alpha	For: GCTATTTTGATCATTGCCGCC Rev: TCATTAAAACGTTCTTCCGACC	Waschke et al., 2006
RiBTub1	β-tubulin	For: AAGCGGAATCTTGTGATTGTTTG Rev: CCCATACCAGCTCCAGTACCA	Lammers et al., 2001
RiMST2	Sugar transporter	For: GTTAATGGTCTTGTCAATATGTTAG Rev: AAATGTTTTCCCAACGATTCATCA	Helber et al., 2011
RiDPD4	DNA polymerase delta subunit 4	For: TACAGCCCGATGTTGAGGC Rev: TGATGGCTTGAGACGTGACC	Sugimura & Saito, 2017
RiPCNA	Proliferating cell nuclear antigen	For: CACGTCCCTCCTCTGCAAAA Rev: GCTCAAACGCGCTTCAAACA	Sugimura & Saito, 2017
RiRNR	Ribonucleutide reductase	For: CGGATTGGGCTCTTCGATGG Rev: ATTGACGCGAAAGCTCCAGA	Sugimura & Saito, 2017
RiMhC	Myosin II heavy chain	For: GTGCGGGATTTCCTAACCGA Rev: TGTGCAGCTTCTCGTCCATT	Sugimura & Saito, 2017
RiMFS	Major facilitator superfamily	For: TCAACCAATGATATCTCCACCTCT Rev: CGAGAGCACAGAGACCAAGT	Sugimura & Saito, 2017
RiGST	Glutathione s-transferase	For: AATGACTATTACAGTTTTCGG Rev: GAATTCTTCCGAAAGGATGTTTG	Waschke et al., 2006
RiSOD	Superoxide dismutase	For: ATTCCACATCCATGAATTCGGTGA Rev: GATAGTACGTCCGATTACAGAGT	González-Guerrero et al., 2010
RiPT1	Plasma membrane phosphate transporter 1	For: AACACGATGTCAACAAAGCAAC Rev: AAGACCGATTCCATAAAAAGCA	Fiorilli et al., 2013
RiPT3	Plasma membrane phosphate transporter 3	For: AAAGGCGTGGAGCAATGA Rev: CGGGAATAATACCGACACCA	Mercy et al., 2017
RiPT6	Plasma membrane phosphate transporter 6	For: AACCGGAGCTTTCGCTTCA Rev: AGCATCGATAGCAGCTCCAC	Mercy et al., 2017
RiPT7	Plasma membrane phosphate transporter 7	For: CCAGTCTCAGGATTCCCAAA Rev: CCGATCGTGACAACACAAAG	Mercy et al., 2017
RiPT5	Plasma membrane phosphate transporter 5	For: CCGCCCGTAGTGTGAATAAA Rev: GAAGCGAATGAGGCAGTAAGAAT	Mercy et al., 2017
RiCOX5b	Cytochrome oxidase	For: TTGTCGGCTGTACTGGGTTC Rev: ACCACATTCAGGGCATCTGT	Mercy et al., 2017
RiAOX	Alternative oxidase	For: AAAATGAACGTATGCACTTGATGAC Rev: GCGTTCCCACCAGGTAGGT	Mercy et al., 2017

II.4 Field experiments

II.4.1 Mass production of acclimatised inoculum

In vivo production of inocula for field experiment were prepared during the growing season 2017 in greenhouse [Loitze, Germany, 52.905286, 10.832907; 32 °C/15 °C (day/night), June-October, natural light, and day] with the normal company production setup. Production was performed in 1 m^2 beds (Figure II.5), made of concrete blocks filled with a layer of 30 cm of mix (75:25 v:v) of fine sand (0.2–1 mm; Euroquarz,) and vermiculite (Agra-Vermiculite, Rhenen, Netherlands), fertilised with 0.8 g/l of Nutricote® mini (N, P, K 13:13:13; Arysta, Cary, United States). Inoculation of the blocks was made adding commercial INOQ inoculum of *R. irregulare* (strain QS81) with a final concentration of 80,000 propagules/L. No other fertilizations were performed in the production of the non-acclimatised *in vivo* inoculum; while the production of the acclimatised inoculum was obtained adding KH_2PO_4 to reach the final mass fraction of 75 mg of P per kg of dry substrate. 1 g of seeds of *P. lanceolata* was sown in each block and covered with 0.5 cm layer of expanded clay. At the end of the growing season, plants were harvested, and roots were air dried and grinded to a size of 1-2 mm, ready to be used as inoculum. Quality of inocula produced are shown in Table II.2.

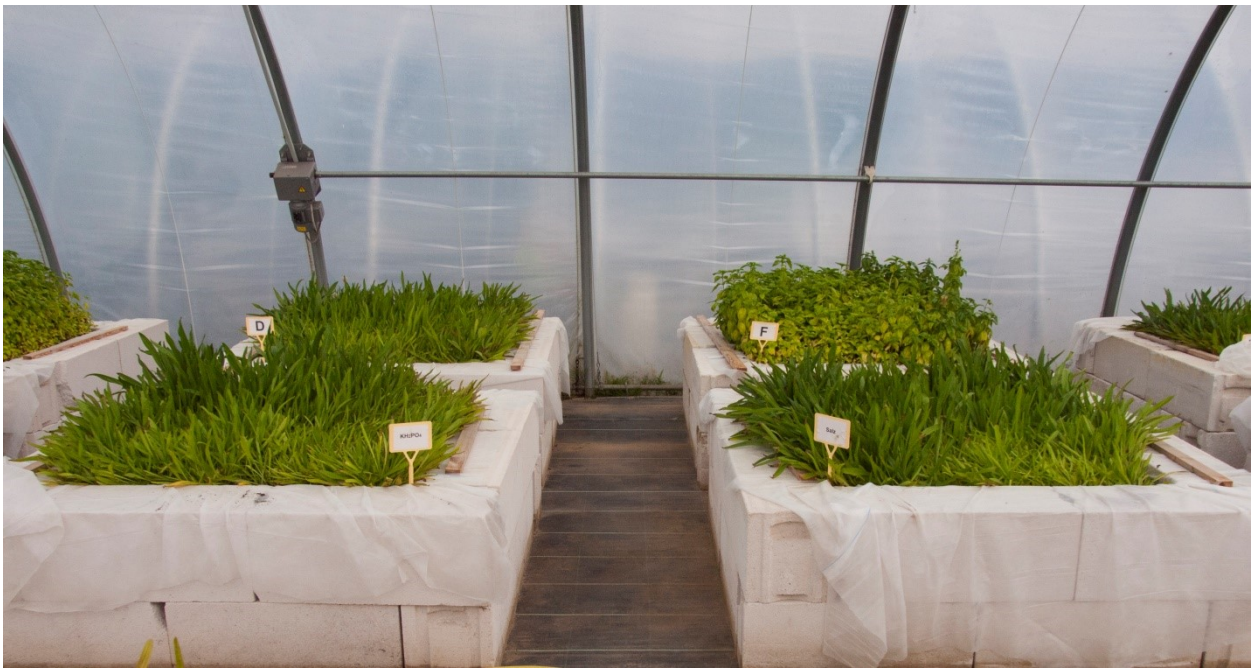

Figure II.5 *In vivo* production system of AM fungi in greenhouse.

Table II.2 Number of propagules for inocula produced in 1m^2 blocks.

Production setup	Total root biomass produced (g)	total number of propagules per root biomass produced
Plantago	821	242 906 533
Plantago + KH_2PO_4	1 026	50 775 521

II.4.2 Growth conditions and inoculation for testing the acclimatisation hypothesis.

With the aim to mimic field conditions, tubers from *S. tuberosum* cv. Jasia were sown in 10 L pots containing non-sterile field soil (Loitze, Germany, 52.905286, 10.832907) and grown outdoor. To test the response to inhibiting [Pi], Pi was applied weekly in half litre of full Hoagland solution (Hoagland & Arnon, 1950) in each pot to mimic fertilization applied in the field during cultivation. These plants were labelled as "full-fert", while the control plants did not receive any further fertilization and were labelled as "no-fert", since the level of nutrient were enough to allow the growth of plants. Irrigation was applied when needed. Characteristics of the soil are described in the Table II.3.

Table II.3. Characteristics of soil used to test the acclimatisation hypothesis in pots grown outdoor. Soil analyses were performed by LUFA Nord-West (Hamlen, Germany).

Parameter	Unit	Quantity
pH		5.5
P	mg/kg	40
K	mg/kg	40
Mg	mg/kg	30
Na	mg/kg	1.9
Total nitrogen	%	0.04
Organic matter	%	0.57
C/N ratio		14
Sand	%	85.5
Silt	%	10.5
Clay	%	4

At sowing, plants were inoculated with 100 mg of root powder-based inoculum diluted in 10 mL of sterile sand. For plant receiving the acclimatised inoculum, the amount of root powder-based inoculum was increased to reach the same propagule number as of the non-acclimatised inoculum. *In vivo* inoculum was acclimatised AM+ or not AM- for high level of Pi. Autoclaved inoculum (2 x 120 °C, 6 h) was added in the same ratio to control plants (NM).

Plants were harvested 8 weeks after emergence of the sprouts. Approximately 1 g of leaves and roots were immediately frozen in liquid nitrogen for further analysis. Fresh and dry weights of shoots, roots and tubers were measured as described above for the greenhouse plants. Sub samples of roots were saved to assess the mycorrhizal rate according to Trouvelot (1986) after trypan blue staining as described in section II.2.2 Analysis of plant growth and root colonization; temperatures and precipitations occurring during the experiment were reported (https://www.wetterkontor.de) and are shown in Figure II.5 Temperature (A) and precipitation (B) during the period of field experiment I. Data from https://www.wetterkontor.de

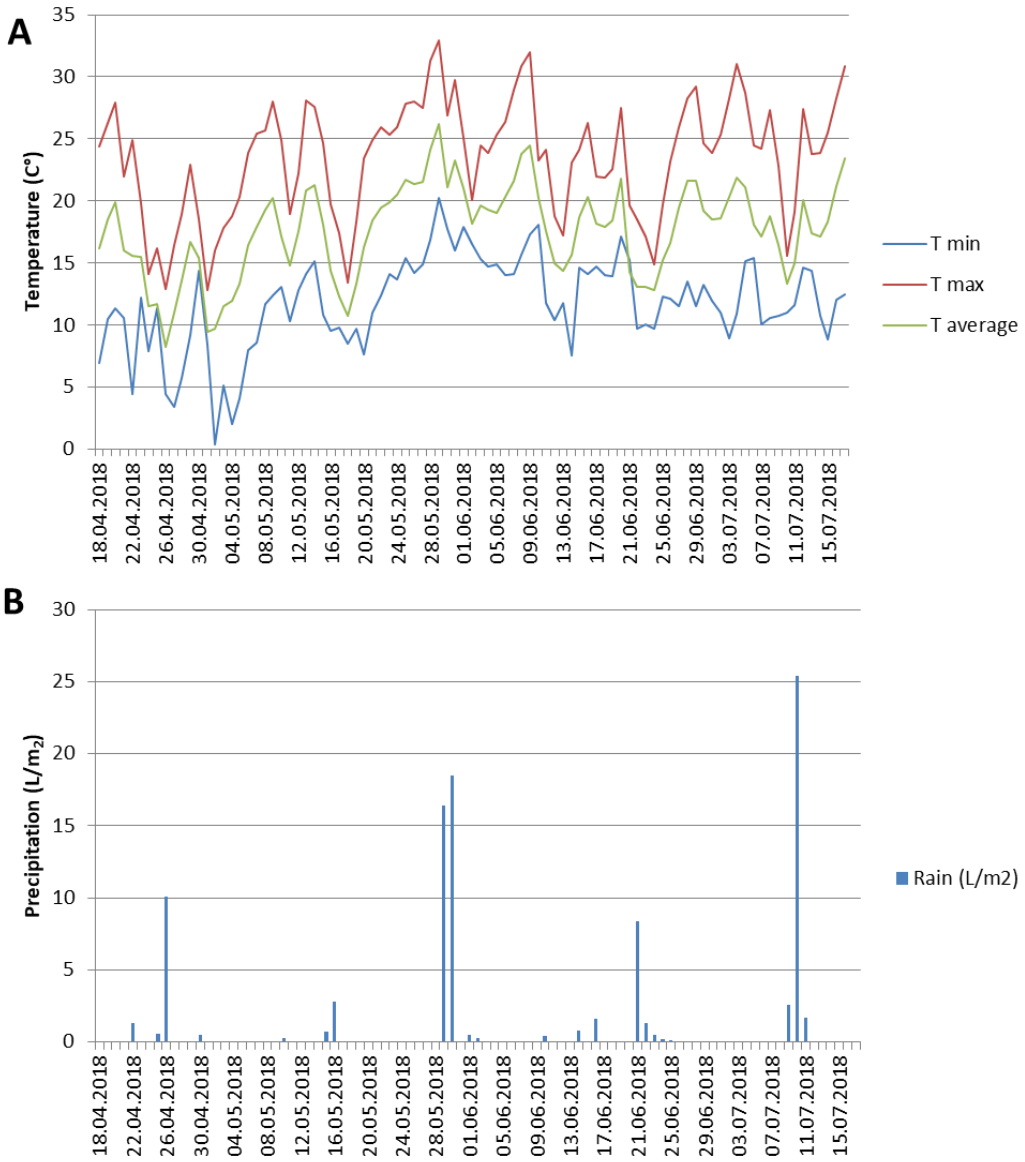

Figure II.5 Temperature (A) and precipitation (B) during the period of field experiment I. Data from https://www.wetterkontor.de

II.4.1 Growth conditions and inoculation for testing the regulation hypothesis.

The experiment was conducted from April 2018 to September 2018 in fields located in Waddeweitz, Lower Saxony, Germany (52°58'23.7"N 10°58'24.6"E), with the commercial starch potato variety Jasia. The previous crop cultivated in the field was winter rye. Soil analyses were performed by LUFA Nord-West (Hamlen, Germany). Soil was classified as loamy-sandy, presented pH values of 4.9 with normal dotation of P (50 mg/kg), high levels of K (130 mg/kg) and low quantity of magnesium (20 mg/kg). The agronomic preparations for all the experiments included tillage (approx. 15 cm) and harrowing before seeding. The seeding machine was used to get a customized seeding pattern for each plot. Every plot was composed by four rows of ten potato with outdistance of 35 cm. Rows were set 75 cm from each other. Each plot was separated by an empty row and by the distance between two potatoes. Irrigation was performed five times during the duration of the experiment and plants never showed severe water stress symptoms.

R. irregulare INOQ strain QS81 was used in the experiment in form of root based in formulation Advantage (INOQ GmbH), diluted in sterile sand. Plant inoculation was performed as described in II.4.2. The plots were either fertilized or non-fertilized, and the fertilized plots received N only (60 kg/ha, foliar application), but no supplementary Pi. Every treatment consisted of nine plots randomly distributed in the experimental field.

Solutions containing 0.55 mM of xylose or pipecolic acid were prepared with tap water and sprayed on leaves of potato plants four weeks after inoculation. Around 50 mL of solution was used for each plant. Control plants were sprayed with solution containing only water.

A mid-term harvest was conducted eight weeks after inoculation to evaluate plant growth in the initial stage and root colonization. Three plants were harvested for each plot and a total of five plots were harvested per treatment. Only plants in the two central rows of a plot were harvested, the three plants were pooled to make one biological replicate. 1 g of roots and shoots were frozen with liquid nitrogen for further analysis. Fresh and dry weights of shoots, roots and newly formed tubers were measured and mycorrhization was assessed as described above.

In the final harvest, only tubers were harvested from the two central rows of every plot to avoid the border effect. In total, 17-20 plants/plot were harvested (depending on the number collected during the mid-term sampling and losses). The potatoes harvested in each plot were stored in separate bags, then sorted in two classes (⌀ < 6.5 cm or ⌀ > 6.5 cm), weighed, and counted. The starch content was measured according to REGULATION (EC) No 2235/2003 on potatoes that showed ⌀ > 6.5 cm. Washed potatoes (⌀ > 6.5 cm) were sampled for black scurf disease scoring (*Rhizoctonia solani*) based on the disease index scale, according to Canadian food inspection, shown in Figure II.8. Temperatures and precipitations occurring during the experiment are reported in Figure II.7 Temperature (A) and precipitations (B) during the period of field experiment II. Climatic data from: https://www.wetterkontor.de

Figure II.6. Picture above: panoramic view of the experimental field. Below: detail of the field experiment plots

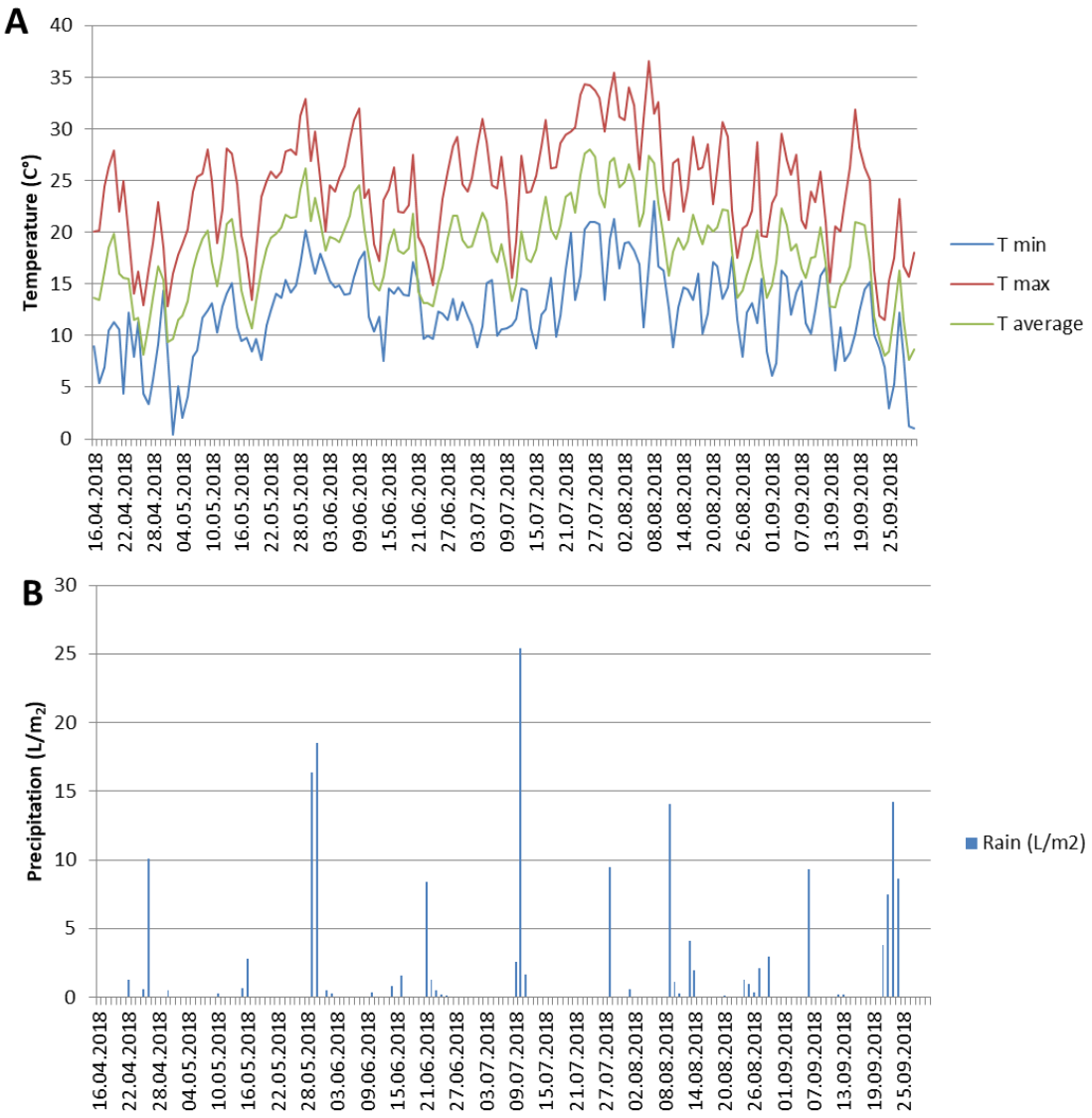

Figure II.7 Temperature (A) and precipitations (B) during the period of field experiment II. Climatic data from: https://www.wetterkontor.de.

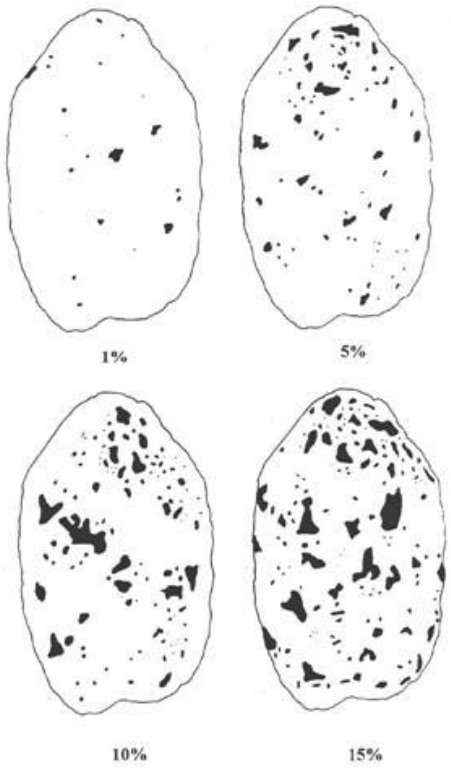

Figure II.8 Table of evaluation of black scurf disease. Evaluation made according to http:// www.inspection.gc.ca/plants/potatoes/guidance-documents/pi-005/chapter-9/eng/1381190301495/1381190302464.

II.5 Statistical analyses

Statistical analyses were performed using the program SPSS v.20 (IBM, Armonk, USA). Two-way and One-way analysis of variances (ANOVA) were carried out, as appropriate, when all the assumptions were satisfied after log or arcsin transformation of values as indicated in figure legends. In case of significant interactions ($P < 0.05$) between factors Tukey honestly significant difference (HSD) was conducted (Tukey, 1953). Non parametric test of Kruskal-Wallis (Kruskal & Wallis, 1952) was performed when homogeneity of variances was not met in our samples. Double tailed T-test was performed when the comparison was limited to only two samples. All data are shown as mean values with standard deviations or standard errors as indicated in figure descriptions.

III. Results

III.1 *In vitro* experiments

The *in vitro* experiments were set up with the aim of obtaining initial validations on the two theses proposed in this study: acclimatisation and regulation.

For the acclimatisation hypothesis (sections III.1.1, III.1.2, III.1.3), experiments were carried out with the fifth generation of the fungus *R. irregulare* strain QS81. The strain grown for 5 generations in the presence of high Pi levels was considered acclimatised and named AM+, while the strain grown on normal MSR medium was named AM-. The experiments in sections III.1.1 were carried out to evaluate the effects on the asymbiotic development of the fungus. In this work, the asymbiotic phase is meant to be that phase of development which occurs independently of the presence of the plant, such as germination and early mycelium development. In contrast, the experiments in section III.1.2 evaluate presymbiotic development, as the synthetic analogue of strigolactone (GR24) mimics the presence of the plant in the growth media. In this case, presymbiotic development is defined as the phase in which recognition between plant and fungus has already begun, but physical contact has not yet occurred. The experiment described in section III.1.3, finally, is based on the symbiotic phase of the plant-fungus relationship, i.e. when the interaction between the two partners is complete.

Section III.1.4 deals instead with the regulation hypothesis. In this case the spores were put in contact with different molecules known as stimulators of the salicylic acid-mediated response, known as systemic acquired resistance (SAR) or the jasmonic acid-mediated response known as induced systemic resistance (ISR).

III.1.1 Spore responses to inhibiting [Pi] (acclimatisation hypothesis)

Response of AM+ and AM- strains in terms of asymbiotic mycelium development patterns at contrasting Pi concentrations is shown in Figure III.1. Analysis of effects of the factors Pi level and Inoculum type and their interactions (Table III.1) showed absence of significant interaction between the two factors in modulating the mycelium response. Simple main effects on branch development were identified with respect of Pi level, that induced the expected inhibition. Investigation within the same Pi level, highlighted higher mycelium branching rate in the AM+ strain compared to the AM- strain at high Pi concentrations. No other difference was observed within the same Pi level for the investigated parameters.

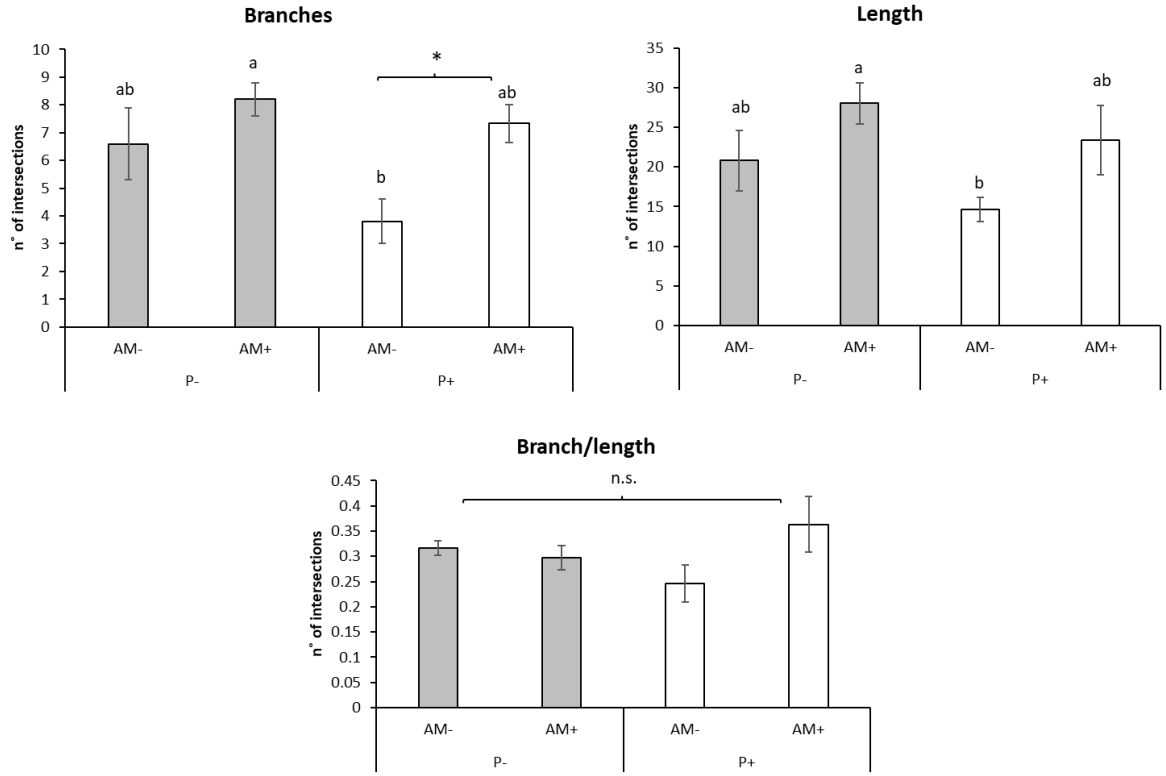

Figure III.1. Hyphal development of AM+ and AM- strains, in presence (P+) or not (P-) of Pi. Asymbiotic mycelium development patterns were studied in acclimatised (AM+) and non-acclimatised (AM-) strains in water in presence (P+) or not (P-) of 2 mM of Pi added as KH_2PO_4. Figures show means and standard deviations. Two-way ANOVA ($P = 0.05$; $n = 5$) showed significant effect of the factors Pi level and Inoculum type for hyphal length and branch number, but their interaction was not statistically significant. Different letters indicate significant difference according to post hoc Tuckey HSD test. Independent T-tests were carried out to assess differences between strains in presence or not of Pi. * = $P < 0.05$.

Table III.1 Significance of source of variation after two-way ANOVA for each parameter. Sources of variance were Pi level (absence or presence of 2 mM Pi), Inoculum type (AM+/AM-), and their interactions. * = $P <$ 0.05; n.s. not significant. AM+: acclimatised strain; AM-: non-acclimatised strain.

Factors and their interaction	**Parameters investigated**		
	Branch	Length	Branch/ Length
Pi level	*	n.s.	n.s.
Inoculum type	*	n.s.	n.s.
Pi level * Inoculum type	n.s.	n.s.	n.s.

III.1.2 Spore responses to strigolactone (GR24) in presence of inhibiting [Pi] (acclimatisation hypothesis)

Analysis of effects of the factors Pi level, GR24 and Inoculum type and their interactions are shown in Table III.2. Significant interaction of all the factors determined the different branching patterns of the presymbiotic mycelia. The other investigated parameters, instead, were not determined by the tripartite interaction among the factors. Single main effect for Pi level and GR24 were identified as determinant of difference in branching and length, confirming the inhibitory effect of Pi and the promoting effect of GR24. Investigation within the same Pi level, showed the expected promotion in branching rate in the AM- in presence of GR24 in P- (Figure III.2). AM+ did not exhibit the branching response to GR24 in P- condition. The branching level of AM+ was not affected by Pi presence, showing values comparable to the one observed in P-, while the presence of Pi induces a general depression in branching in all the other conditions, despite the presence of GR24. Concerning hyphal length, a difference between the two strain was observed in P- in presence of GR24 which exerted a stronger promotion effect to AM- compared to AM+. Concerning the branch/length ratio, no interaction effects were recognized following the analysis, and no simple main effect was identified.

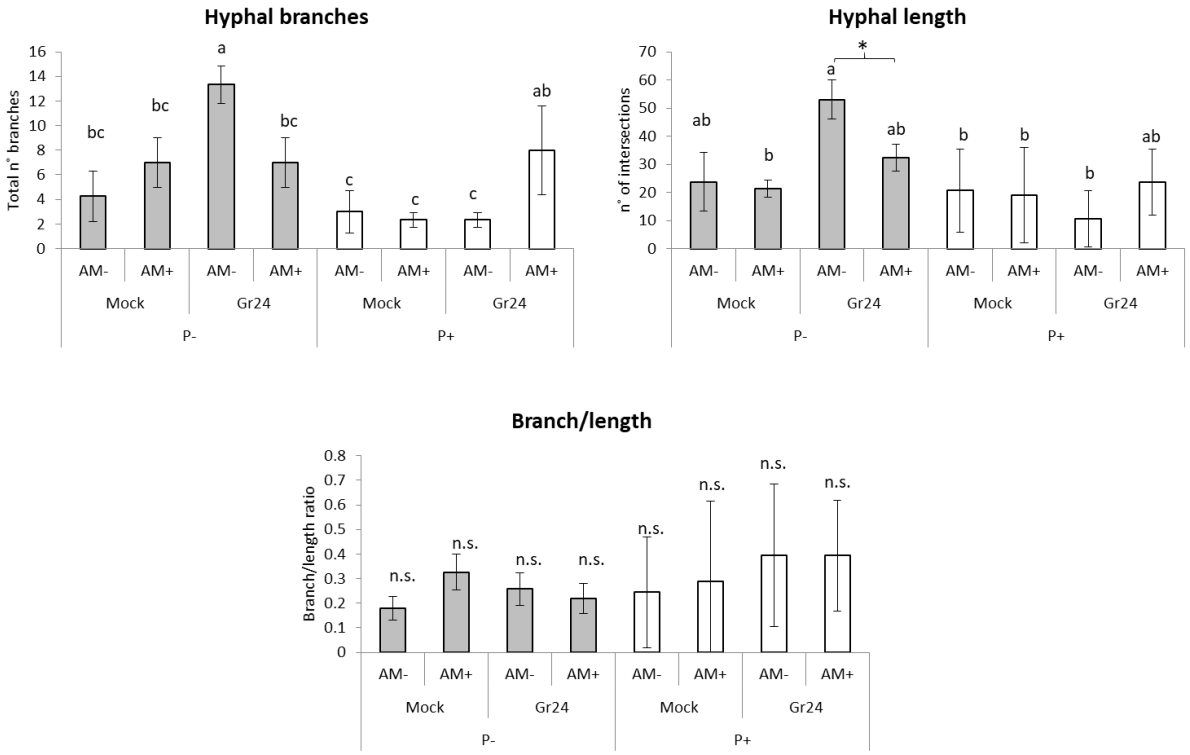

Figure III.2 Hyphal development of AM+ and AM- strains, in presence (P+) or not (P-) of Pi and strigolactones (GR24). Presymbiotic mycelium development patterns were studied in acclimatised (AM+) and non-acclimatised (AM-) strains in water in presence or not of strigolactones (GR24) and in presence (P+) or not (P-) of 2 mM of Pi added as KH_2PO_4. Figures show means and standard deviations. Three-way ANOVA (P = 0.05; n = 5) showed significant effect of the factors Pi level, GR24 and Inoculum type for hyphal length and branching, but their interaction was not statistically significant. Different letters indicate significant difference according to post hoc Tuckey HSD test.

Table III.2 Significance of source of variation after three-way ANOVA for each parameter. Sources of variance were Pi level (absence or presence of 2 mM Pi), Inoculum type (AM+/AM-), GR24 (presence of GR24), and their interactions. * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.0005$; n.s. not significant. AM+: acclimatised strain; AM-: non-acclimatised strain; GR24: synthetic strigolactone.

Factors and their interaction	Parameters investigated		
	Branch	Length	Branch/ Length
Pi level	*	*	n.s.
Inoculum type	n.s.	n.s.	n.s.
GR24	*	*	n.s.
Pi level * Inoculum type	*	n.s.	n.s.
Pi level * GR24	n.s.	n.s.	n.s.
Inoculation * GR24	*	n.s.	n.s.
Pi level * Inoculum type * GR24	**	n.s.	n.s.

III.1.3 AM+/AM- strain development in ROC with inhibiting [Pi] (acclimatisation hypothesis)

Results of symbiotic growth patterns of AM+ and AM- strains at contrasting Pi condition are shown in Figure III.3. Effects of the different factors: Pi level and Inoculum type, and their interaction are shown in Table III.3. Both parameters were influenced by the interaction of the factors, indicating that both, Pi level and Inoculum type, influenced the number of spores and the hyphal density. Investigation within the same Pi level showed a strong reduction of spore number of AM+ compared to AM- in MSR medium. The level observed in AM+ was comparable to the number of spores observed in MSR + 2 mM Pi when the Pi inhibition induces a reduction in AM-. Contrary to what observed for spore number, AM+ did not show a reduction in hyphal density compared to AM- in MSR medium. Moreover, the addition of Pi in the medium, did not reduce the hyphal density of AM+, while it exerted the expected inhibition on AM-.

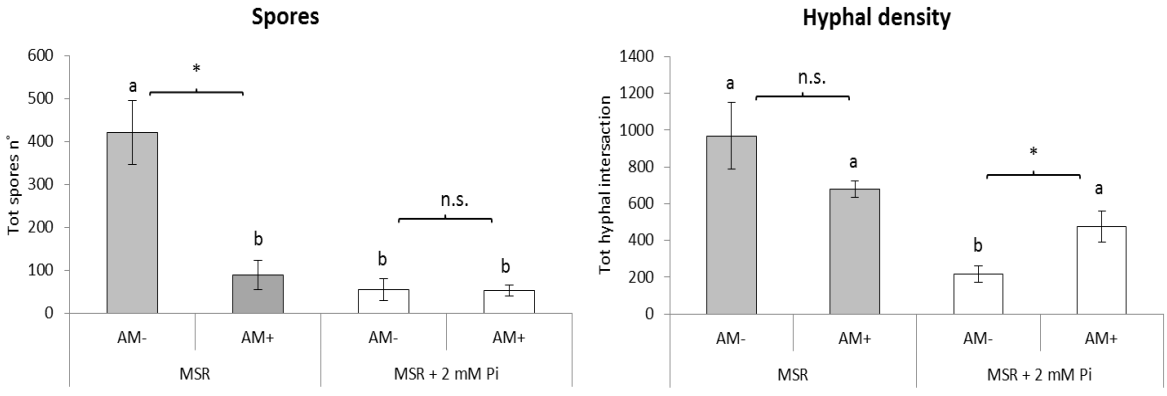

Figure III.3 Total spore number and hyphal development of AM+ and AM- strains, in presence of low (MSR) and high Pi (MSR + 2 mM Pi). Symbiotic mycelium development was studied for acclimatised (AM+) and non-acclimatised (AM-) strains in solid MSR medium with or without 2 mM of Pi in ROC system. Two-way ANOVA ($P = 0.05$; $n = 3$) showed significant interaction between Pi levels and Inoculum type. Different letters indicate significant difference according to post hoc Tuckey HSD test.

Table III.3 Significance of source of variation after two-way ANOVA for each parameter. Sources of variance were Pi level (normal MSR medium or MSR medium added with 2 mM of Pi), Inoculum type (AM+, AM-) and their interactions. * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.0005$; n.s. not significant. AM+: acclimatised strain; AM-: non-acclimatised strain.

Factors and their interaction	Parameters investigated	
	Spore number	Hyphal density
Pi level	**	**
Inoculum type	*	n.s.
Pi level* Inoculum type	*	*

III.1.4 Evaluation of the effect of inducer molecules on R. irregulare asymbiotic development (regulation hypothesis)

Germination rate of spores of *R. irregulare* at four different time points, growing in presence of different regulators, are shown in Figure III.4. Germination of spores was observed after 7 days post inoculation (dpi) of the plates and only spores growing in presence of azelaic acid did not show any germination at this time point. The second observation, made at 14 dpi, showed a significant lower germination in the SAR-associated molecules pipecolic acid and azelaic acid compared to control. However, germination level showed no difference in the last two observation time points (21 dpi and 28 dpi), reaching overall values compared between 35% and 55%.

Investigation of asymbiotic mycelium development was performed at 28 dpi and is shown in Figure III.5. Reduction in length was observed in spores treated with pipecolic acid, compared to control and xylose treatment, while no other differences were observed in the development of asymbiotic mycelium for the investigated parameters in the different conditions (Figure III.5).

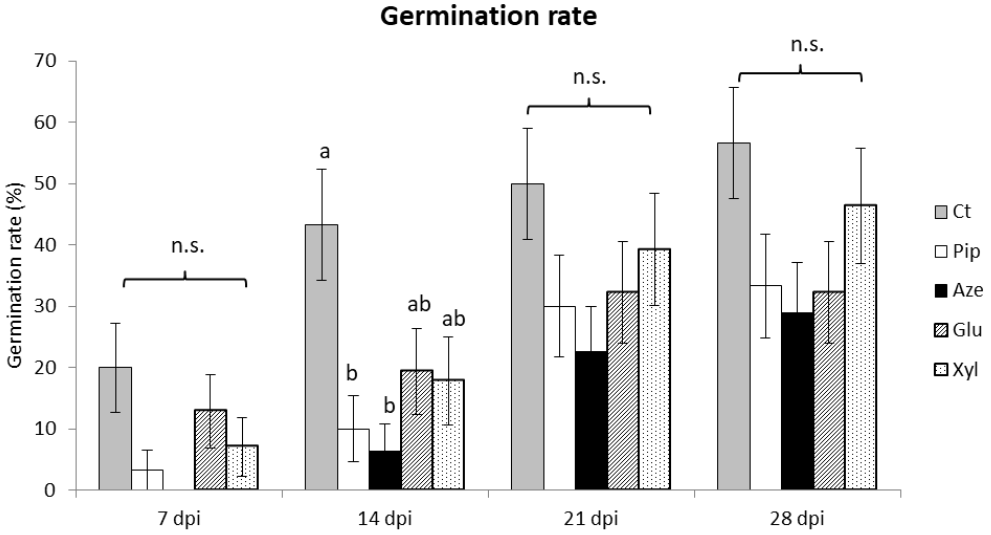

Figure III.4 Effect of several inducer molecules on spore germination of *R. irregulare*. Pipecolic acid (Pip), azelaic acid (Aze), glucose (Glu) and xylose (Xyl) were tested in water agarose medium at concentration of 0.55 mM. Non-treated spores were noted as control (Ct). Shown are means ($n = 30$) and standard deviations. Treatments with the same letter are not significantly different ($P < 0.05$, Kruskal-Wallis, pairwise comparison for 7 and 14 dpi sets, One-way ANOVA for 21 and 28 dpi sets).

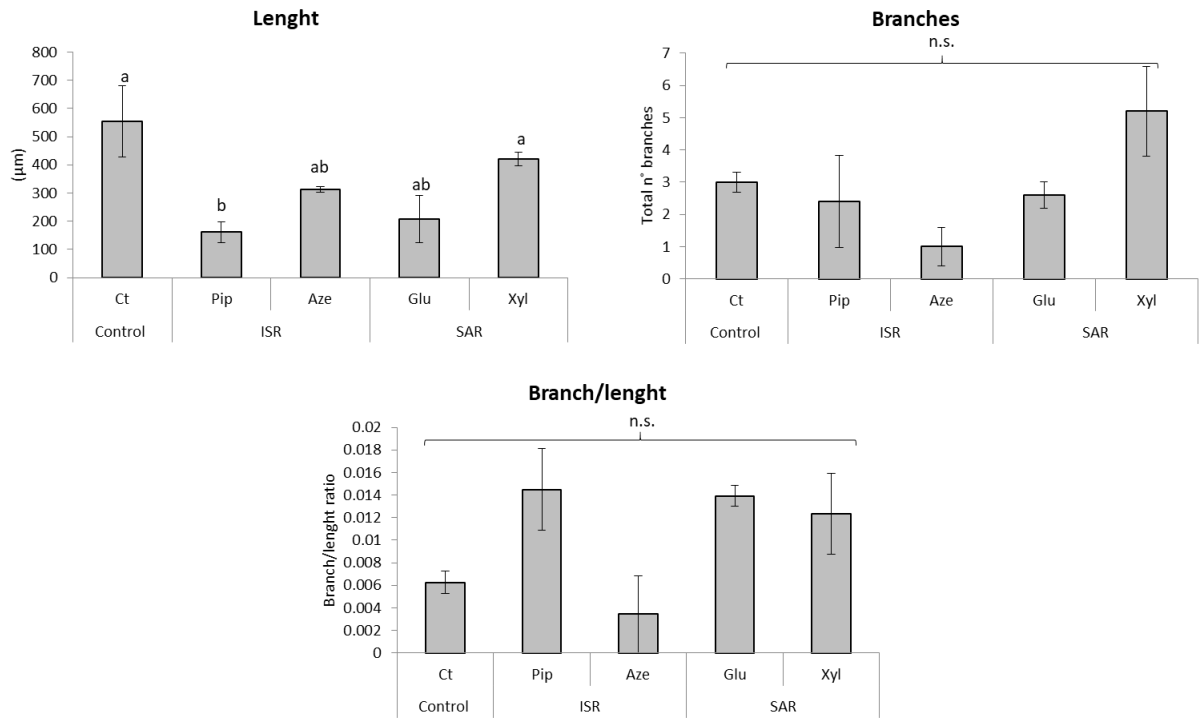

Figure III.5 Effect of inducer molecules on asymbiotic mycelium development of *R. irregulare*. Pipecolic acid (Pip), azelaic acid (Aze), glucose (Glu) and xylose (Xyl) were tested in water agarose medium at concentration of 0.55 mM. Non-treated spores were noted as control (Ct). (A) shows length of the longest hyphae in the germinated spores, (B) shows the total number of branches in the longest hyphae, (C) shows the ratio between number of branches and length of the hyphae. Shown are means (n = 5) and standard deviations. Treatments with the same letter are not significantly different (P < 0.05, Kruskal-Wallis, pairwise comparison). Statistical tests were performed after log transformation of values.

III.2 Greenhouse experiments

Following the first indications obtained from *in vitro* experiments, the two hypotheses: acclimatisation and regulation were tested in the greenhouse to evaluate the effects in a controlled environment.

For the acclimatisation hypothesis (III.2.1 and III.2.2), the experiments were carried out using the spores of acclimatised (AM+) and non-acclimatised (AM-) strain as inoculum. In the experiment described in section III.2.1 (performed in 2017), the first generation of the AM+ and AM- strains was used, while in the results described in section III.2.2, the fifth generation of the AM+ and AM- strains was used (performed in 2018). Both experiments were aimed at measuring the effects on plant growth and colonisation capacity of the acclimatised strain in the presence of high Pi concentrations. In the experiment described in section III.2.2, more information was collected as the acclimation process was considered completed.

Sections III.2.3 relate to the regulation hypothesis, in which case the molecules tested *in vitro* were tested in the greenhouse. To obtain more information for use in the field, two modes of application were tested: foliar and soil, at two different concentrations. The main interest of these experiments was to see if certain regulators were able to help the colonisation of the fungus even in the presence of high Pi levels. At this stage we were not interested in the effects on plant growth, as the cultivation system adopted was not representative of the reality in the field.

III.2.1 Effects of inoculation of first generation of AM+/AM- strains on growth of potato (acclimatisation hypothesis)

Investigation of the effects of the factors Inoculum type and Pi level and their interaction are shown in (Table III.4). No interaction was observed for any of the investigated parameters. A simple main effect of Pi level was observed for shoot fresh weight (SFW) and total FW, while the simple main effect of Pi level was observed for F% and a% where it was associated with the well-known inhibition of fungal parameters. Growth parameters and root colonization of potato plantlets inoculated with first generation of AM+ or AM- strain is shown in Figure III.6. Investigation within the same Pi fertilization level evidenced a lack of difference in plant total fresh weight (FW), while investigation of the fungal parameters highlighted a difference between AM+ and AM- for F% in P10, were AM+ showed a significant lower frequency of colonization compared to AM- (Figure III.6 C). No differences between strains were detected for the other fungal parameters within the same Pi fertilization level (Figure III.6 D, E).

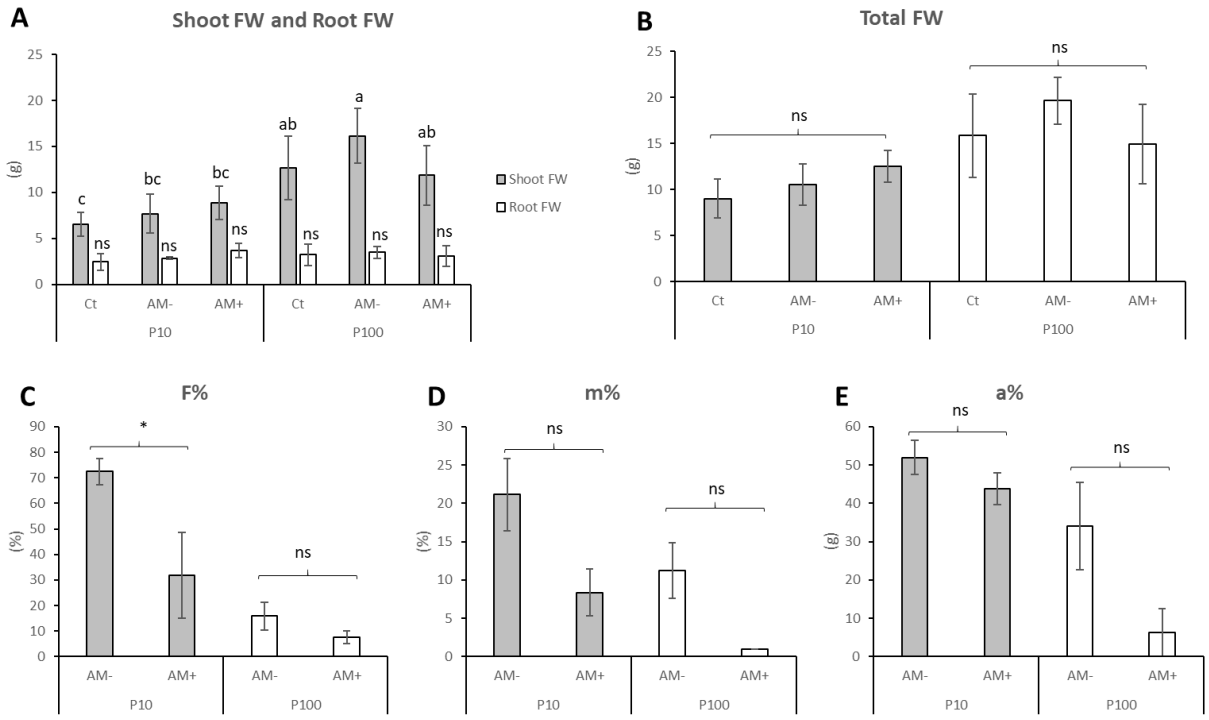

Figure III.6 Plant and fungal parameters of potato plantlets inoculated with AM+ or AM- strains, in presence at two Pi fertilization levels. Shoot fresh weight (SFW), root fresh weight (RFW) (A) and total fresh weight (Total FW; B) of potato plants grown at different Pi fertilization levels (P10: 10 mg P/kg dry sand; P100: 10 mg P/kg dry sand), not inoculated (Ct) or inoculated with acclimatised (AM+) or non-acclimatised (AM-) strain of *R. irregulare*. Fungal parameters: frequency of colonization (F%), intensity of mycorrhization in the root fragment (m%), arbuscule abundance in the root fragment (a%) according to Trouvelot et al., (1986) are shown in C, D, E. Figures show means (n = 5), and standard deviations. Treatments with the same letter are not significantly different ($P < 0.05$; Tukey HSD, ANOVA). Two-tailed T-test (n = 5; P = 0.05) was performed for fungal parameters, comparing the strains within the same Pi fertilization level.

Table III.4 Significance of source of variation after two-way ANOVA for each parameter. Sources of variance were Pi level (P10, P100) and inoculum type (Ct/AM+/AM-) and their interactions: Pi level * inoculum type for the following parameters: shoot fresh weight (SFW), root fresh weight (RFW), total fresh weight (FW), mycorrhizal frequency (F%), intensity of mycorrhization in the root fragment (m%), arbuscule abundance in the root fragment (a%). * = $P < 0.05$; n.s. not significant. P10: 10 mg P/kg dry sand; P100: 100 mg P/kg dry sand; Ct: without inoculation; AM+: acclimatised strain; AM-: non-acclimatised strain.

Factors and their interaction	Parameters investigated					
	SFW	RFW	Total FW	F%	m%	a%
Pi level	*	n.s.	*	*	n.s.	*
Inoculum type	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Pi level* Inoculum type	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

III.2.2 Effects of inoculation of fifth generation of AM+/AM- strain on growth of potato (acclimatisation hypothesis)

Investigation of the fifth generation of AM+ or AM- strains concerning effects of the factors Inoculum type and Pi level and their interaction on plant parameters are shown in (Table III.6). Interaction between the factors determined differences in root fresh weights (FW) and in root and total DW. Growth parameters and roots colonization of potato plantlets inoculated with fifth generation of AM+ or AM- strain is shown in Figure III.7. Plants inoculated with AM+ strain, showed higher total DW (Figure III.7 D) in P100, compared to control plants and AM- inoculated plants, while no differences were observed in P10. Investigation of the effects of the factors Inoculum type and Pi level and their interaction on colonization parameters are shown in (Table III.6). Significant interaction was observed only for F%. Analysis within the same Pi level, showed a significant higher mycorrhizal frequency in AM+ compared to AM- in P100 (Figure III.7 E), while AM- was associated with significant higher m% compared to AM+ in P10 (Figure III.7 F). The other fungal parameters did not differ from each other within the different Pi fertilization (Figure III.7 E, F, G). Pi content was significantly higher in AM- compared to Ct and AM+ in P10 (Figure III.8); while P uptake was significantly higher in AM+ compared to Ct in P100. Analysis of shoot P content indicate an accumulation of Pi in all plants grown in P100, indicating luxury consumption for this element.

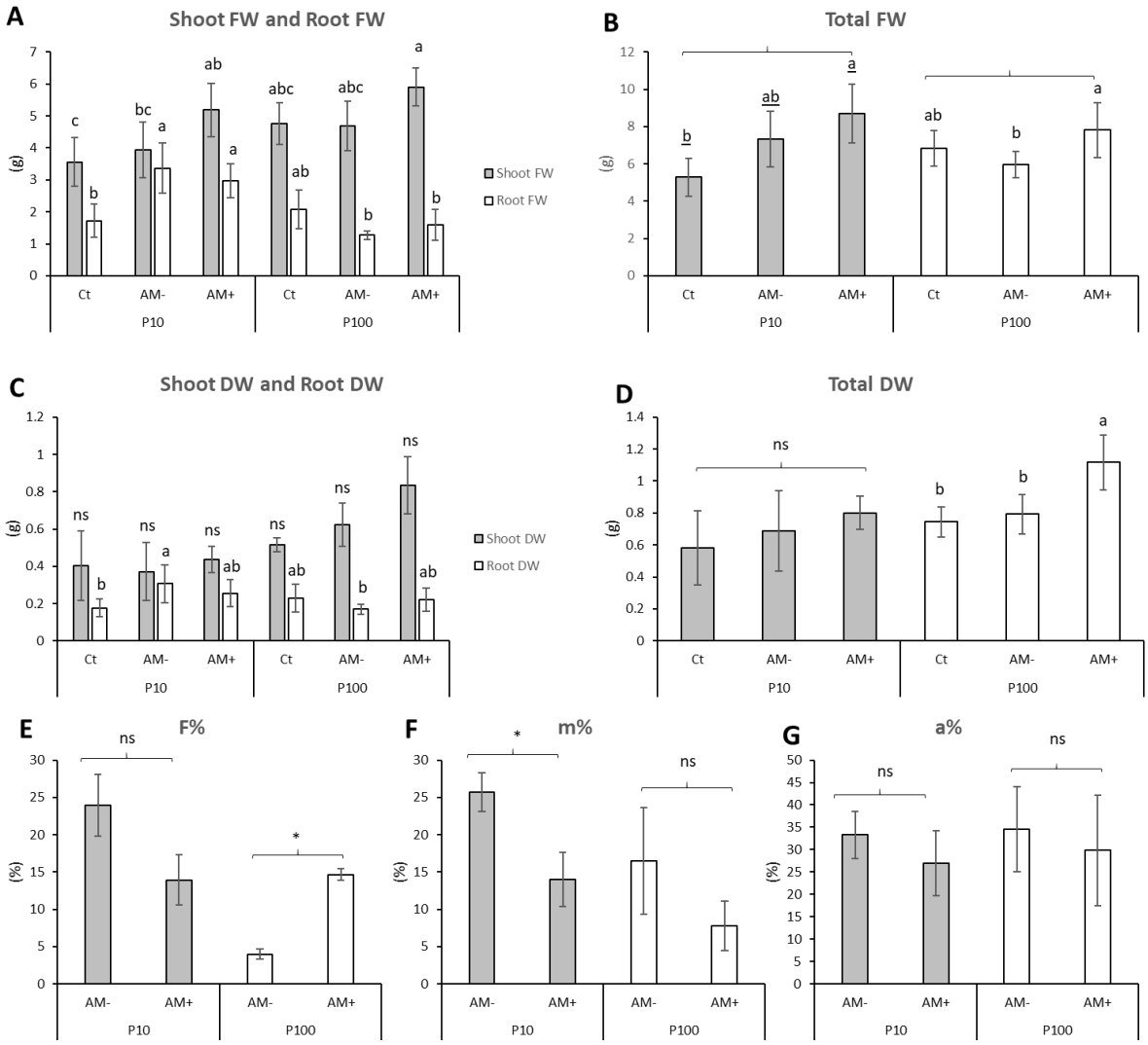

Figure III.7 Plant and fungal parameters of potato plantlets inoculated with fifth generations of AM+ or AM- strains, at two Pi fertilization levels. Root and shoot fresh weight (Shoot FW, Root FW; A) Total fresh weight (Total FW; B); root and shoot dry weight (Shoot DW, Root DW; C); total dry weight (Total DW; D) of potato plantlets grown at two Pi fertilization (P10: 10 mg P/kg dry sand; P100: 10 mg P/kg dry sand) inoculated with acclimatised (AM+) or non-acclimatised (AM-) strain of *R. irregulare*. Fungal parameters: frequency of colonization (F%), intensity of mycorrhization in the root fragment (m%), arbuscule abundance in the root fragment (a%) according to Trouvelot et al., (1986), are shown in E, F, G. Figures shows means (n = 5), and standard deviations. Treatments with the same letter are not significantly different ($P < 0.05$; Tukey HSD, ANOVA). Two-tailed T-test (n = 5; P = 0.05) was performed for fungal parameters, comparing the strains within the same Pi level.

Table III.5 Significance of source of variation after two-way ANOVA for each parameter. Sources of variance were Pi level (P10, P100) and inoculum type (Ct, AM+, AM-) and their interactions: Pi level * inoculum type for the following parameters: Shoot fresh weight (SFW); root fresh weight (RFW); total fresh weight (TFW), shoot dry weight (SDW); root dry weight (RDW) and total dry weight (TDW). * = $P < 0.05$; n.s. not significant. / = lack of homogeneity of variances. P10: 10 mg P/kg dry sand; P100: 100 mg P/kg dry sand; Ct: without inoculation; AM+: acclimatised strain; AM-: non-acclimatised strain.

Factors and their interaction	Parameters investigated					
	SFW	RFW	TFW	SDW	RDW	TDW
Pi level	*	*	n.s.	/	n.s.	n.s.
Inoculum type	*	n.s.	*	/	n.s.	n.s.
Pi level* Inoculum type	n.s.	*	*	/	*	*

Table III.6 Significance of source of variation after two-way ANOVA for each parameter. The Sources of variance were Pi level (P10, P100) and Inoculum type (Ct/AM+/AM-) and their interactions: Pi level * inoculum type for the following parameters: shoot fresh weight (SFW), root fresh weight (RFW), total fresh weight (FW), mycorrhizal frequency (F%), intensity of mycorrhization in the root fragment (m%), arbuscule abundance in the root fragment (a%). * = $P < 0.05$; n.s. not significant. / = lack of homogeneity of variances. P10: 10 mg P/kg dry sand; P100: 100 mg P/kg dry sand; Ct: without inoculation; AM+: acclimatised strain; AM-: non-acclimatised strain.

Factors and their interaction	Parameters investigated		
	F%	m%	a%
Pi level	*	/	n.s.
Inoculum type	n.s.	/	n.s.
Pi level* Inoculum type	*	/	n.s.

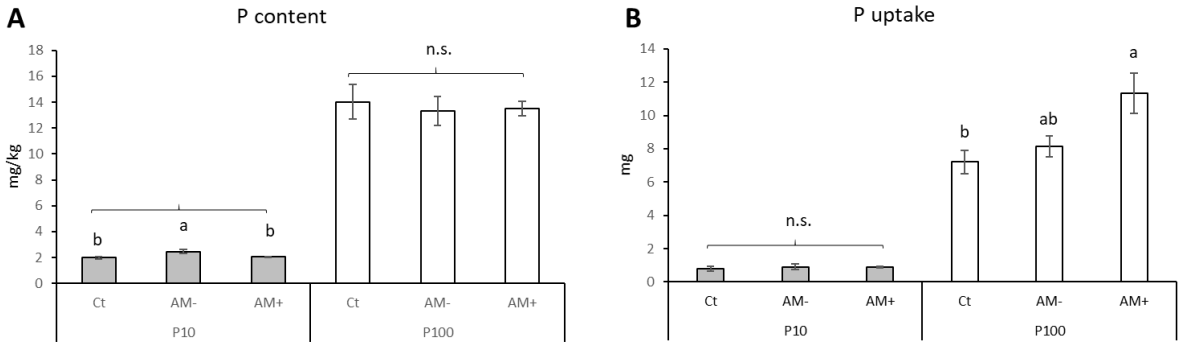

Figure III.8: P content and shoot P uptake of potato plantlets inoculated with AM- and AM+ strains, at two Pi fertilization levels. P content (A) and P uptake (B) in shoots of potato plants grown at different Pi fertilization levels (P10: 10 mg P/kg dry sand; P100: 10 mg P/kg dry sand) inoculated, or not (Ct) with acclimatised (AM+) or non-acclimatised (AM-) strain of *R. irregulare*. Figures shows means (n = 5) and standard errors. Treatments with same letter are not significantly different ($P < 0.05$; Tukey HSD, ANOVA). Two-tailed T-test (n = 5; P = 0.05) was performed for fungal parameters, comparing the strains within the same Pi fertilization level with the own control. * < 0.05.

III.2.3 Evaluation of the effect of inducer molecules on R. irregulare colonization and plant growth in greenhouse (regulation hypothesis)

III.2.3.1 Glucose

Total FW of mycorrhizal and non-mycorrhizal potato plantlets grown in greenhouse in presence of two Pi fertilization levels and three concentration of glucose (Glu 0, Glu I and Glu II), applied either in soil or on leaves are shown in Figure III.9 and Table III.9 A and B. Analysis of effects of the factors Pi level, Inoculation and Glucose and their interactions are shown in Table III.7. Total plant FW, when glucose was applied in soil, was not affected by the tripartite interaction of the factors. However, all the bipartite interactions between factors (Pi level * Inoculation; Pi level * Glucose soil; Inoculation * Glucose soil) were identified as variables affecting total plant FW. Total plant FW, when glucose was applied on leaves, was not affected by the tripartite interaction of the factors. Moreover, the factor Glucose in case of application on leaves never interacted with the other factors for the determination of total plant FW, while, as for soil application, Inoculation and Pi level strongly interacted with each other. The growth promoting effect of Pi fertilization was confirmed comparing the different total plant FW of the two control (Ct) groups.

Fungal parameters for plants treated with glucose in soil are shown in Figure III.9 C and E. Two-way ANOVA analysis (Table III.7) showed significant interaction between the two factors Glucose and Pi level for m% and A%. A single main effect of Pi level was always observed for all fungal parameters, confirming the negative effect of Pi fertilization for colonization. The factor Glucose, when applied in soil, showed significant single main effect for M% and for arbuscule presence (a% and A%). Specifically, investigation within the same Pi fertilizer concentration showed that at higher Pi fertilization, the higher dose of glucose promoted m%, a% and A% compared to control (Figure III.9 C and E). Two-way ANOVA analysis of leaf-treated plants did not show any interaction between the factors. Pi fertilization level confirmed its role for fungal parameters, while glucose leaf-application did not influence any fungal parameter.

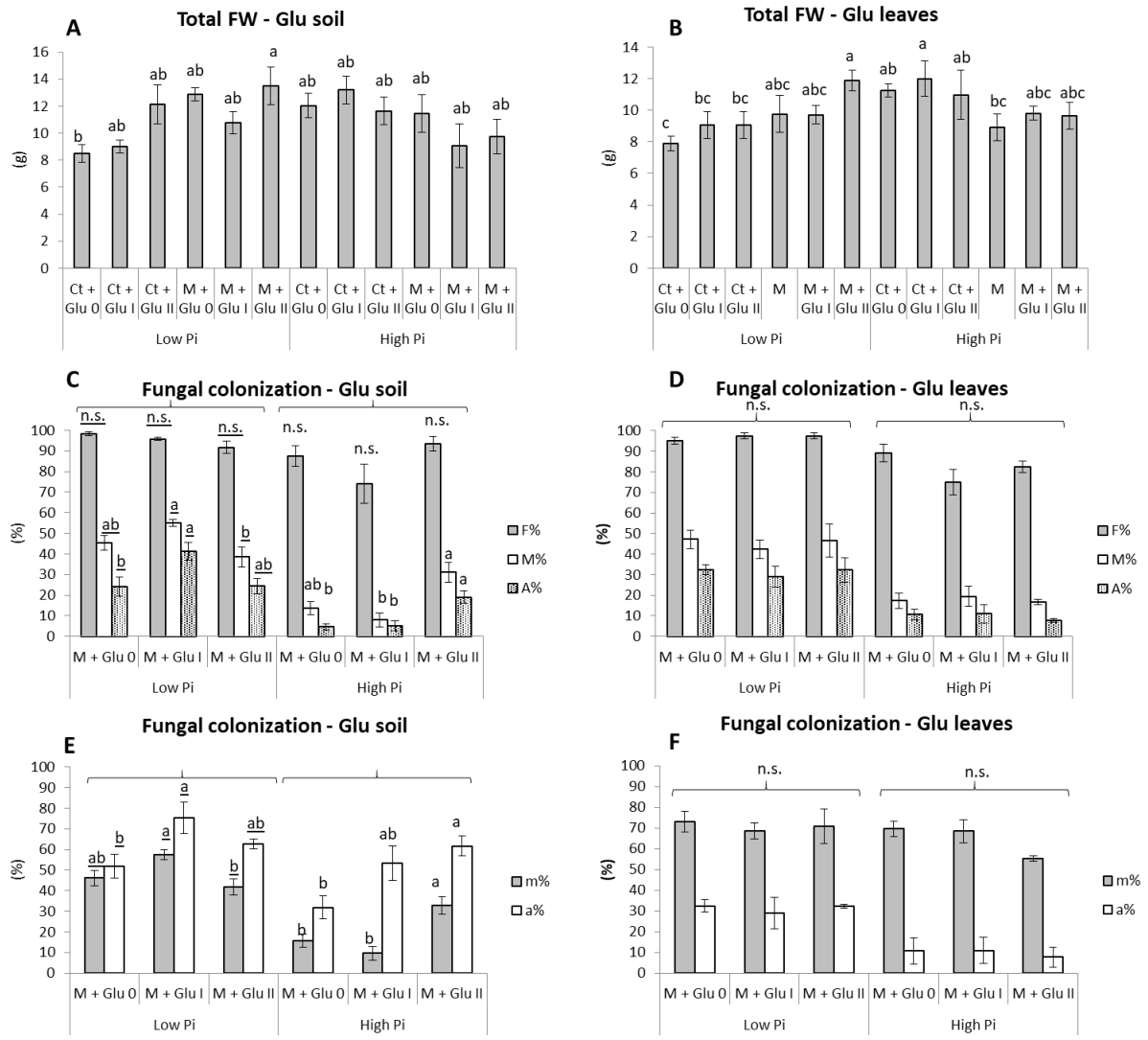

Figure III.9 Effect of glucose (Glu) applied in soil or on leaves on plant growth and root colonization at two Pi fertilization levels. Total fresh weight (A, B) and root colonization (C, D, E, F) of potato plantlets treated with glucose in soil or leaves at 0.05 mM (Glu I) and 0.50 mM (Glu II) or not treated, inoculated (M) or not (Ct) with AM fungi, in presence of two Pi fertilization levels: Low Pi (10 mg P/Kg dry sand) and High Pi (100 mg P/Kg dry sand). Fungal parameters: frequency of colonization (F%), intensity of colonization in the root system (M%), intensity of mycorrhization in the root fragment (m%), arbuscule abundance in the root fragment (a%) and arbuscule abundance in the root system (A%) according to Trouvelot et al., (1986), are shown in C, D, E, F. Shown are means (n = 5) and standard deviations for Total FW or standard error for fungal colonization. Treatments with the same letter are not significantly different ($P < 0.05$). Tukey HSD test were performed for figure A and B. Two different Tukey HSD tests, one for low Pi and one for High Pi, were performed for each parameter of fungal colonization. Effects of factors and interaction among the factors for the investigated parameters are shown in Table III.7.

Table III.7 Significance of source of variation after three-way or two-way ANOVA for each parameter. Sources of variance were Pi level (Low Pi, High Pi), Inoculation (Ct/M), Glucose (Glu 0, Glu I, Glu II) and their interactions. * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.0005$; n.s. not significant. Investigated parameters were: total fresh weight (FW), mycorrhiza frequency (F%), mycorrhiza intensity (M%), and arbuscule abundance (A%), accordingly to Trouvelot et al., 1986. Low Pi (10 mg P/Kg dry sand) and High Pi (100 mg P/Kg dry sand); Ct: without inoculation; M: inoculated; Glu 0: without glucose; Glu I: treated with 0.05 mM of glucose; Glu II: treated with 0.50 mM of glucose.

Glucose applied in soil – Plant parameters					
Factors and their interaction	Parameter investigated				
	Total FW				
Pi level	n.s.				
Inoculation	n.s.				
Glucose soil	*				
Pi level * Inoculation	***				
Pi level * Glucose soil	**				
Inoculation * Glucose soil	**				
Pi level * Inoculation * Glucose soil	n.s.				
Glucose applied on leaves – Plant parameters					
Factors and their interaction	Parameter investigated				
	Total FW				
Pi level	*				
Inoculation	n.s.				
Glucose leaves	*				
Pi level * Inoculation	***				
Pi level * Glucose leaves	n.s.				
Inoculation * Glucose leaves	n.s.				
Pi level * Inoculation * Glucose leaves	n.s.				
Glucose applied in soil – Fungal parameters					
Factors and their interaction	Parameter investigated				
	F%	M%	m%	a%	A%
Pi level	*	***	***	*	***
Glucose Soil	n.s.	***	n.s.	**	*
Pi level * Glucose soil	n.s.	n.s.	***	n.s.	*
Glucose applied on leaves – Fungal parameters					
Factors and their interaction	Parameter investigated				
	F%	M%	m%	a%	A%
Pi level	***	*	*	n.s.	*
Glucose leaves	n.s.	n.s.	n.s.	n.s.	n.s.
Pi level * Glucose leaves	n.s.	n.s.	n.s.	n.s.	n.s.

III.2.3.2 Xylose

Total FW for plants treated with three concentrations of xylose (Xyl 0, Xyl I and Xyl II) are shown in Figure III.10 A and B. The evaluation of the effects of the different factors and their interactions via three-way ANOVA showed a significant interaction of all the three factors in determining the observed differences with both xylose application methods (Table III.8).

Fungal colonization of plants treated with xylose in soil is shown in Figure III.10 C and E. Table III.8 shows only a simple main effect for Pi levels determining the values of all fungal parameters, but no interaction between the factors Pi level and Xylose. Analysis of fungal parameters within the same Pi fertilization level did not show significant differences. Fungal colonization in roots of plants treated with xylose on leaves is shown in Figure III.10 D and F. Here, the two factors Pi levels and Xylose interacted with each other for all fungal parameters (Table III.8). Specifically, at high Pi fertilization level, application of xylose at higher dose induced a promotion of m%, M% and A% compared to the other treatments (Figure III.10 D) and of a% compared to the lower tested dose (Figure III.10 F).

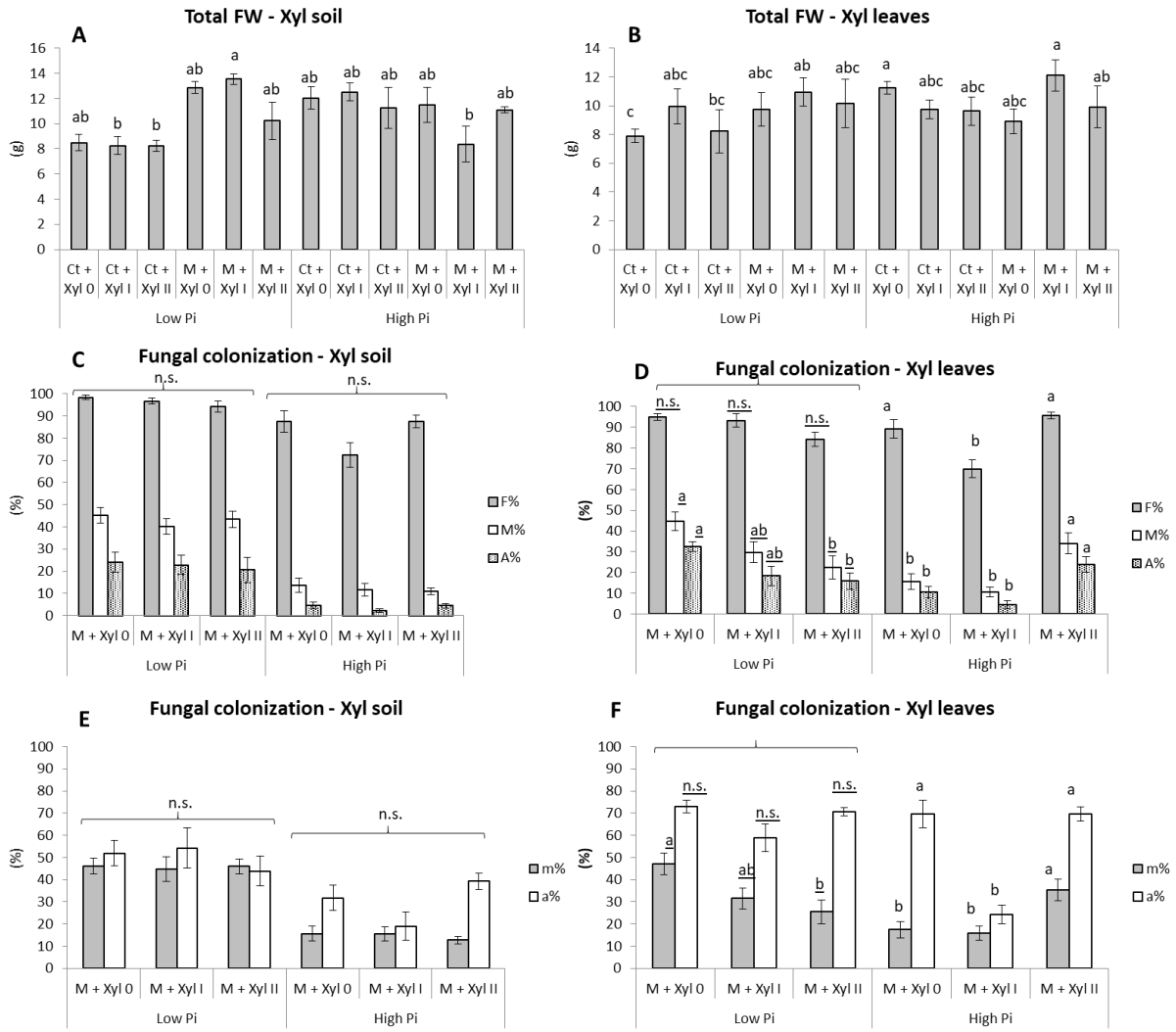

Figure III.10 Effect of xylose (Xyl) applied in soil or on leaves on plant growth and root colonization at two Pi fertilization levels. Total fresh weight (A, B) and root colonization (C, D) of potato plantlet treated with xylose in soil or leaves at 0.05 mM (Xyl I) and 0.50 mM (Xyl II) or not treated, inoculated (M) or not (Ct) with AM fungi, in presence of two Pi fertilization levels: Low Pi (10 mg P/Kg dry sand) and High Pi (100 mg P/Kg dry sand). Fungal parameters: frequency of colonization (F%), intensity of colonization in the root system (M%), intensity of mycorrhization in the root fragment (m%), arbuscule abundance in the root fragment (a%) and arbuscule abundance in the root system (A%) according to Trouvelot et al., (1986), are shown in C, D, E, F. Shown are means (n = 5) and standard deviations for total FW or standard error for fungal colonization. Treatments with the same letter are not significantly different ($P < 0.05$). Kruskal-Wallis test was performed for figure A and B. Two different Tukey HSD tests, one for Low Pi and one for High Pi, were performed for each parameter of fungal colonization. Effects of factors and interaction among the factors for the investigated parameters are shown in Table III.8.

Table III.8 significance of source of variation after two-way ANOVA for each parameter. Sources of variance were Pi level (Low Pi, High Pi), Inoculation (Ct/M), Xylose (Xyl 0, Xyl I, Xyl II) and their interactions. * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.0005$; n.s. not significant. Investigated parameters were: total fresh weight (FW), mycorrhiza frequency (F%), mycorrhiza intensity (M%), and arbuscule abundance (A%), accordingly to Trouvelot et al., 1986. Low Pi (10 mg P/Kg dry sand) and High Pi (100 mg P/Kg dry sand); Ct: without inoculation; M: inoculated; Xyl 0: without xylose; Xyl I: treated with 0.05 mM of xylose; Xyl II: treated with 0.50 mM of xylose.

Xylose applied in soil – Plant parameters					
Factors and their interaction	Parameter investigated				
	Total FW				
Pi level	*				
Inoculation	*				
Xylose soil	n.s.				
Pi level * Inoculation	***				
Pi level * Xylose soil	**				
Inoculation * Xylose	n.s.				
Pi level * Inoculation * Xylose soil	***				
Xylose applied in leaves – Plant parameters					
Factors and their interaction	Parameter investigated				
	Total FW				
Pi level	*				
Inoculation	*				
Xylose leaves	*				
Pi level * Inoculation	*				
Pi level * Xylose leaves	*				
Inoculation * Xylose	n.s.				
Pi level * Inoculation * Xylose leaves	*				
Xylose applied in soil – Fungal parameters					
Factors and their interaction	Parameter investigated				
	F%	M%	m%	a%	A%
Pi level	***	***	***	*	*
Xylose Soil	n.s.	n.s.	n.s.	n.s.	n.s.
Pi level * Xylose soil	n.s.	n.s.	n.s.	n.s.	n.s.
Xylose applied on leaves – Fungal parameters					
Factors and their interaction	Parameter investigated				
	F%	M%	m%	a%	A%
Pi level	n.s.	*	*	*	*
Xylose leaves	*	n.s.	n.s.	***	*
Pi level * Xylose leaves	*	**	*	*	**

III.2.3.3 Pipecolic acid

Total FW of plants treated with two concentrations of pipecolic acid (Pip I and Pip II) in soil are shown in Figure III.11 A. Three-way ANOVA analysis (Table III.9) confirmed the contribution of all the factors Pi level, Pipecolic acid and Inoculation in the determination of the FW of plants. Specifically, at low Pi fertilization, the presence of mycorrhiza alone, resulted in the expected promotion of plant biomass compared to Ct. On the contrary, inoculated plants treated with Pip I at high Pi fertilization showed a significant reduction of FW compared to Ct and all the other treatments. Total FW of plants treated with Pip on leaves is shown in Figure III.11 B. The effects of the three tested parameters Pi levels, Pipecolic acid and Inoculation on plant FW and their interactions (Table III.9) were in most cases not significant, and only an effect of the interaction between Inoculation and Pi level was detect. In general, no differences in plant FW were observed along the treatments.

Fungal colonization data for soil application of pipecolic acid are shown in Figure III.11 C and E. No interaction between Pi level and Pipecolic acid (application in soil) was observed with respect to fungal parameters (Table III.9). A strong simple main effect of Pi level was detected for all the fungal parameters. The factor Pipecolic acid application showed to have a simple main effect only on F% (Table III.9). Differences in fungal colonization within the same Pi fertilization level highlighted a general reduction associated with the higher pipecolic acid concentration for A% and m% compared to control and the lower pipecolic acid concentration. Fungal colonization of leaf-treated plants, instead, showed a strong interaction of the factors in determining the observed results (Table III.9). Values of fungal colonization within the same Pi fertilization level are shown in Figure III.11 D and F. Pip II applied with high Pi fertilization level induced an overall decrease of colonization values compared to control with the same Pi fertilization level, except for a%. Investigation within the same Pi fertilization level, indicated a progressive reduction of fungal colonization associated with the increasing concentration of pipecolic acid suggesting a dose effect of the compound at this condition (Figure III.11 D and F). At low Pi fertilization, the effect of leaf treatment followed a different pattern, with both pipecolic acid levels responsible for mycorrhizal inhibition compared to control. Inhibition that appeared stronger for Pip I compared to Pip II.

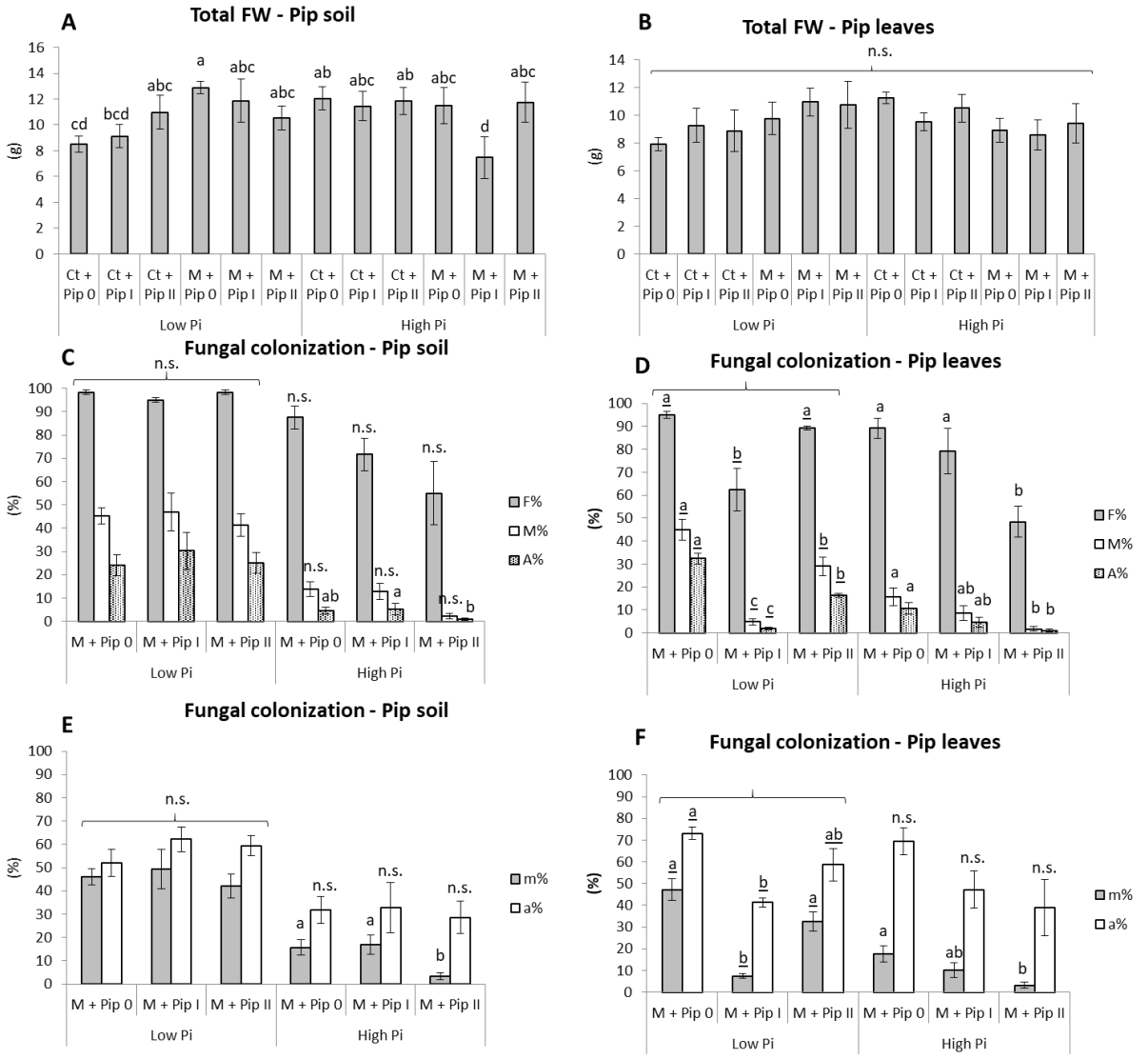

Figure III.11 Effect of pipecolic acid (Pip) applied in soil or on leaves on plant growth and root colonization at two Pi fertilization levels. Total fresh weight (A, B) and root colonization (C, D) of potato plantlet treated with Pipecolic acid in soil or leaves at 0.05 mM (Pip I) and 0.50 mM (Pip II) or not treated, inoculated (M) or not (Ct) with AM fungi, in presence of two Pi concentrations: Low Pi (10 mg P/Kg dry sand) and High Pi (100 mg P/Kg dry sand). Fungal parameters: frequency of colonization (F%), intensity of colonization in the root system (M%), intensity of mycorrhization in the root fragment (m%), arbuscule abundance in the root fragment (a%) and arbuscule abundance in the root system (A%) according to Trouvelot et al., (1986), are shown in C, D, E, F. Shown are means (n = 5) and standard deviations for Total FW or standard error for fungal colonization. Treatments with the same letter are not significantly different ($P < 0.05$). Tukey HSD test were performed for figure A and B. Two different Tukey HSD tests, one for low Pi and one for high Pi, were performed for each parameter of fungal colonization. Effects of factors and interaction among the factors for the investigated parameters are shown in Table III.9.

Table III.9 Significance of source of variation after three-way ANOVA for each parameter. Sources of variance were Pi level (Low Pi, High Pi), Inoculation (Ct/M), Pipecolic acid (Pip 0, Pip I, Pip II) and their interactions. * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.0005$; n.s. not significant. Investigated parameters were: total fresh weight (FW), mycorrhiza frequency (F%), mycorrhiza intensity (M%), and arbuscule abundance (A%), accordingly to Trouvelot et al., 1986. Low Pi (10 mg P/Kg dry sand) and High Pi (100 mg P/Kg dry sand); Ct: without inoculation; M: inoculated; Pip 0: without pipecolic acid; Pip I: treated with 0.05 mM of pipecolic acid; Pip II: treated with 0.50 mM of pipecolic acid.

Pipecolic acid applied in soil – Plant parameters					
Factors and their interaction	Parameter investigated				
	Total FW				
Pi level	n.s.				
Inoculation	n.s.				
Pipecolic acid soil	**				
Pi level * Inoculation	***				
Pi level * Pipecolic acid soil	*				
Inoculation * Pipecolic acid soil	*				
Pi level * Inoculation * Pipecolic acid soil	**				
Pipecolic acid applied on leaves – Plant parameters					
Factors and their interaction	Parameter investigated				
	Total FW				
Pi level	n.s.				
Inoculation	n.s.				
Pipecolic acid leaves	n.s.				
Pi level * Inoculation	***				
Pi level * Pipecolic acid leaves	n.s.				
Inoculation * Pipecolic acid leaves	n.s.				
Pi level * Inoculation * Pipecolic acid leaves	n.s.				
Pipecolic acid applied in soil – Fungal parameters					
Factors and their interaction	Parameter investigated				
	F%	M%	m%	a%	A%
Pi level	*	***	***	***	***
Pipecolic acid Soil	*	n.s.	n.s.	n.s.	n.s.
Pi level * Pipecolic acid soil	n.s.	n.s.	n.s.	n.s.	n.s.
Pipecolic acid applied on leaves – Fungal parameters					
Factors and their interaction	Parameter investigated				
	F%	M%	m%	a%	A%
Pi level	n.s.	***	***	n.s.	***
Pipecolic acid leaves	**	***	***	**	***
Pi level * Pipecolic acid leaves	**	***	***	n.s.	***

III.2.3.4 Azelaic acid

Total FW of potato plantlets grown in presence of three concentrations of azelaic acid (Aze 0, Aze I and Aze II) in soil are shown in Figure III.12 A. Three-way ANOVA analysis (Table III.10) highlighted the lack of the trifold interaction effect in the determination of total FW. However, significant interaction of Pi level with Inoculation and Pi level with Azelaic acid were observed. Moreover, all factors showed significant simple effects with respect to total FW. Total FW of plants sprayed with azelaic acid on leaves are shown in Figure III.12 B. Also here (Table III.10), no significant trifold interaction of the factors in the determination of FW was observed. However, all factors showed to determine FW both, as single main effect and in twofold interactions among the factors. Non-mycorrhizal plants (Ct) treated with high azelaic acid concentrations (Aze II) at high Pi fertilization showed the highest plant FW compared to all other treatments.

Fungal colonization data for soil application of azelaic acid are presented in Figure III.12 C and E. Investigation of effects on root colonization underlined a simple main effect of Pi in the determination of all fungal parameters (Table III.10). Simple main effects were observed for Pi levels, which induced the well-known inhibition in AM fungi development. Simple main effect of Azelaic acid was observed even for the intensity of mycorrhization, that was influenced in both parameters (M% and m%). No significant interaction between the factors emerged from the analysis. Investigation of colonization parameters within the same Pi level did not highlight the presence of any differences in any of the investigated parameters. Fungal colonization data for leaf application of azelaic acid are presented in Figure III.12 D and F. Also here, Azelaic acid showed a simple main effect, but for A% (Table III.10), while Pi levels had a more intense main effect observed for M%, m% and A%. No significant factor interaction was observed and differences within the same Pi level were also not detected.

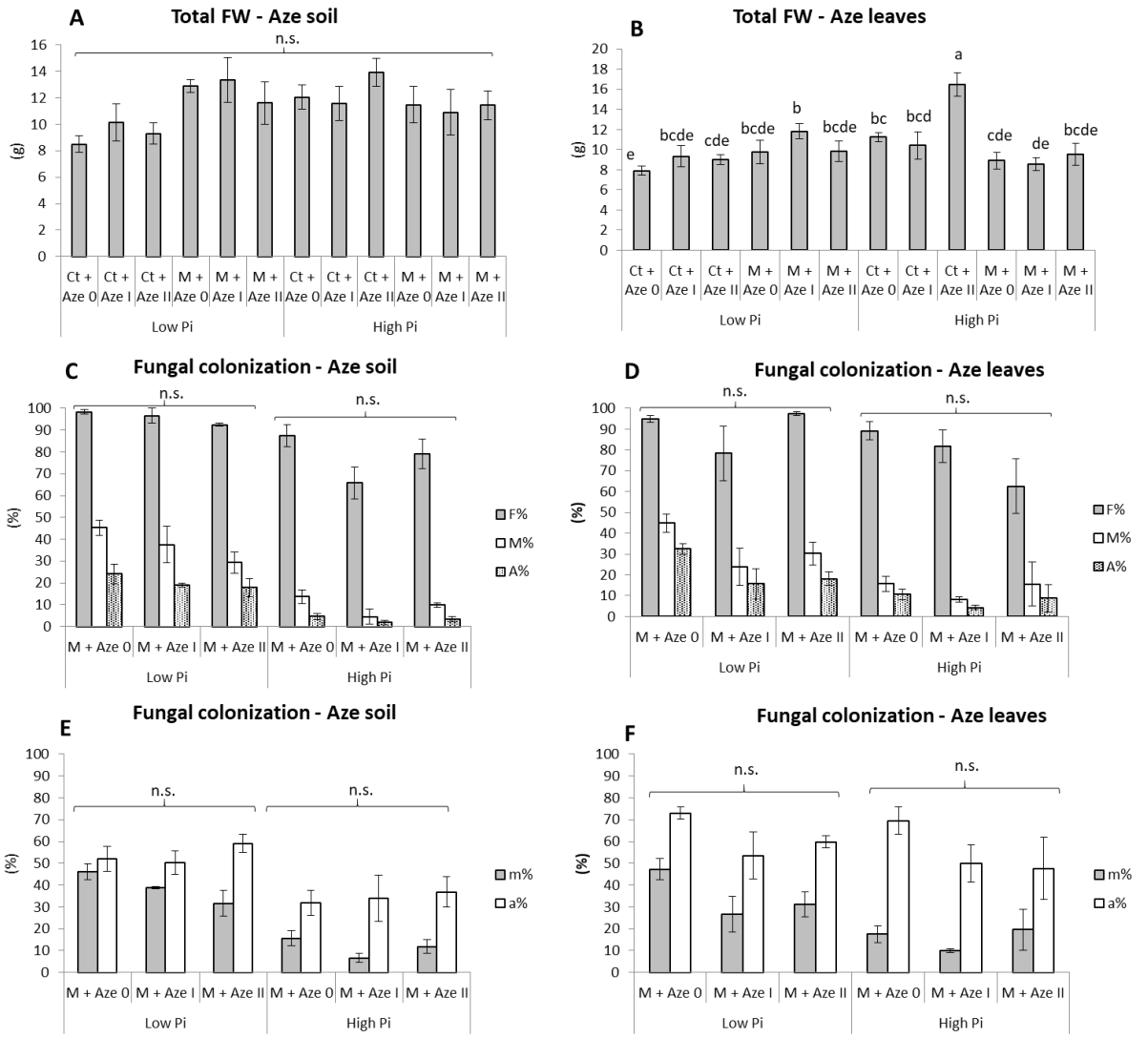

Figure III.12 Effect of Azelaic acid (Aze) applied in soil or on leaves on plant growth and root colonization at two Pi fertilization levels. Total fresh weight (A, B) and root colonization (C, D) of potato plantlet treated with azelaic acid in soil or leaves at 0.05 mM (Aze I) and 0.50 mM (Aze II) or not treated, inoculated (M) or not (Ct) with AM fungi, in presence of two Pi concentrations: Low Pi (10 mg P/Kg dry sand) and High Pi (100 mg P/Kg dry sand). Fungal parameters: frequency of colonization (F%), intensity of colonization in the root system (M%), intensity of mycorrhization in the root fragment (m%), arbuscule abundance in the root fragment (a%) and arbuscule abundance in the root system (A%) according to Trouvelot et al., (1986), are shown in C, D, E, F. Shown are means (n = 5) and standard deviations for Total FW or standard error for fungal colonization. Treatments with the same letter are not significantly different ($P < 0.05$). Kruskal-Wallis test was performed for A and B. Two different Tukey HSD tests, one for low Pi and one for high Pi, were performed for each parameter of fungal colonization. Effects of factors and interaction among the factors for the investigated parameters are shown in Table III.10.

Table III.10 significance of source of variation after two-way ANOVA for each parameter. Sources of variance were Pi level (Low Pi, High Pi), Inoculation (Ct/M), and azelaic acid levels (Aze 0, Aze I, Aze II) and their interactions. * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.0005$; n.s. = not significant. Investigated parameters were: total fresh weight (FW), mycorrhiza frequency (F%), mycorrhiza intensity (M%), and arbuscule abundance (A%), accordingly to Trouvelot et al., 1986. Low Pi (10 mg P/Kg dry sand) and High Pi (100 mg P/Kg dry sand); Ct: without inoculation; M: inoculated; Aze 0: without azelaic acid; Aze I: treated with 0.05 mM of azelaic acid; Aze II: treated with 0.50 mM of azelaic acid.

Azelaic acid applied in soil – Plant parameters					
Factors and their interaction	Parameter investigated				
	Total FW				
Pi level	*				
Inoculation	*				
Azelaic acid soil	**				
Pi level * Inoculation	***				
Pi level * Azelaic acid soil	*				
Inoculation * Azelaic acid soil	n.s.				
Pi level * Inoculation * Azelaic acid soil	n.s.				
Azelaic acid applied on leaves – Plant parameters					
Factors and their interaction	Parameter investigated				
	Total FW				
Pi level	*				
Inoculation	*				
Azelaic acid leaves	*				
Pi level * Inoculation	***				
Pi level * Azelaic acid leaves	*				
Inoculation * Azelaic acid leaves	***				
Pi level * Inoculation * Azelaic acid leaves	n.s.				
Azelaic acid applied in soil – Fungal parameters					
Factors and their interaction	Parameter investigated				
	F%	M%	m%	a%	A%
Pi level	***	***	***	**	***
Azelaic acid soil	n.s.	*	*	n.s.	n.s.
Pi level * Azelaic acid soil	n.s.	n.s.	n.s.	n.s.	n.s.
Azelaic acid applied on leaves – Fungal parameters					
Factors and their interaction	Parameter investigated				
	F%	M%	m%	a%	A%
Pi level	n.s.	*	*	n.s.	*
Azelaic acid leaves	n.s.	n.s.	n.s.	n.s.	*
Pi level * Azelaic acid leaves	n.s.	n.s.	n.s.	n.s.	n.s.

III.3 Molecular investigations

The investigations carried out on the two hypotheses on which this work is based on, showed interesting phenotypic patterns, however, investigations at molecular level were necessary to better understand the phenomena observed.

With regards to the acclimatisation hypothesis, the differences observed at the level of phenotypic development suggested a certain ability of the fungus to respond to the presence of Pi. The hypothesis is that the phenomenon underlying the observed differences is acclimatisation, which involves the differential expression of certain genes. In the absence of time to sequence and analyse the entire RNA, some target genes were selected based on literature to test whether their differential expression was the basis of the observed differences. The starting material from which the RNA was extracted were the roots of plants from the greenhouse experiment with the fifth generation of the acclimatised (AM+) and non-acclimatised (AM-) strain (section III.2.2).

Regarding the regulation hypothesis, the differences observed at the level of colonisation in the plants grown in the greenhouse led to the selection of two molecules, an activator of the SAR pathway (Pipecolic acid), capable of reducing mycorrhizal colonisation; and an activator of the ISR pathway (Xylose) able to promote mycorrhizal colonisation. To confirm that the selected molecules act as inducers of one or other defence response, the genes related to these responses were analysed (section III.3.5), starting from the roots of the plant treated with Pipecolic acid and Xylose (section III.2.3).

III.3.1 RNA accumulation of genes involved in cell cycle regulation of AM+ and AM- strains (acclimatisation hypothesis)

Analysis of RNA accumulation of genes encoding factors involved in cell cycle regulation, mitosis and replication are shown in Figure III.13. Two-Way ANOVA analysis showed the absence of interaction effects between the factors Pi levels and Inoculum type for any of the investigated genes (Table III.11). A significant simple main effect of Pi level was identified in determination of the expression of *RiDPD4* encoding a DNA polymerase delta subunit 4 and *RiPCNA* encoding a proliferating cell nuclear antigen (Table III.11). However, no differences were observed in the expression of genes between the two strains within the same Pi level, indicating absence of difference in regulation between the AM+ and AM- strains.

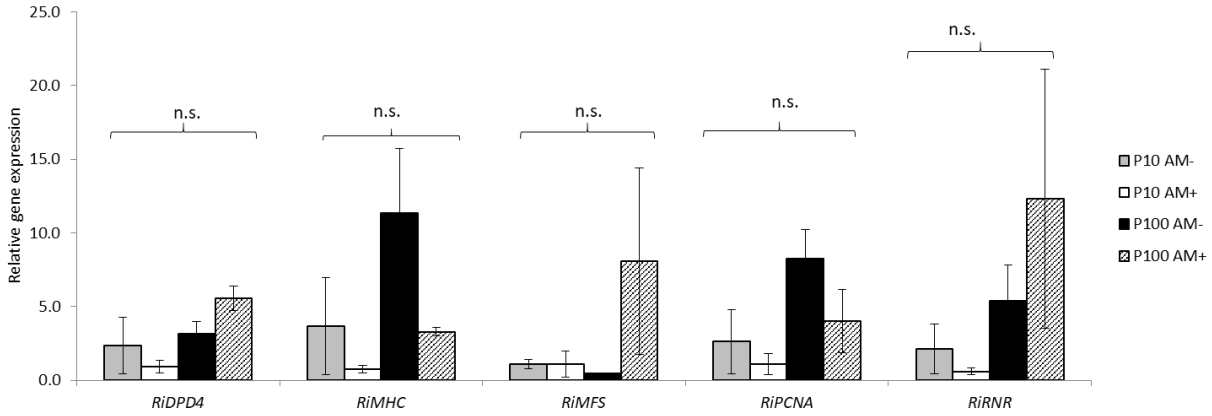

Figure III.13 Relative gene expression of acclimatised (AM+) and non- acclimatised (AM-) strains in presence of mycorrhiza-non-inhibiting (P10) or mycorrhiza-inhibiting Pi levels (P100). Two-tailed T-test ($n = 3$, $P < 0,05$) was performed to test difference in the expression of single genes between AM+ and AM- at P10 or P100 conditions. Shown are means and standard deviations. 'n.s'. indicates no significant differences. Selected genes code for DNA polymerase delta subunit 4 (*RiDPD4*), myosin II heavy chain (*RiMHC*), major facilitator superfamily (*RiMFS*), proliferating cell nuclear antigen (*RiPCNA*), and ribonucleutide reductase (*RiRNR*).

Table III.11 Significance of source of variation after two-way ANOVA for each gene. Sources of variance were Pi level (P10, P100), inoculum type (AM+/AM-) and their interaction. * = $P < 0.05$; n.s. = not significant. P10: 10 mg P/kg dry sand; P100: 100 mg P/kg dry sand; Ct: without inoculation; AM+: acclimatised strain; AM-: non-acclimatised strain.

Factors and their interaction	**Parameters investigated**				
	RiDPD4	*RiMHC*	*RiMFS*	*RiPCNA*	*RiRNR*
Pi level	*	n.s.	n.s.	*	n.s.
Inoculum type	n.s.	n.s.	n.s.	n.s.	n.s.
Pi level * Inoculum type	n.s.	n.s.	n.s.	n.s.	n.s.

III.3.2 RNA accumulation of genes involved in antioxidant activities of AM+ and AM- strains (acclimatisation hypothesis)

RNA accumulation of genes encoding enzymes involved in the scavenging of reactive oxygen species are shown in Figure III.14. Two-Way ANOVA analysis indicated the absence of interaction between the factors in modulating gene expression (Table III.12). Simple main effect of Pi level was identified to determine the expression of *RiGST* in the two fertilizations regimes tested (Table III.12). However, no differences emerged between the two strains under the same Pi condition, neither for *RiGST* encoding a glutathione S-transferase nor for *RiSOD* encoding a superoxide dismutase (*RiGST*).

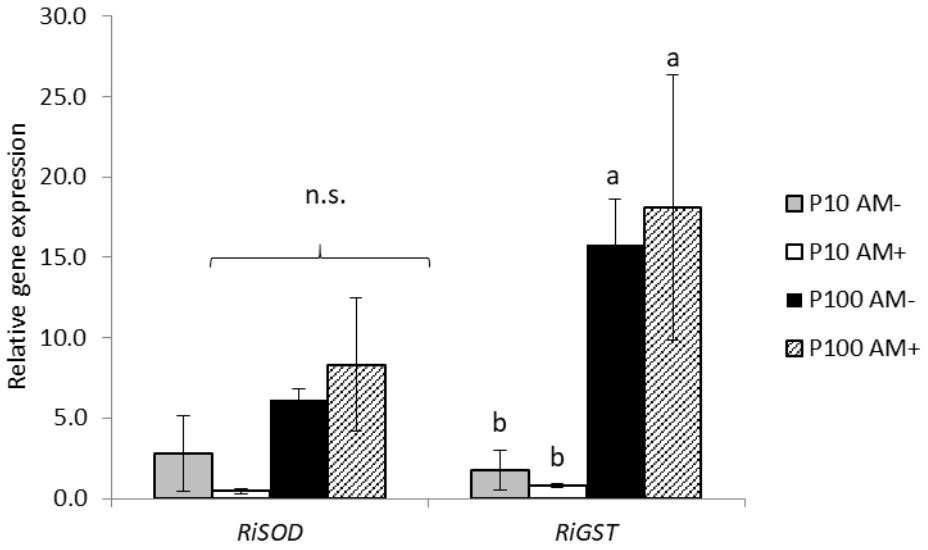

Figure III.14 Relative gene expression of acclimatised (AM+) and non- acclimatised (AM-) strains in presence of mycorrhiza-non-inhibiting (P10) or mycorrhiza-inhibiting Pi levels (P100). Two-tailed T-test ($n = 3$, $P < 0.05$) was performed to test difference in the expression of single genes between AM+ and AM- at P10 or P100 conditions. Shown are means and standard deviations. 'n.s.' indicates no significant differences. Selected genes code for a superoxide dismutase (*RiSOD*) and a glutathione S-transferase (*RiGST*).

Table III.12 significance of source of variation after two-way ANOVA for each gene. Sources of variance were Pi level (P10, P100), inoculum type (AM+/AM-) and their interaction. * = $P < 0.05$; n.s. = not significant. P10: 10 mg P/kg dry sand; P100: 100 mg P/kg dry sand; Ct: without inoculation; AM+: acclimatised strain; AM-: non-acclimatised strain.

Factors and their interaction	**Parameters investigated**	
	RiGST	*RiSOD*
Pi level	*	n.s.
Inoculum type	n.s.	n.s.
Pi level * Inoculum type	n.s.	n.s.

III.3.3 RNA accumulation analysis of genes involved in respiration of AM+ and AM- strains (acclimatisation hypothesis)

RNA accumulation of genes coding for AOX and COX are shown in Figure III.15. Two-Way ANOVA analysis showed absence of interaction between the factors Pi level and Inoculum type in modulating the expression of the investigated genes. Simple main effect was observed for Pi level, as factor able to modulate the expression of *RiAOX* encoding an alternative oxidase (Table III.13), but no differences emerged between the two strains. No other differences were observed between the investigated factors and their interactions in *RiCOX* expression encoding a cytochrome oxidase.

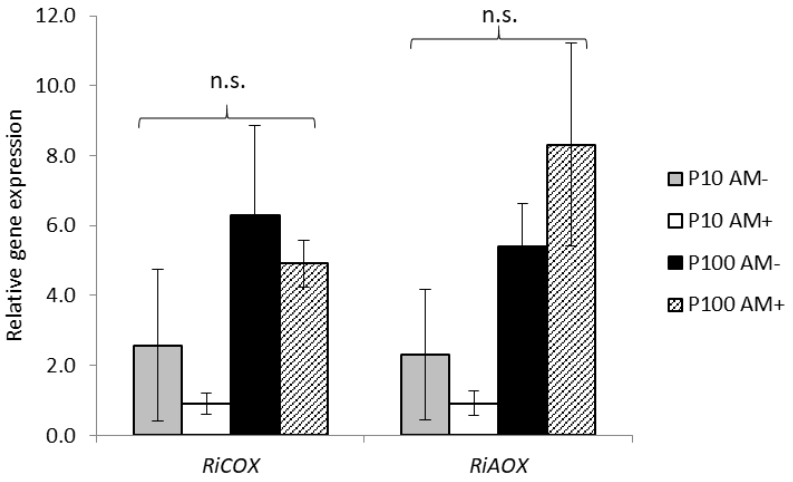

Figure III.15 Relative gene expression of acclimatised (AM+) and non- acclimatised (AM-) strains in presence of mycorrhiza-non-inhibiting (P10) or mycorrhiza-inhibiting Pi levels (P100). Two-tailed T-test (n = 3, P < 0.05) was performed to test difference in the expression of single genes between AM+ and AM- at P10 or P100 conditions. Shown are means and standard deviations. 'n.s.' indicates no significant differences. Selected genes code for a cytochrome oxidase (*RiCOX*) and an alternative oxidase (*RiAOX*).

Table III.13 significance of source of variation after two-way ANOVA for each gene. Sources of variance were Pi level (P10, P100), inoculum type (AM+/AM-) and their interaction. * = P < 0.05; n.s. = not significant. P10: 10 mg P/kg dry sand; P100: 100 mg P/kg dry sand; Ct: without inoculation; AM+: acclimatised strain; AM-: non-acclimatised strain.

Factors and their interaction	**Parameters investigated**	
	RiCOX	*RiAOX*
Pi level	n.s.	*
Inoculum type	n.s.	n.s.
Pi level * Inoculum type	n.s.	n.s.

III.3.4 RNA accumulation of genes encoding transporters of AM+/AM- strains (acclimatisation hypothesis)

Regulation of transcription of genes encoding for phosphate transporters (*RiPT1-7*) and sugar transporter (*RiMST*) between plant and AM fungus are shown in Figure III.16 and Figure III.17. Two Way ANOVA analysis (Table III.14) showed significant interaction of Pi level and Inoculum type for gene expression of *RiPT6*. This gene was differentially regulated even within the same Pi level. Simple main effect of Pi level was observed as determining *RiPT5* and *RiPT7* expression showing a general induction of these genes. *RiPT1* and *RiPT3* expression was not influenced by the interaction of the studied factors, nor by their simple main effects. Also, the expression of the sugar transporter RiMST2 was affected by acclimatisation, in which the AM- strain possessed a higher expression of *RiMST2* compared to the AM+ strain at high Pi concentrations in the fertilizer.

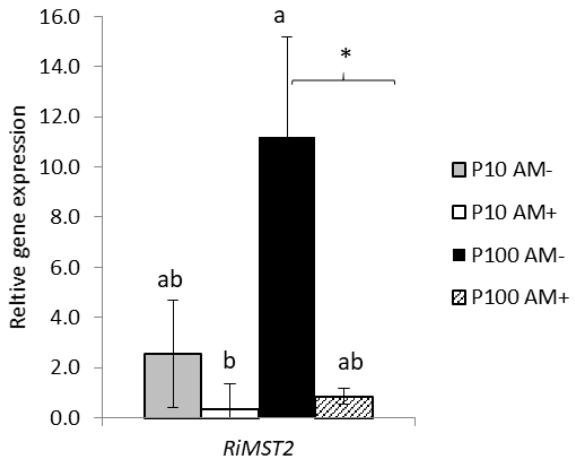

Figure III.16 Relative gene expression of acclimatised (AM+) and non- acclimatised (AM-) strains in presence of mycorrhiza-non-inhibiting (P10) or mycorrhiza-inhibiting Pi levels (P100). Two-tailed T-test (n = 3, P < 0.05) was performed to test difference in the expression between AM+ and AM- in low or high Pi. Shown are means and standard deviations. 'n.s.' indicates no significant differences. Selected gene codes for a sugar transporter (*RiMST2*).

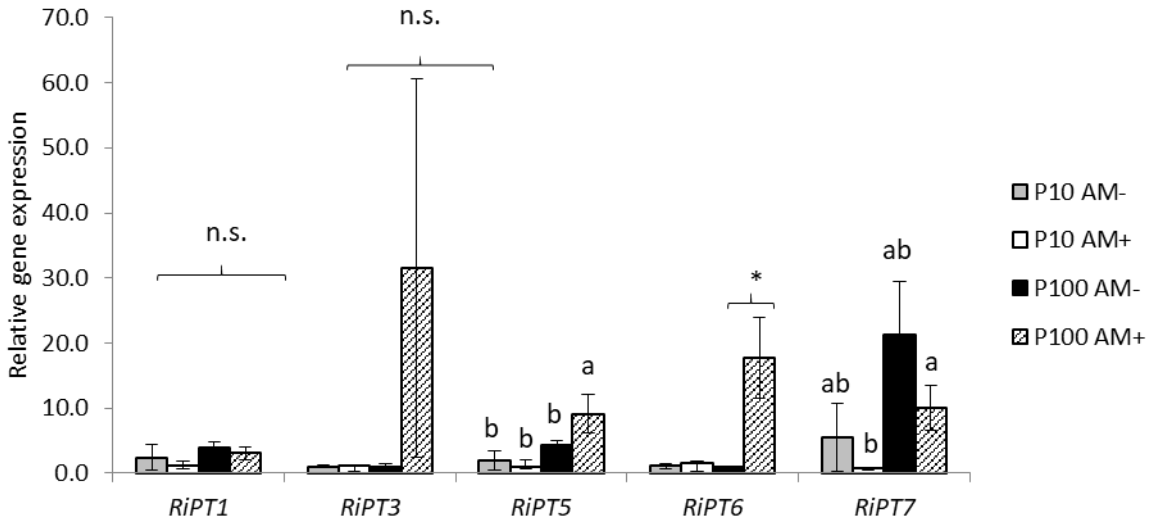

Figure III.17 Relative gene expression of acclimatised (AM+) and non- acclimatised (AM-) strains in presence of mycorrhiza-non-inhibiting (P10) or mycorrhiza-inhibiting Pi levels (P100). Two-tailed T-test (n = 3, P < 0.05) was performed to test difference in the expression of single genes between AM+ and AM- in low or high Pi. Shown are means and standard deviations. 'n.s'. indicates no significant differences. Selected genes code for plasma membrane phosphate transporter (RiPT1, 3, 4, 5, 6, 7)

Table III.14 significance of source of variation after two-way ANOVA for each gene. Sources of variance was Pi level (P10, P100), inoculum type (AM+/AM-) and their interaction. * = $P < 0.05$; n.s.= not significant. P10: 10 mg P/kg dry sand; P100: 100 mg P/kg dry sand; Ct: without inoculation; AM+: acclimatised strain; AM-: non-acclimatised strain.

Factors and their interaction	Parameter investigated					
	RiMST2	*RiPT1*	*RiPT3*	*RiPT5*	*RiPT6*	*RiPT7*
Pi level	n.s.	n.s.	n.s.	*	*	*
Inoculum type	*	n.s.	n.s.	n.s.	*	n.s.
Pi level* Inoculum type	n.s.	n.s.	n.s.	n.s.	*	n.s.

III.3.5 Evaluation of defence related gene activation (regulation hypothesis)

Data on relative expressions of genes encoding the pathogenesis-related proteins PR2 and PR6 investigated for the JA defence pathway or encoding the pathogenesis-related protein PR1, PR3 and a glutathione S-transferase investigated for the SA defence pathway are shown in Figure III.18. Two of the three genes selected for the SA response were significantly differentially regulated after pipecolic acid spray application, suggesting SA-related activation. *StPR1* were instead not significantly differently expressed compared to control or xylose-treated plant. Investigation of gene associated to JA response showed no significant difference among the treatments.

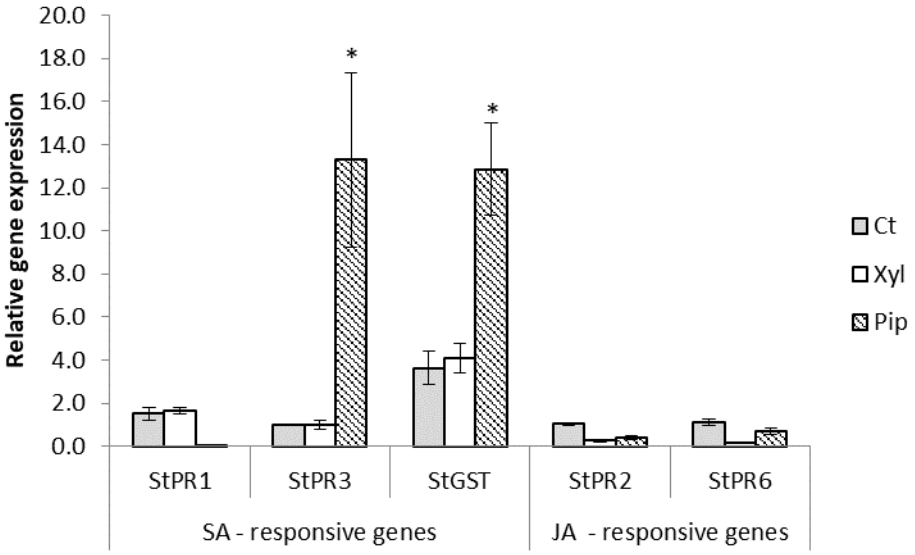

Figure III.18 Relative gene expression for SA- or JA- responsive genes in inoculated plants sprayed with pipecolic (Pip) acid or xylose (Xyl) on leaves. Two-tailed T-test ($n = 3$, $P < 0,05$) was performed to test difference in the expression of single genes between xylose- or pipecolic-treated plants compared to control (Ct) in presence of high Pi fertilizer concentrations. Shown are means and standard deviations. * indicates significant differences compared to control. Selected genes code for the pathogenesis-related proteins PR1, PR2, PR3 and PR6 and for a glutathione S-transferase.

III.4 Field experiments

The two hypotheses proposed and tested in this work had the aim of identifying a strategy to be implemented under field conditions. The experiments described below test the effectiveness of the proposed hypotheses under field conditions.

For the acclimatisation thesis, it was decided to operate under semi-field conditions in 10-litre containers filled with local soil and left out in the open. The choice of pots was necessary to better control the fertilisation and inoculation of the plants. Inoculation was carried out with spores of the acclimatised (AM+) and non-acclimatised (AM-) strains. Since natural soil was used, the presence of native mycorrhizae was also observed.

The regulation thesis, on the other hand, was carried out in the open field with the support of a local farmer. Due to the randomised organisation of the experiment, inoculation was carried out by hand, using a commercial inoculum. The treatment with the regulators molecules was carried out with a knapsack sprayer one month after sowing. A first sampling of the roots was carried out to evaluate the colonisation after two months, the period in which the colonisation was considered to be maximum. The final harvest was carried out together with the farmer, and commercial parameters were evaluated for the chosen potato cultivar.

III.4.1 Field evaluation of acclimatised inoculum (acclimatisation hypothesis)

Growth parameters and root colonization of potato plantlets inoculated with *in vitro*-acclimatised inoculum (AM+) or with *in vitro*-non-acclimatised inoculum (AM-) is shown in Figure III.19. No differences in any component of plant biomass were observed with respect of the investigated parameters (Figure III.19 A, B, C, D). Analysis of the factors Inoculum type and Fertilization regime did neither reveal an interaction between the factors nor a significant influence of inoculation on any aspect of plants biomass (Table III.15). However, a single main effect of Fertilization regime was detected for SFW, SDW, total FW, total DW and yield indicating that Pi generally induced a promotion of plant growth.

Data on fungal colonization are shown in Figure III.19 E, F, G, H and I. A differential colonization pattern was observed among the different strains in the two fertilizer regimes (Figure III.19 E, F, G, H). In non-fertilized soil, the AM+ strain showed significant higher M% compared to the control, significant higher A% compared to both, control and the AM- strain, and higher a% compared to the AM- strain. No differences were observed for the parameters F% and m% at this fertilizer soil condition. At full fertilization conditions, the AM+ strain showed significant higher values for F% and M% compared to control and AM- strain-inoculated plants. No differences were observed for the other fungal parameters among the three treatments with full fertilization. No interactions between factors were observed in determining the fungal value after Two-Way ANOVA analysis (Table III.15). Figure III.19Higher colonization was not associated with any differences in P uptake and P level (Figure III.19) with respect to different inocula and different fertilization regimes.

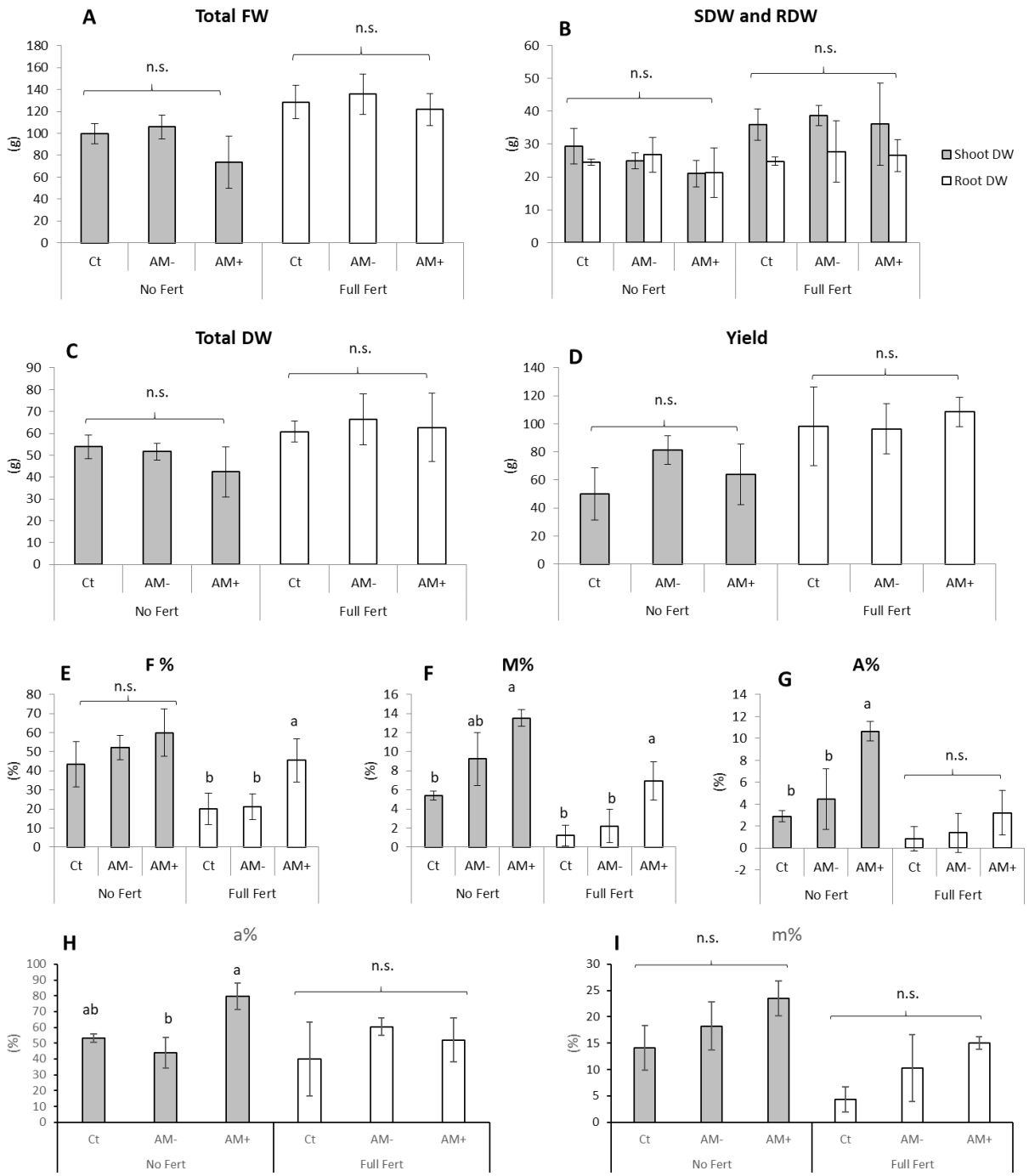

Figure III.19 Plant and fungal parameters of potato plantlets inoculated with AM+ or AM- strains at different fertilization regimes. Total fresh weight (FW), shoot and root dry weight (SDW and RDW), total dry weight and yield (A-D) of potato plants grown without additional fertilization (No Fert) or with additional fertilization (Full Fert) and inoculated with an acclimatised (AM+) or a non-acclimatised (AM-) strain of *R. irregulare,* or non-inoculated (Ct). Root colonization parameters F%, M%, A%, m% and a% are shown in E, F, G, H and I, respectively. Figure shows means (n = 5), and standard deviation. Treatments with the same letter are not significantly different ($P < 0.05$; Tukey HSD, ANOVA).

Table III.15 Sources of variance were Pi level (No Fert, Full Fert) and Inoculum type (AM+/AM-) and their interactions. * = $P < 0,05$; n.s. = not significant. No Fert: without additional fertilization; Full Fert: with additional fertilization; Ct: non-inoculated; AM-: inoculated with non-acclimatised strain; AM+: inoculated with acclimatised strain.

Factors and their interaction	Parameters investigated						
	SFW	RFW	Total FW	SDW	RDW	Total DW	Yield
Pi level	*	n.s.	*	*	n.s.	*	*
Inoculum type	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Pi level * Inoculum type	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table III.16 Sources of variance were Pi level (No Fert, Full Fert) and Inoculum type (AM+/AM-) and their interactions. * = $P < 0,05$; n.s. not significant. No Fert: etc. (see above) No Fert: without additional fertilization; Full Fert: with additional fertilization; Ct: non-inoculated; AM-: inoculated with non-acclimatised strain; AM+: inoculated with acclimatised strain.

Factors and their interaction	Parameters investigated				
	F%	M%	m%	a%	A%
Pi level	n.s.	n.s.	n.s.	n.s.	n.s.
Inoculum type	n.s.	n.s.	n.s.	n.s.	n.s.
Pi level * Inoculum type	n.s.	n.s.	n.s.	n.s.	n.s.

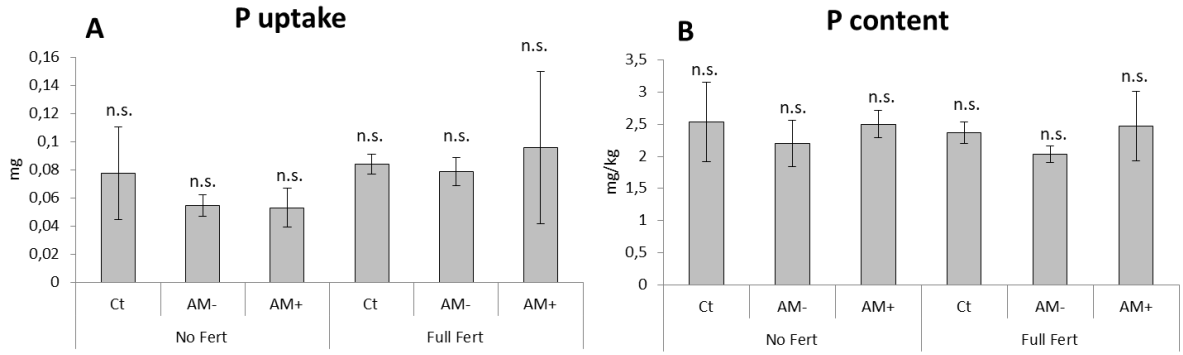

Figure III.20 P concentrations and shoot P content of potato plantlet inoculated with AM- and AM+ strains, at different fertilization regimes. P uptake per plant (A) and shoot P content (B) in shoots of potato plants grown without additional fertilization (No Fert) or with additional fertilizer (Full Fert) and inoculated with an acclimatised (AM+) or a non-acclimatised (AM-) strain of *R. irregulare,* or non-inoculated (Ct). Figure shows means ($n = 3$), and standard error. Treatments were not significantly different (n.s.; $P < 0.05$; Tukey HSD, ANOVA).

III.3.4 Evaluation of regulators effect at field conditions (regulation hypothesis)

No fungal colonization was observed in the mid-term harvest of potato plants in fertilized and non-fertilized soils. Stained roots did not show any presence of fungal hypha, or fungal structure even if inoculum and regulators were applied (data not shown). The occurrence and amount of native mycorrhiza assessed via most probable number (MPN) test is shown in Table III.17. Trace of inoculation was observed only in the first dilution, and the MPN of propagules per litre was quantified with a value of 0.17.

Table III.17 Most probable number test of field soil.

Dilution fold	Repetitions	Positive samples	Total propagules/litre
1	5	2	0.17
10	5	0	
100	5	0	
1000	5	0	
10000	5	0	

Data on final harvest in the non-fertilized and fertilized field are presented in Figure III.21 and III.22. No significant differences were observed in terms of mass of tuber production or starch content among the different treatments at both fertilization treatments.

Comparison of harvest data between fertilized and non-fertilized field are shown in Figure III.23. Fertilization of the soil induced an overall increase of yield of around 20% compared to non-fertilized soil. The increase of the yield is to be attributed to the contribution of big tubers, which doubled their weight compared to the tubers harvested from non-fertilized soil. The small tubers biomass remained constant in the two fertilization regimes. In terms of starch accumulation, the increase of biomass observed in fertilized soil led to a significant dilution effect, therefore concentration of starch was significantly higher in tubers from non-fertilized soil. However, in terms of total starch production, fertilization exerted a significant positive effect with 13% higher overall starch production compared to the production in non-fertilized soil due to the higher yield.

The effect of inducer molecules sprayed on plant's leaves was investigated for the effect in reducing *R. solani* incidence on tubers. Data of disease rate are shown in Figure III.24. No significant differences were observed in the amount of sclerotia in the tuber among the different treatments in the two fertilized regimes.

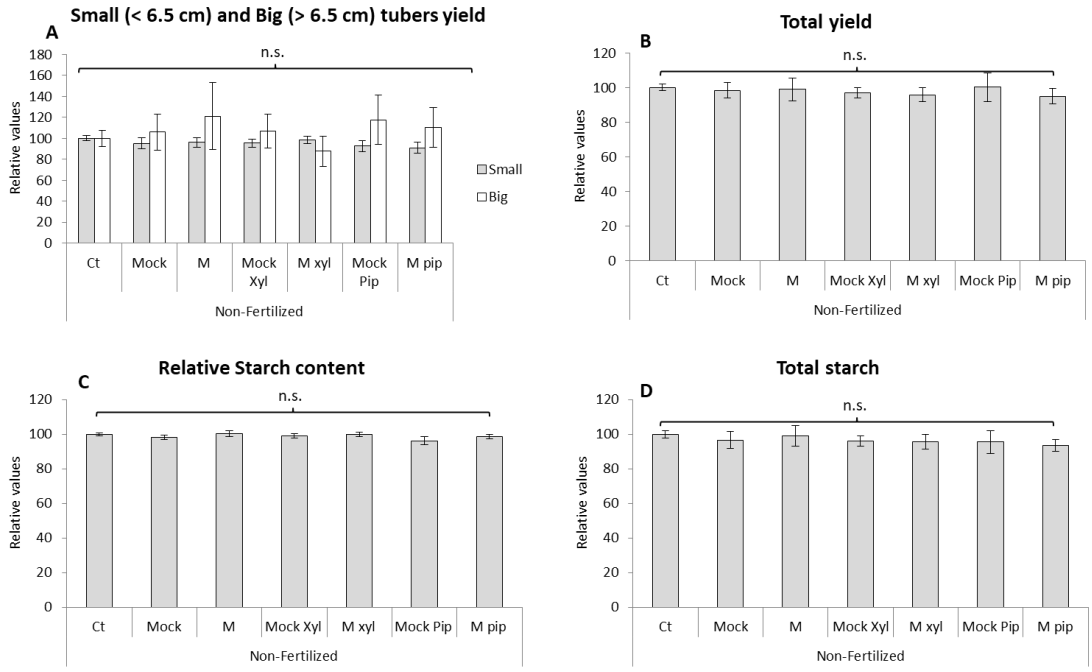

Figure III.21 Harvest parameters in non-fertilized soil. A: yield for small tubers (Ø < 6.5 cm) and big tubers (Ø > 6.5 cm); B: total yield; C: relative starch content of tubers; D: total starch yield (relative starch content * total yield) of plants grown in the field that did not received additional fertilization (Non-fertilized). Plants were: not inoculated and not treated with any solution on leaves (Ct), inoculated with autoclaved inoculum (Mock), inoculated (M); inoculated with autoclaved inoculum and sprayed with Xylose solution (Mock Xyl); inoculated and sprayed with Xylose solution (M Xyl); inoculated with autoclaved inoculum and sprayed with Pipecolic acid solution (Mock Pip); inoculated and sprayed with Pipecolic acid solution (M Pip). Values are normalized to the Ct group accordingly to the slope of the field. Shown are means (n = 6-9) and standard deviations. Treatments are not significantly different (n.s.) after ANOVA test (P = 0.05).

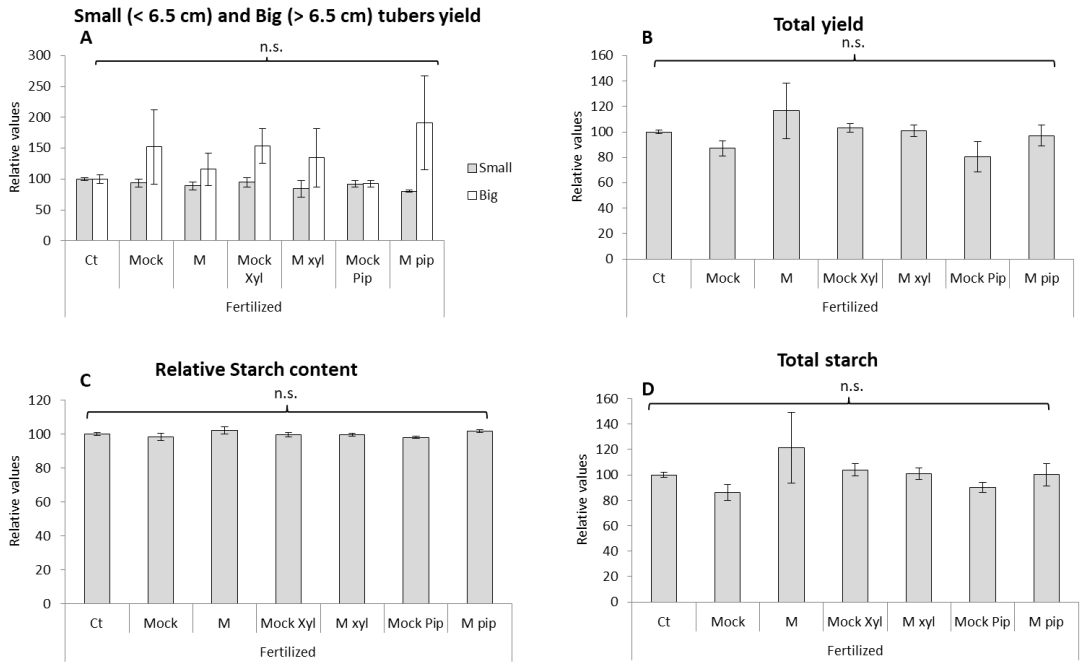

Figure III.22 Harvest parameters in fertilized soil. A: yield for small tubers (Ø < 6.5 cm) and big tubers (Ø > 6.5 cm); B: total yield; C: relative starch content of tubers; D: total starch yield (relative starch content * total yield) of plants grown in the field that received additional fertilization (Fertilized). Plants were: not inoculated and not treated with any solution on leaves (Ct), inoculated with autoclaved inoculum (Mock), inoculated (M); inoculated with autoclaved inoculum and sprayed with Xylose solution (Mock Xyl); inoculated and sprayed with Xylose solution (M Xyl); inoculated with autoclaved inoculum and sprayed with Pipecoli acid solution (Mock Pip); inoculated and sprayed with Pipecolic acid solution (M Pip). Values are normalized to the Ct group according to the slope of the field. Shown are means (n = 6-9) and standard deviations. Treatments are not significantly different (n.s.) after ANOVA test (P = 0.05).

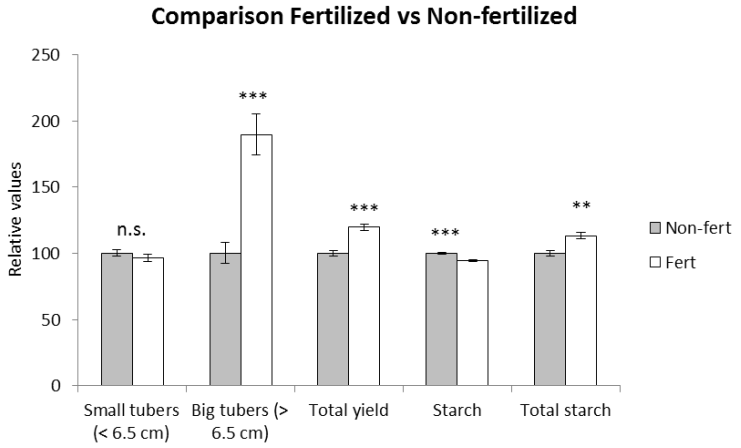

Figure III.23 Comparison of yield data between fertilized and non-fertilized soil. Data on small tubers (Ø < 6.5 cm), big tubers (Ø > 6.5 cm), total yield (small tubers + big tubers), starch content of tubers (starch) and total starch yield (relative starch content * total yield). Values are normalized to the Ct group according to the slope of the field. Shown are means (n = 6-9) and standard deviations. Two tailed independent T-test (* P < 0.05; **P < 0.005; *** P < 0.0005).

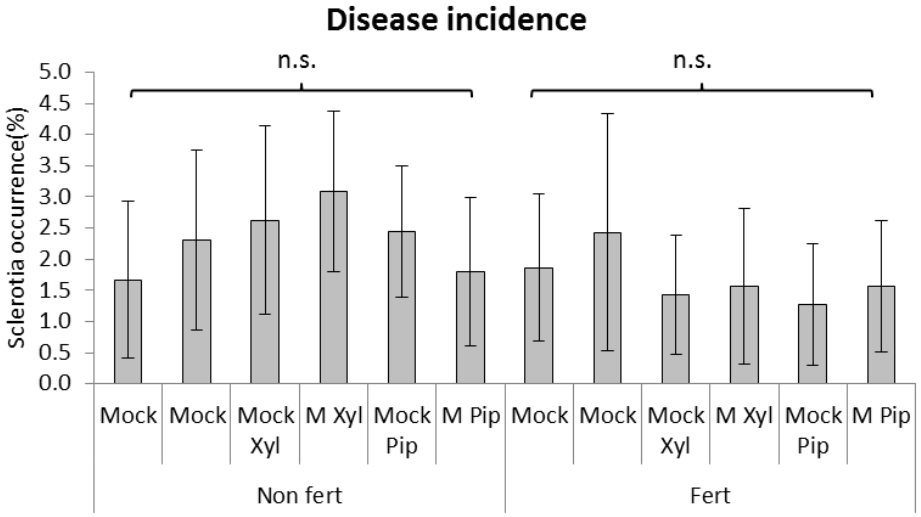

Figure III.24 Disease score in field trial. Percentage of occurrence of sclerotia on tubers of plants grown in the field that received additional fertilization (Fert) or not (Non fert). Plants were: not inoculated and not treated with any solution on leaves (Ct), inoculated with autoclaved inoculum (Mock), inoculated (M); inoculated with autoclaved inoculum and sprayed with Xylose solution (Mock Xyl); inoculated and sprayed with Xylose solution (M Xyl); inoculated with autoclaved inoculum and sprayed with Pipecolic acid solution (Mock Pip); inoculated and sprayed with Pipecolic acid solution (M Pip). Shown are means (n = 9) and standard deviations. Treatments are not significantly different (n.s.) after ANOVA test.

IV. Discussion

IV.1 Acclimatisation as strategy to improve inoculum performance under practical conditions.

IV.1.1 In vitro acclimatisation of Rhizoglomus irregulare strain QS81

Growth of the AM fungal *R. irregulare* strain QS81 for five generations in root organ cultures with additional phosphate resulted in an "acclimatised" strain (AM+). This strain and its counterpart non-acclimatised strain (AM-) were used for testing the hypothesis that propagation under these conditions can result in an AMF strain which shows fungal development patterns and impact on plant performances less affected by the presence of high amounts of phosphate.

Effects of acclimatisation were investigated during different developmental stage of the strain QS81. The asymbiotic development is characterized by elongation and branching of mycelium, representing the first steps following the germination of the spores before root contact (Giovannetti et al., 1993). The development of the asymbiotic mycelium is considered as an important factor influencing the success of the following root colonization and, in general, the symbiosis with the plant. Mycelium branching during presymbiotic development was described as one of the event following host recognition before physical contact and hyphodia formation (Giovannetti et al., 1993; Tamasloukht et al., 2003). In the current experiments, the presence of inhibiting [Pi] already reduced asymbiotic branching capacity of AM-, suggesting a direct negative action of Pi already in this preliminary phase of interaction (Figure III.1). On the contrary, AM+ strain maintained the same branching frequency independent of Pi levels, with significant higher values compared to the AM- strain. The constant branching frequency observed in AM+ spores can be considered as positive trait, since it maintains unaltered the chance to get in contact with the host, even at hostile conditions. Mycelium branching, in this phase, is usually mediated by the presence of the plant, and it can represent the first moment of interaction between the plant and the fungus. Therefore, observation made about response to high Pi only, may be insufficient to understand the behaviour of AM+ strain in this delicate stage, if not coupled with signals coming from the plant. The dialogue between plant and fungus is mediated by a plethora of chemical compounds. Specifically, roots exudates showed to be able to modulate the response of AM fungi before physical contact, and many components have been identified, like: volatiles (Balaji et al., 1995; Bécard et al., 1992), flavonoids (reviewd by Vierheilig et al., 1998), mannitol (Kuwada et al., 2005) and CO_2 (Bécard & Piché, 1989). However, among them the phytohormone strigolactone was shown, in the last decade, to play a crucial role in the stimulation of fungal metabolism and branching (Akiyama et al. 2005; Besserer et al. 2006). For this reason, the synthetic analogues GR24 was added to the medium with the aim to mimic the presence of the plant (Figure III.2). As expected, addition of GR24 in the medium was associated with branching response in AM- in absence of Pi, confirming previous findings (Akiyama et al., 2005; Besserer et al., 2008; Besserer et al., 2006). Interestingly AM+, exposed to GR24, in absence of Pi, did not show a branching response. Moreover, in presence of 2 mM of Pi, AM+ maintained the same response to GR24, while AM- exhibit the well-known epistatic effect of Pi on strigolactone (Foo et al., 2013). Taken together these observations may suggest that AM+ strain is less susceptible to Pi branching inhibition and that perception of GR24 in this strain is independent by Pi levels; since AM+ branching is constant in the two Pi conditions, not showing branching promotion in Pi absence, neither branching inhibition in presence of 2 mM of Pi, as observed in AM- strain. Interestingly, the differences observed for the branching were not observed in other parameters, such as: total mycelium length and branch/length ratio. This observation suggests that differences between the strains are not a consequence of different metabolic

activity leading to a general growth promotion of the fungus, but mainly on mycelium architecture that is shaped differentially in the two strains accordingly to the environmental conditions and the exposure to Pi in the previous generations.

Asymbiotic and presymbiotic phases are fundamental for the establishment of the symbiosis, but the symbiotic phase, including nutrient exchange, determines the outcome of the interaction for both partners. ROC *in vitro* system allows to follow the developmental patterns of extra-radical mycelium and spore formation at phenotypical level. Results highlighted that Pi exert an inhibitory effect in terms of spore production, not only directly, when added to the medium, but even indirectly, acting in the subsequent generation of AM fungi exposed to inhibiting [Pi] (Figure III.3). Moreover, the observations confirmed that acclimatisation process induces changing in developmental pattern of AM+ compared to AM-. Unfortunately, almost no information is available on the events that determine the dynamic of development of spores and mycelium of AM fungi (Declerck et al., 2001; Jakobsen et al., 1992). Reduction in spore production was observed in presence of high [Pi] (Daft and Nicolson, 1972; Douds and Schenck, 1990) and our results confirmed these observations for both strains. Interestingly, a consistent reduction in spore production of AM+ strain was observed in low [Pi]. This fact is consistent with previous findings on acclimatised AM fungi for Zn (Bui and Franken, 2018) where spores number of acclimatised strain is reduced compared to control in absence of the stressor. However, in case of Zn, number of spores in acclimatised strain recovers to level comparable to control in presence of the stressor. This fact was explained with the "need hypothesis", which state that fungi growing under high metal concentration, like Zn, calibrate cell homeostasis to the higher levels of the metal, resulting in higher demand for Zn (Antosiewicz, 1990). However, in the case of Pi, spore number did not recover to normal values in presence of inhibiting [Pi], leading to a rejection of the need hypothesis. This underline the difference between P, that is not a metal, and Zn in the physiology and phenotypic development of AM fungi, suggesting that acclimatisation for these two elements is based on different mechanisms.

AM fungi lack genes encoding for fatty acid synthases and cannot produce fatty acids (Tisserant et al., 2013; Trepanier et al., 2005), which are instead translocated from the plant to the fungus during symbiosis (Keymer et al., 2017) and used for the production of the lipid bodies of the spores. Different genes have been identified as responsible for lipids biosynthesis and transport in arbuscocytes (RAM1, RAM2, FatM, and STR/STR; Wang et al., 2012; Bravo et al., 2017; Gutjahr et al., 2012; Gobbato et al., 2013; Pimprikar et al., 2016; Keymer et al., 2017) and it was shown that *R. irregulare* own in its genome only the genes for their elongation. The observed reduction of spore numbers may be a consequence of alteration in the fungal lipid metabolism and/or translocation towards the developing spores. Alternatively, the transfer of fatty acids from the plant towards the fungus could be inhibited leading to reduced fungal lipid biosynthesis. A generally reduced carbon flow from the plant towards the fungus under conditions of high phosphate fertilization has been observed (Konvalinková et al., 2017) and the low expression of the sugar transporter gene *RiMST2* (see discussion below) indicates a reduced carbon uptake by the AM+ strain in the root at least for hexoses. Independent of finding the reasons for reduced spore formation of the acclimatised strain, the property could lead to problems in the mass production of phosphate-acclimatised inocula, the final goal of the approach. This problem could be overcome by providing plants with enhanced lipid transfer capacities as recently proposed by Gargouri et al., (2021). On the contrary to what observed for spores, extra-radical mycelium proliferation showed no differences at low phosphate but was more intense at high phosphate concentrations then mycelium development of AM- strain. Literature reports conflicting observations on extra-radical growth of hyphae in presence of elevated Pi levels. Growth promotion correlated with high [Pi] were observed in root-free compartments (Li et al., 1991; Olsson and Wilhelmsson, 2000; Cavagnaro et al., 2005), while negative effects on growth were recorded in other observations (Abbott et al. 1984; de Miranda and

Harris, 1994). Therefore, it is impossible to make a clear generalization of effect of Pi on mycelium development. Results of the current experiments suggest that the strain QS81 is susceptible to phosphate inhibition of extra-radical mycelium development, but that this effect is alleviated by the acclimatisation process.

IV.1.2 Greenhouse performance of acclimatised AM inoculum

AM+ inoculum was expected to show higher colonization rate and improved plant growth in presence of high [Pi] in soil. Inoculation with the first and the fifth generations showed contrasting results, with reduced roots colonization observed in the first AM+ generation and with positive effects in terms of plant growth and root colonization, associated to the fifth generation of the AM+ strain. Plants grown at low [Pi] in soil and inoculated with the first generation of the AM+ strain were associated with reduction of colonization frequency compared to the AM- strain, indicating that propagation in a medium enriched with Pi, partially reduced the ability of the fungus to colonize the roots of the host. In presence of high [Pi] in soil, significant inhibition of colonization levels was observed in both strains. The two inocula, however, did not lead to significant differences in terms of plant growth, suggesting that, at functional level, the two strains were still comparable (Figure III.6).

Inoculation with fifth generation of AM+ and AM- spores was, instead, associated with different responses compared to the first generation (Figure III.7). Higher colonization level, in terms of m% was associated with AM- at low [Pi] in soil. However, in presence of high [Pi], AM+ showed higher F% compared to AM-, partially counteracting the Pi inhibition and showing the same F% at both Pi levels. The observed increased in F% can relate to the observations made during the *in vitro* tests, were the AM+ strain showed an asymbiotic and a presymbiotic mycelium growing longer and with more branches compared to the AM- strain. At this stage it is difficult to say whether the higher colonisation frequency observed is therefore exclusively due to the higher probability that the mycelium of the AM+ strain has of encountering the roots of the host plant; or whether it is due to an actual colonisation capacity linked to other factors. However, the lack of differences in terms of m% and a% between the roots colonized with AM+ and AM- strains would seem to suggest that the first possibility is the most likely. Previous findings associated increased Pi levels with a shifting from Arum- to Paris-type arbuscules (Mercy et al., 2017). Arum-type arbuscules are fast growing, finely branched and offer higher surface for nutrient exchange (Smith and Read 2008). Paris-type, instead, are slow growing and formed by hyphal coils with reduced, if any, branching (Smith and Read 2008). Data from the experiments confirmed a general reduction in frequency of mycorrhiza, but the occurrence of arbuscules remained unaltered in the experiment and occurrence of coils were not observed. Finally, a general reduction of colonization was observed in both AM+/AM- between the two generations. This fact can be associated to the different growing period of the experiment, or to the well-known phenomena, often observed in AM fungi and pathogenic fungi growing *in vitro,* of reduced ability of host colonization after several generation under monoxenic conditions (Butt et al., 2006; Marx & Daniel, 1976; Plenchette et al., 1996). In this second greenhouse experiment, an effect of inoculum on plant growth was, moreover, observed. Higher plant fresh and dry weight were measured after inoculation with the AM+ strain at inhibiting [Pi] in the soil, compared to the AM- strain. The higher biomass was observed jointly with higher infection frequency of the AM+ strain in host roots. The relation between plant growth and colonization levels is not linear. In fact, despite a higher colonization level has been correlated with improved plant growth in some studies (Merryweather & Fitter, 1995; Mullen & Schmidt, 1993), others reported that this was not always the case (McGonigle and Fitter, 1988; Sanders and Fitter, 1992; West et al., 1993), suggesting that the relationship between

colonization and plant growth also depends on factors like the specific plant-fungus interactions and the growing conditions. Stronger relation exists between the length of external mycelium and mineral uptake capacity of AM fungi (Graham et al., 1982; Munkvold et al., 2004; Sanders et al., 1977), but data show that also this parameter is not always directly correlated (Smith et al., 2000). Length of extra-radical mycelium was not measured in this experiment; it is therefore not possible to evaluate eventual differences among the strains regarding this parameter. However, in previous experiments with potato, the strain QS81 showed good relationship between root colonization levels and plant growth (Mercy et al., 2017), and this pattern was also observed in other field experiments (Lekberg & Koide, 2005; Pellegrino et al., 2015). Moreover, the *in vitro* results suggest that AM+ have better extra-radical mycelium (ERM) growth compared to AM- in presence of high [Pi]. Increased P uptake of plants inoculated with the AM+ strain as compared with the non-inoculated plants and plants inoculated with the AM- strain at high Pi conditions might be based on improved ERM development which would allow a higher Pi uptake and transport rate in the hyphae. For the sake of completeness, it should be noted that all the plants growth in high [Pi] showed accumulation of Pi in the tissues, therefore reducing the importance of AM fungi in foraging Pi activity. P content on dry shoot is usually around 0.2%, and these levels were observed already at low [Pi] in soil, while, in presence of elevated [Pi], plants exhibit concentrations ranging between 1.2% and 1.4%. This accumulation indicates that [Pi] was not the factor limiting plant growth. In this respect, AM+ effect concerning Pi nutrition may be of limited interest, since plants growing at high [Pi] in soils are usually not Pi limited. However, the impact of the ERM on soil characteristics can support also other mycorrhizal functions (Bitterlich et al., 2020) which would explain the general growth promotion by the AM+ strain at high [Pi] in the soil. Such functions could be the reduction abiotic stresses induced by other limiting factors occurring during the cultivation period, like improving photosynthetic efficiency (Sheng et al., 2008) counteracting sub-optimal sun irradiation, reducing thermal stress (Bunn et al. 2009) during temperature fluctuation, or improving water availability (Bitterlich et al. 2018) at slight and temporary drought conditions.

IV.1.3 Genetic investigation of the acclimatisation process

The effect on plant growth observed in the described experiment associated with the inoculation of the two strains in the presence of high [Pi] in the soil, rise the need to shed light on the mechanisms of these phenomena. The observed differences between the AM+ and AM- strains could be reflected by differential expression of genes involved in particular cellular processes. Four different hypotheses (hypothesis V, VI, VII, VIII, respectively) were therefore formulated and tested.

IV.1.3.1 Acclimatisation is based on different regulation of the cell cycle.

In order to get first insights into the molecular mechanisms underlying the differences in the interactions of the AM+ and the AM- strain with the plant, particular hypotheses were formulated concerning the involved processes and tested by analysing the RNA accumulation of different set of genes. First, the expression pattern of four genes involved in DNA replication (*RiDPD4* and *RiRNR*), cell cycle (*RiPCNA*) and mitosis (*RiMHC*) were investigated (Figure III.13). A previous study (Sugimura and Saito, 2017) indicated the inhibition of these genes in *R. irregulare* grown at high [Pi], compared to *R. irregulare* grown at low [Pi]. Cell cycle regulates the differentiation of multicellular eukaryotes, and it is responsible for the synchronization of cell division in order to form specific organs and tissues (Cools and De Veylder, 2009; Kipreos, 2005; Théry and Bornens, 2006). Regulation of cell cycle has been shown to play crucial role in the development of the infection structures like appressoria in pathogenic fungi (Saunders et al. 2010), and it regulates the polarized growth of filamentous fungal hyphae (Momany, 2002; Steinberg and Perez-Martin,

2008; Whiteway and Bachewich, 2007). Cell cycle is finely regulated by multiple signals, both intracellular and extracellular (Sakaue-Sawano et al., 2008), like Pi levels in plant during AM fugal symbiosis (Sugimura and Saito, 2017). Still, it is questionable whether the inhibition of cell cycle is the cause of reduced growth of AM fungi or just a consequence of the fungal growth inhibition determined by other factors influenced directly by Pi levels. Expression of *RiDPD4* and *RiPCNA* was influenced by Pi levels, as revealed by two-way ANOVA analysis, confirming what was observed in the previous study (Sugimura & Saito, 2017). In addition, the expression of *RiDPD4* and *RiMHC* reached higher values in the AM+ strain than in the AM- strain and this was significant at high phosphate fertilization levels. This correlates with the increased branching activities of the hyphae of the AM+ strain, but a cause-consequence relation can only be speculated.

IV.1.3.2 Acclimatisation is based on different regulation of the antioxidant response.

The second tested hypothesis postulated that the acclimatisation process of AM fungi to high phosphate levels is based on the differential expression of genes involved in the depletion of reactive oxygen species (ROS) as it has been shown for Zn acclimatisation (Bui and Franken, 2018). Activity of ROS-depleting genes were correlated with tolerance to biotic and abiotic stress in plants (Chamnongpol et al., 1998; Rizhsky et al., 2002; Wong et al., 2004). It is, however, questionable whether higher phosphate concentrations can be considered as a stress factor for the fungus, although it is associated with AM fungal growth depression. Oxidative burst coupled with ROS production in regions of AM fungal entry points, in cells containing intra-radical hyphae (Puppo et al., 2013) and in arbuscule-harbouring cells (Campos et al., 2015) is considered as an element able to control the colonization at spatial and temporal level (Salzer et al. 1999) potentially reducing AM fungi development when plants tissue have sufficient Pi content. In this frame, higher scavenging activity of the AM+ strain may counteract the detrimental effect of plant ROS production in limiting root colonization. The results showed phosphate-induced RNA accumulation for the genes encoding SOD and GST in both strains and this was significant for *RiGST*. This indicates that high phosphate levels induce ROS production also in the current experiment, but acclimatisation did not lead to a different response (Figure III.14). The hypothesis has to be rejected on the basis of this result. The role of ROS depletion, however, should not be totally excluded. There are many more genes involved in this process and, the GST gene family alone has numerous members in AM fungi (Waschke et al., 2006).

IV.1.3.3 Acclimatisation is based on different regulation of fungal respiration?

For the third hypothesis, the expression of genes involved in the respiration pathway for acclimatisation was investigated (Figure III.15). Despite not deeply studied, shifting the fungal respiration pathway from COX to AOX were shown to play a role in spore germination and branching during the presymbiotic phase (Campos et al., 2015; Mercy et al., 2017). Modulation of fungal genes for AOX or COX were associated with different development patterns in mycorrhiza with potato (Mercy et al., 2017). In general, AOX activation is considered as element of flexibility in fungal metabolism, allowing the adaptation of development to different stress conditions (Campos et al., 2015). Moreover, it was shown that AOX generally regulates fungal development and pathogenicity, and may contribute to fungal ecological fitness (Grahl et al., 2012; Thomazella et al., 2012; Xu et al., 2012). Although one cannot necessarily deduce from RNA accumulation to enzymatic activities for the genes *RiAOX* and *RiCOX* (Bedini et al., 2018), it is interesting that a similar general increase could be observed at high phosphate fertilization levels as for the genes involved in ROS depletion. It could be speculated that the increased *RiAOX* and *RiCOX* expression reflects higher mitochondrial activities due to higher phosphate availability. This could lead to increased ROS production which in turn up-regulates genes involved in antioxidative activities. Comparing the two strains, a general trend can be observed that the activities of genes involved in respiration and ROS depletion

are reduced in the AM+ strain under low phosphate levels but increased at high phosphate levels. The new state of AM+ strain might allow a stronger response to changing phosphate availabilities, but this needs to be confirmed by further studies.

IV.1.3.4 Acclimatisation is based on higher functional capacity of the AM+ strain

Finally, the role of nutrient exchange between the partners was investigated. AM fungi provide phosphate to plants via the ERM and uptake into the hyphae is mediated by fungal plasma membrane phosphate transporters (Smith and Read, 2008). Phosphate transporters are expressed both in intra-radical mycelium (IRM) and in the ERM (Balestrini et al., 2007; Benedetto et al., 2005; Tisserant et al., 2012). The tested samples contain mainly IRM, consequently reducing the possibility to elucidate the role of these genes outside of the roots. Previous findings showed that the ERM expression of *RiPT1* is influenced by phosphate levels, while the IRM expression is not affected by it (Benedetto et al., 2005). Our data confirmed this observation for IRM for both strains and showed that this is true also for the other PT-encoding genes in the AM- strain (Figure III.17). In the AM+ strain, however, three PT-encoding genes were induced at high phosphate level and this was significant for *RiPT5* and *RiPT6*. PTs usually transport phosphate into cells against a concentration gradient. It is therefore difficult to assign a particular role in the AM+ strain, because upregulation of PT expression implies phosphate translocation from the plant towards the fungus. At high phosphate levels, P content of plants are, however, not changed while P uptake is higher in plants inoculated with the AM+ strain. It can only be speculated that PT5 and PT6 activities keep phosphate in a cycling system where it is also more available for the plant partner. The sugar transporter gene *RiMST2* is expressed in the IRM and down-regulation leads to reduced colonization of the roots (Helber et al., 2011). It is therefore assumed that *RiMST2* is one element in the sugar translocation from the plant to the fungus. In our experiments, the expression of *RiMST2* was significantly affected by the inoculum type (Figure III.16). The AM+ strain showed a much lower expression than the AM- strain, and the difference was most obvious at high phosphate levels suggesting that the two strains may have different sugar uptake capacities. This could shift the fungus-plant balance towards the plant explaining decreased spore production and increased growth-promoting effects of the AM+ strain at both fertilization levels. A similar shift in the balance but in the opposite direction has been observed for tomato plants. The expression of a sucrose transporter at the periarbuscular membrane, which presumably back-transports sucrose into the plant cell away from the fungus, was down-regulated. This down-regulation led to increased fungal development and disappearance of the plant growth-promoting effect (Bitterlich et al., 2014). In conclusion, RNA accumulation analyses during symbiosis point to several processes which could be changed in the AM+ strain compared to the AM- strain. The acclimatised strain seems to be generally more active concerning DNA replication and cell cycle (correlating with hyphal branching) and concerning respiration and ROS depletion (reflecting phosphate-induced mitochondrial activity). Interestingly, genes involved in nutrient exchange processes show the clearest difference between the acclimatised and the non-acclimatised strains. If the differential expression patterns are causative or a consequence for the observed differences in the symbiotic functions remains to be shown. It will be also important to investigate if the acclimatisation process is accompanied by genetic or epigenetic changes or if it is simply a consequence of the phenotypic plasticity of AM fungi and some of these points are address in the next section.

IV.1.3.5 Consideration about the acclimatisation of AM fungi

In this text it was decided to use the term acclimatisation as it was considered the most relevant and the most easily understandable. Even though the main aim of these experiments was to test a method for improving the quality of the produced inoculum and not test the occurrence of acclimatisation phenomena, it is pertinent here to say a few words about the term acclimatisation and its meaning in AM fungi. The term

acclimatisation has been employed by several authors in experiments with AM fungi, and generally the term has been used about the response by the fungus in the presence of a stress for a more or less long period of time (Addy et al., 1998; Sharifi et al., 2007; Bui & Franken, 2018). However, only Bui and Franken (2018) investigated the presence of alterations in gene expression on a temporal level, while the other authors limited themselves to observing fungal phenotypic differences and/or induced effects on the colonized plants. The term acclimatisation, as described in the introduction, lies somewhere between regulation (faster process) and adaptation (slower process). Like any definition of natural processes, these definitions have blurred boundaries and, overlapping phenomena may frequently happen. Furthermore, the subdivision: regulation, acclimatisation, adaptation is generally defined for simple organisms, such as unicellular algae (Giordano, 2013; Raven & Geider, 2003), which have a simple genetic organisation. In the case of AM fungi, things become more complicated, since the genetic organisation of these organisms, characterised by the simultaneous presence of several nuclei which sometimes present homokaryotic characteristics, sometimes dikaryotic, places certain limits on the definitions described above (Corradi & Brachmann, 2017). The five generations used in the experiment showed to be enough in this kind of fungi to be able to induce some genetic change, with a range of 0.5 to 1% observed after 4 generations (Ehinger et al., 2009), suggesting that some adaptation process may already occur in this relative short period of time. Similarly, the impossibility of reproducing an AM fungus from a single nucleus, as well as the observation that spores produced in a clonal manner present significant changes in phenotype (Ehinger et al., 2012) with different effects on the colonized plant (Angelard et al., 2010) place limits on the possibility of falling within the definition of acclimatisation as stated by the cited authors. In addition to that, other processes may have been involved in the experiments conducted, such as maternal effects or phenotypic plasticity. Maternal effect is defined as: phenotypic changes in offspring determined by the environment of the mother (Burton & Metcalfe, 2014; Donelson et al., 2018; Donohue, 1999), while phenotypic plasticity is defined as the ability of one genotype to produce multiple phenotypes depending on environmental conditions (West-Eberhard, 1989) and they both may have occurred during the experiments. This leads to the conclusion that a precise definition of the observed process is far from obvious and would require an understanding of the genetic organisation of these fungi that is not yet shared by the scientific community. However, the aim of this work was to exploit the characteristic of these fungi to adapt to unfavourable conditions and to be able to induce benefits to the colonised plant. The elements underlying this characteristic, although fascinating, are beyond the scope of this work. Therefore, the term acclimatisation was used all along the text even though it may be not the mechanism generating the observed variation, since the process followed was the same used from other experimental approaches from the literature on acclimatisation.

IV.1.4 Performance of acclimatised inoculum under practical conditions

Inoculation with the fifth generation of acclimatised *in vitro* inoculum induced higher plant biomass and higher root colonization frequency in potato grown in greenhouse. However, the final aim of the experiment was to produce an acclimatised inoculum able to work under practical field conditions, and in this perspective, results obtained in greenhouse represented only the proof of concept of the development of the acclimatised inoculum. In fact, results obtained in controlled conditions often fail to be confirmed in field. Therefore, it was crucial to produce and test the acclimatised inoculum in a "real" scenario important for commercialisation. Production of *in vivo* produced inoculum ensure an economic way to produce large amounts of AM fungal propagules, while *in vitro* production of inoculum represents a yet too expensive and not economically feasible method for field application. For this reason, *in vivo* acclimatisation of AM fungal inoculum was performed, and its effects tested under field-like conditions. Due to time reasons, it was only possible to test the first generation of the *in vivo* acclimatised inoculum.

Plants inoculated with the *in vivo* acclimatised inoculum generally showed higher root colonization at both fertilizer conditions and performed better than the non-acclimatised inoculum which did not show significant increase in any parameter compared to AM fungi naturally occurring in the soil (Figure III.19). In fully fertilized soil, the AM+ strain *in vivo* acclimatised for one generation showed increased infection frequencies but no difference in relative spread (m%) and relative arbuscule abundance (a%) similar to the *in vitro* obtained strain acclimatised for five generations. Without additional fertilization, however, the *in vivo* acclimatised strain interestingly showed a higher absolute spread (M%) and arbuscule abundance (a% and A%) and this was in clear contrast to the observations with the *in vitro* strain. This suggests that *in vivo* and *in vitro* acclimatisation act in different way on AM fungal propagules. In fact, *in vivo* acclimatisation appeared able to induce a general stimulation in colonization capacity of the fungus, not specifically targeting the presence of the stressor. Fungal spread in root tissues is fine-tuned by numerous signals produced by the plant, like oxidative burst and specific phytohormone levels (Bedini et al., 2018; Martín-Rodríguez et al., 2016), but it is currently not possible to state whether the observed higher colonization is based on the ability of the AM+ inoculum to modulate the described plant signals occurring in the roots, or if it is linked with other factors. As example, at the present time it cannot be excluded that the higher colonization capacity may be consequence of selection of beneficial bacteria during *in vivo* acclimatisation living in close contact with AM fungal spores, adapted to high [Pi] and therefore able to induce beneficial effect on fungal growth (Barea et al., 2005; Bharadwaj et al., 2012; Pivato et al., 2009), inducing elongation and branching of mycelium (Lumini et al., 2007). The occurrence of these microorganisms in *in vitro* cultivation is extremely limited, if not eliminated, due to the surface sterilization of spores.

As discussed before, level of colonization is a parameter depending on the growing conditions for the fungus in the roots of host plant, and a higher root colonization is not always correlated with plant growth promotion. Also here, the presence of more colonized roots in presence of acclimatised inoculum, was not associated with plant growth promotion effects, confirming the inaccuracy of root colonization to define the effect of the fungus in plant growth. Nevertheless, it is even possible that the harvesting time, set at 8 weeks post inoculation, may represent a too narrow time window to allow a proper evaluation of growth promoting effect of the inoculum. In fact, at harvest time, the tuberization was just started and the new potato tubers were still in development. Although, the lack of differences in P (Figure III.20) content and in the biomass of plants, suggest that at functional levels neither the acclimatised nor the non-acclimatised inoculum add any benefit for the plant to the natural occurring inoculum.

A further factor, which may explain the different degree of colonisation and the absence of growth promotion in the field experiment is due to the different environmental conditions between the field and the greenhouse. Starting from the bottom, the growth substrate is completely different. While in the greenhouse a 100% sterilised sand substrate was used, in the field natural soil was used. The natural soil in the experiment, although also sand-based, has significant differences from the substrate used in the greenhouse, both at a physical and chemical level. On a physical level, the substrate used in the greenhouse had only a homogeneous primary structure, determined by the size of the sand particles. The natural soil, on the other hand, consists of a more complex primary structure, formed by the different fractions of the soil particles (sand, silt, clay) (Brewer & Sleeman, 1960). A secondary structure can also be found in natural soils, determined by the arrangement of the various primary fractions (Brewer & Sleeman, 1960). This arrangement is largely determined by the presence of plants (Bodner et al., 2014) and microorganisms, such as AM fungi (Rillig & Mummey, 2006), which can produce substances such as glomalin (Bedini et al., 2009; Rillig & Steinberg, 2002). Primary and secondary structure greatly influence the hydraulic properties of the soil (De Gryze et al., 2006; Querejeta, 2017), as well as nutrient, gas and water fluxes (Wu et al., 2012). The presence of macroaggregates and protein and organic compounds, present in natural soils, plays an important

role in cation exchange (Helling et al., 1964), so for the same fertilisation, nutrient availability for plants grown in greenhouses or in the field can change substantially. Moreover, at a biological level, the natural soil has a microbial population already present, and already adapted to the soil and climate conditions of the area, which can interact along the entire symbiotic spectrum from negative to positive interactions with the applied inoculum and the sown plant. Moreover, mycorrhizal communities already present can significantly influence the growth of the host plant (Turrini et al., 2018). To these differences present in the soil, must be added the climatic differences to which the plants are subjected, while plants in the greenhouse are in controlled environment in which fluctuations are lower, plants grown in the field have to deal with environmental mutability. It is therefore clear that the transition of plant growth from the greenhouse to the field involves the introduction of a very wide range of components that can determine the growth response of plants and their relationship with AM fungi. The action of these factors therefore limits the possibility of correctly seeing and evaluating the effect of external inoculation with AM.

IV.2 Use of regulators as strategy to improve inoculum performance in practical conditions.

IV.2.1 Compatibility tests between R. irregulare and selected regulators

Use of regulators as promoter of mycorrhizal response in plants represents a new application strategy to be tested and evaluated in the field. The signal molecules tested can induce physiological changes on plants acting as signal (Aranega-Bou et al., 2014; Jung et al., 2009; Navarova et al., 2012; Ramon et al., 2008; Rolland et al., 2006; Shah, 2009) and the modulation of plant physiology induced by these molecules was expected to modify the plant-mycorrhiza interaction, too (Bedini et al., 2018). With the aim to implement the use of these molecules on practical conditions, a first issue to be addressed was the compatibility between the selected molecules and the fungal propagules, especially in case they are in close contact after mixing in the substrate or integrated in the same coating for seeds. There are no reports concerning the study of these molecules on AM fungal spores under *in vitro* condition in axenic and/or monoxenic systems. For this reason, the effects of selected inducer on germination rate and development of *R. irregulare* spores was investigated. Results suggested an overall compatibility between the selected molecules and the spores of *R. irregulare,* with some distinctions. Pipecolic aid and azelaic acid, regulators of Systemic Acquired Resistance (SAR), inhibited germination in the first two weeks (Figure III.4). No studies exist on the relationship between spore germination rate and colonization; however, germination is a fundamental pre-requisite for colonization and reduced level of germinating spores reduces the chance of meet between presymbiotic mycelium and host roots. Despite the differences in germination tend to disappear at 21 dpi, it is important to note that the first two weeks represent a crucial moment for the establishing of colonization in some cultivations. For example, plantlets coming from *in vitro* propagation highly benefit from colonization during hardening time, occurring in this time-frame (Krishna et al., 2005). Moreover, for commercial purpose and in short cycle crops, the faster the colonization, the faster the benefits will be provided to the host plant. Concerning mycelium development, reduced length of mycelium was observed in presence of pipecolic acid compared to control. The growth ability of mycelium can be associated with the colonization capacity, in fact, a mycelium able to grow more increase the chance to reach the roots of the host. However, the chances of physical contact between presymbiotic mycelium and host roots is also a function of the degree of branching of the mycelium (Bonfante & Genre, 2010), and this parameter was not affected between control and regulator molecules. Concerning the selected molecules for Induced Systemic Resistance (ISR), the data showed an overall good compatibility at the tested concentration, with no

difference in germination rate compared to control. In conclusion, selected molecules showed to be compatible in axenic *in vitro* conditions with spores of *R. irregulare*. Reduction of germination rate observed at 14 dpi with SAR-inducing molecules and reduction of mycelium length occurring after pipecolic acid treatment represent a non-desirable trait that can be probably avoided with application of regulators on leaves (Figure III.5).

IV.2.2 Selection of most effective regulators of AM fungi colonization

Compatibility of selected regulators were confirmed by *in vitro* experiments. However, in order to define a proper mode of use and to confirm the efficiency in shaping mycorrhizal response, greenhouse tests were run to define the best application way and the most effective concentration. Two concentrations of regulators were tested via application in soil or on leaves, in presence of promoting and inhibiting phosphate levels. As largely discussed previously, high [Pi] in substrate drastically and systematically reduce the root colonization by AM fungi and is often associated with lower mycorrhizal responsiveness (Breuillin et al., 2010; Mercy et al., 2017). On the contrary, under limited [Pi], AM fungi can better colonize roots and thus promote nutrient acquisitions and plant responses (Smith and Read, 2008). Data obtained in this experiment confirmed the inhibiting effect on fungal colonization by high [Pi], with an overall reduction of colonization parameters in non-treated plants. Higher mycorrhizal colonization was instead observed with low [Pi] and associated with improved plant growth. However, the focus of the experiment was to evaluate the effects of the regulators in presence of high [Pi], since it was the target of the inoculum improvement and a situation likely to occur in the field. The attention has been directed mainly on colonization patterns, rather than on plant growth promotion provoked by regulator application. Despite the plant growth promotion represent one of the parameters of interest for growers when applying products like AM fungal inoculum, at this stage of the study the attention was mainly focused on confirming the mode of action of regulators at the root/fungus interface.

Glucose was selected as a potential inductor of mycorrhiza via induction of favourable metabolic context which involves an ISR-like system. Considering the results, the efficacy of this molecule in promoting AM fungal colonization via stimulation of plant physiology is questionable. Glucose is an important element of plant metabolism, and it is directly involved in energy production of plants. Moreover, glucose can be directly uptake by plant roots, therefore, soil application of this molecule may result in higher energy availability of the plant (Kuzyakov & Jones, 2006). This observation is confirmed by the increase in biomass observed with the higher glucose soil application in soil at low [Pi], while the absence of growth promotion with the lower concentration is probably a consequence of too low amounts, unable to stimulate significant growth effects (Figure III.9). Stimulation of plant growth would have been expected even with glucose application at higher [Pi], however, the lack of differences in plants growth in that case, is probably a consequence of the occurrence of other limiting factors, stabilizing the plant growth to the observed values. Direct uptake of glucose via roots is supported by the higher colonization observed at high [Pi] and application of higher glucose amounts. Previous experiments reports that application of glucose in substrate leads to increase its concentration in the roots, and this was connected with promotion of arbuscule development (Wu et al., 2015). However, according to this explanation, higher number of arbuscule should have been observed even at low [Pi]. Nevertheless, it is possible to speculate that, at low Pi level, the maximum colonization capacity of the fungus was already reached, and that the plants efficiently controlled the development of fungal development via different strategy, like oxidative burst (Campos et al., 2015; Puppo et al., 2013; Salzer et al., 1999), or via other chemical stimuli or anatomical adaptations (Koide & Schreiner, 1992) to avoid detrimental effects deriving from uncontrolled spread of the fungus. If the

evaluation of the effect of glucose as regulator in soil is made difficult by its role as energy source, foliar application may give more information on its role as "signal molecule" only. However, recent studies showed that glucose can be uptake even from leaf stimulating plant growth (Flütsch et al., 2020). This can explain the simple main effect detected by the two-way ANOVA analysis with regards to plant biomass. At root level the leaves application of glucose was not associated with any measurable effect. These observations suggested a direct action of glucose as energy source for plants rather than as a signal molecule. Effect of glucose as elicitor have been described (Trouvelot et al., 2014), but no visible effects where observed in our experiments, maybe due to the experimental conditions, or for the plant-fungus combination selected. However, due to the lack of consistent results, no further experiment has been performed with this molecule.

Xylose was selected as the second ISR-inducing molecule expected to promote root growth of the fungus in presence of inhibiting [Pi]. Observed results suggested the possibilities to use this molecule as regulator for improved root colonization. Xylose application in soil may be suspected to act as energy source for plant and fungus as observed for glucose. Xylose, indeed, can be absorbed directly by plant roots, but in less efficient way compared to glucose (Helber et al., 2011). Moreover, its assimilation is not associated with increasing concentrations in root tissues (Jones & Darrah, 1996), unlike glucose, being therefore less available for hyphal direct foraging in roots. Xylose is a fundamental element of the pentose phosphate pathway, responsible for production of important components of plant metabolism, as well as energy and redox potential (Zahnley & Axelrod, 1965). The lack of differences in plant growth, observed with soil application of xylose (Figure III.10), suggests a reduced effect of this molecule in inducing plant growth promotion by acting as free energy source. Interestingly, fungal hyphae can uptake directly xylose and assimilate it (Helber et al., 2011), representing, therefore, an additional source of carbon available for the fungus. Although a direct fungal uptake of xylose from the substrate cannot be excluded, no measurable effects in the investigated parameters were observed, suggesting that soil application of xylose does not induce strong effects with respect to plant and fungal growth. Leaf application of xylose, instead, showed good results as root colonization improved when applied at the higher dose in presence of inhibiting [Pi]. The observed promotion of fungal growth in terms of root colonization can be attributed to ISR induction stimulated by the presence of xylose, as suggested by the possible activation of the jasmonic acid (JA) pathway (discussed in IV.2.3 Genetic validation of mode of action of selected regulators). Way of action appeared to be dose dependent, acting as fungal growth promoter at the higher concentration, but not at the lower one. Lack of promoting root colonization at low [Pi] may be again a consequence of controlling fungal development induced by the plant, as described for glucose (Koide & Schreiner, 1992), limiting the spread of the fungus above a certain level. At this point of investigation, the attention was focused on definition of optimal application way of the regulator. Results of xylose suggest that this molecule can effectively improve root colonization, and it was selected for further application.

Pipecolic acid was selected as molecule able to elicit SAR responses in plants and therefore as expected to decrease fungal colonization. Soil application of pipecolic acid was responsible for plant biomass differences, and at mycorrhiza-inhibiting Pi concentrations, a reduction of colonization was observed (Figure III.11). This reduction of colonization can be a consequence of the induction of SA pathway activation (see discussion below) that inhibit fugal colonization and stimulate plant growth response (Durrant & Dong, 2004; Claeys et al., 2014; Eraslan et al., 2008). However, the most interesting results were observed with leaf application. In this case, the activation of the SA pathway was confirmed (discussed in IV.2.3 Genetic validation of mode of action of selected regulators) and the results observed at root level confirmed the hypothesis. Response to this molecule seemed dose dependent and the higher dose always showed the stronger inhibition together with the higher Pi levels. Interestingly, the data showed that at low [Pi], the

lower dose induced a stronger inhibition of root colonization. Interaction between Pi levels and pipecolic acid application has been confirmed by the two-way ANOVA analysis, suggesting a complex interaction of these two elements with respect to fungal colonization. However, this aspect was not investigated, and the observed results allowed us to select this molecule as SAR inducer and use it for further experiments.

Azelaic acid was selected as a potential SAR inductor which may inhibit the mycorrhizal colonization according to the formulated hypothesis. This statement cannot be confirmed due to the lack of consistent results for the fungal parameters obtained from leaf or soil applications of this molecule (Figure III.12). Simple main effect on plant biomass for azelaic acid was observed with both modes of application, and higher biomasses were observed after leaf application. This suggests a plant growth-promoting effect of the molecule, probably via stimulation of phytohormones like SA and GA3 associated with the SAR system (Durrant & Dong, 2004) able to reduce stress and to promote plant growth (Claeys et al., 2014; Eraslan et al., 2008). Interestingly, this promoting effect disappeared after inoculation of the plant which may be due to inhibition of the SAR pathway due to a reprogramming of the plant's defence system induced by the presence of the fungus (Bedini et al., 2018; Pozo et al., 2002; Pozo & Azcón-Aguilar, 2007). Due to the lack of differences in fungal colonization, no further investigations were made with these plants, and the reason for plant growth promotion remains unknown.

The discussed results indicated xylose and pipecolic acid as molecules fitting the theoretical scheme of the hypothesis. Both molecules exerted their effect when applied on leaves at the higher concentration tested at high [Pi]. Therefore, they were selected as candidates for the next experiments, although correlations between root colonization and plant growth were not detected. Greenhouse conditions are, however, not directly comparable with field conditions for potato cultivation and the lack of correlation was therefore not considered for the decision to test the effects of xylose and pipecolic acid as potential regulators in the field. Weekly application of fertilizer avoids the formation of nutrient depletion zone around the roots, and coupled with the limited exploitable volume, they represent a bias in the effect of AM fungi in plant growth promotion. Moreover, the short time of the experiment, and the late development of the selected cultivar, did not permit to fully evaluate the effects of the inoculation in the whole cycle as occurring in the field. As last, the growth parameters of interest of farmers and growers involves tubers production and size, and, regarding these parameters, the developmental stage of the plants in the greenhouse did not give any information. In conclusion, the screening of the regulators allowed for the selection of the two best candidates (one for ISR and one for SAR) and the decision about the mode of application and the applied concentration.

IV.2.3 Genetic validation of mode of action of selected regulators

Xylose and pipecolic acid were selected based on fungal phenotypical response promoting or inhibiting root colonization to be tested in the field. They showed contrasting response in AM fungal development when applied on leaves at [Pi] comparable with conditions occurring in a typical cultivated potato field. With the aim to confirm the action of these molecules as regulators able to promote the ISR or SAR system, transcriptomic analysis was performed on plants showing the desired phenotypic response in the greenhouse experiment. Application of the molecules showed differential expression of SA-responsive genes after pipecolic acid application (Figure III.18). The expression of these genes was consistent with many observation made on this kind of plant response (e.g. Durrant & Dong, 2004) confirming the activation of the SAR response. *StPR1* was not differentially expressed compared to the other treatments and this can be explained with reprogramming of plant defence mediated by AM fungi (Dumas-Gaudot et al., 1996; García-Garrido & Ocampo, 2002; Güimil et al., 2005). ISR response was expected to be activated in xylose

treated plants. The absence of expression of JA-related genes was, however, not surprising, since the activation of this state, by definition, does not imply modification of defence gene expression (Pieterse et al., 1996; Pieterse et al., 2002), but is based on enhanced sensitivity to these plant hormone (De Vleesschauwer et al., 2006; Pieterse et al., 1998; Pieterse & Van Loon, 2004). Activation of ISR response cannot be confirmed in absence of direct JA quantification. However, the non-activation of defence-related genes and the phenotypic response of colonization suggest a positive activation of the ISR pathway, or at least the induction of plant metabolic context favourable for AM fungi.

IV.2.4 Field test of regulators effect on root colonization and plant growth

Potato production can benefit from mycorrhiza inoculation (Rouphael et al., 2015). In the region, where our experiments have been carried out, several tests reported the difficulties to establish proper mycorrhizal symbiosis in the field (INOQ internal communication). For this reason, application of regulators for AM fungi coupled with inoculation is of high importance and it was expected to overcome the Pi inhibiting activity in the field. Despite the promising results obtained in greenhouse experiments, xylose application was not sufficient to induce colonization in potato, and in general AM colonization was not detected in any of the inoculated or non-inoculated plants treated or not with regulators. Natural occurring inocula was expected to colonize the control plants. Field soil was investigated with respect of the number of propagules via MPN test. Results of the test indicate an extremely low number of propagules for the field soil, and this can explain the absence of colonization observed in control plants (Figure III.17). The extremely low number of propagules observed with the MPN test is probably a direct consequence of the conventional management of the field during the last years, with co-occurrence of mineral fertilization, fungicide application and hyphal network destructive practice, like deep tillage. All these practices are known to reduce the AM fungal population in the field (Avio et al., 2013; Treseder, 2004; Dodd & Jeffries, 1989).

Soil [Pi] in the field was compatible with [Pi] in our greenhouse trials and no other source of Pi were added during the experiment. Therefore, the application of the inoculum was expected to be able to induce root colonization. Instead, root colonization was not observed in any of the inoculated plants, suggesting the co-occurrence of other inhibiting factors limiting AM fungal development in the field. These limitations could be based on allelopathy and/or microbiostasis. Allelopathic effects were observed several times in soil. They are derived from substances produced by plants and other soil microorganisms and they have been shown to be able to act in negative way on AM fungi development (Rose et al., 1983; Souto et al., 2000). In contrast, microbiostasis is a soil character that blocks the growth of microorganism. It can be induced by biotic or abiotic factors, and can also reduce the growth of AM fungi (Ho & Ko, 1985, 1986; Veen et al., 1997). Unfortunately, the presence of these factors was not investigated in the experiment, and it is therefore impossible to state whether the observed outcome was a consequence of the activity of these or of other factors. Nevertheless, the lack of colonization made it impossible to evaluate whether selected regulators can modulate AMF colonization and/or responses under field conditions.

Despite it was impossible to answer the main question of the study, it remained interesting to investigate whether regulator application played a role in modulating *R. solani* incidence in the tubers, because they were described as elicitors of plant defence (Aranega-Bou et al., 2014; Navarova et al., 2012; Ramon et al., 2008; Shah, 2009; S. Trouvelot et al., 2014), *R. solani* is a fungal pathogen attacking tubers, underground stems, and stolons of potato plants. It is responsible for black scurf on tubers, representing one of the major disease for this crop (Anderson, 1982). Activation of SA-related defence (Hadi & Balali, 2010) and JA-related defence pathway (Taheri & Tarighi, 2010) were show to be active against the pathogen.

Nevertheless, no difference in disease incidence was observed following regulator applications, and the occurrence of the pathogen was overall quite low (Figure III.24). Any possible effect of the molecule was probably obscured by the unusual dry and hot weather conditions occurring during the season, where the experiment was conducted, negatively influencing the development of the pathogen, that prefers cold and wet conditions (Anderson, 1982).

In summary, it became clear from the data of the experiment that [Pi] is not the only factor determining the success of AM application in the field. A number of factors can co-exist in the field which negatively influence the colonization of roots by AM fungi and the evaluation the effects of inoculation. Application of AM fungi in field must take in consideration all the possible variables to ensure that these microorganisms exert their positive effect on crops. A brief analysis about the strategies that can be followed to ensure a successful application is outlined in the next sections.

IV.3 Considerations about improvement of mycorrhizal inoculum quality for field application

In the previous sections of this chapter two strategies have been proposed to improve AM inoculum quality for field application. Exposure to elevated [Pi] proved to be a factor able to induce changing in AM fungal phenotypic development, able even to improve plant growth in certain conditions. The efficacy of this strategy, unfortunately, was not proven in field where phenotypic fungal differences observed in acclimatised strain, where not associated with benefits for plant growth. The acclimatisation strategy seems to act mainly on the extraradical mycelium, that proliferate at higher rate causing higher colonization frequency. Probably the development inside the root is then controlled by the plant which limit the expansion of the fungus at high [Pi], and the explanation for this phenomenon can probably be found in genes related to phosphorus and sugar transporters. Nonetheless, at the current stage drawn conclusion on the acclimatisation of *R. irregulare* risks to be speculative and some work and several knowledge gaps need to be filled prior to master such strategy.

Similar conclusions can be drawn for the second strategy tested. Selected regulators proved to be compatible and able to induce the metabolic modification expected, inducing changes in colonization degrees in greenhouse tests. However, a positive response in terms of plant growth was missing, and more importantly, modulating the colonization or any other parameters related to the plant or the fungus in the field could not be detected.

The main limitation of the current work is that it was focussed on a single stress parameter for the mycorrhizal symbiosis. Pi fertilization in soil is surely an important factor limiting AM fungal performance in field, however, other limiting factors can be even more important. It is therefore necessary to develop inocula that are as responsive as possible to the needs of the field, combining its development with the adoption of new agronomic strategies, able to ensure the sustainable intensification of agriculture. From an industrial point of view, the described experiments failed to produce an inoculum suitable for open field conditions. However, from a purely scientific point of view, they leave many intriguing questions open about the acclimatisation potential of AM fungi in relation to Pi. Furthermore, the data collected can contribute to the understanding of the cause of Pi inhibition in AM fungal symbiosis.

IV.3.1 Nature of mycorrhizal Pi inhibition

With respect to overcoming Pi inhibition, it is possible to see the two proposed hypotheses (acclimatisation and regulation) as alternatives to each other. The first implied a direct action of Pi to the fungus in symbiosis inhibition, while the second suggested a dominant role of plant in determine the fate of symbiosis in presence of high [Pi]. The observations made in the experiments suggest that Pi inhibition is, at least partially, due to direct effects of Pi on AM fungi, and that acclimatisation can overcome this inhibition. Still, it is questionable if the observed higher colonization is always reflected in improved benefit for the host plant, and this suggest that other component play a role in the determination of positive plant/fungus interactions resulting in plant growth promotion. This last aspect was the core of the second tested hypothesis, implying a role of plant in determining the inhibition of symbiosis due to presence of elevated [Pi]. The literature review on the role of phytohormones and defence pathway, used as basis for the experiments (Bedini et al., 2018), showed to be true at root level, where colonization patterns matched the expected scenarios. However, the lack of responses by plants, in terms of growth, confirmed that colonization levels is unbound to the nutrient trade and growth benefits for the host plant. Coupled with the observations made with the acclimatised strain, the plant-fungus regulation indicates the possible presence of two levels of regulation of the symbiosis. The first involves the development of the AM fungus inside and outside of the roots, while the second involves the metabolic events regulating the nutrient trade and other aspects that can determine plant growth promotion. Development of the fungus in the roots appeared to be regulated by different factors and among them the following two items were observed: (i) the capacity of the fungus to develop a proper mycelium, even in presence of high [Pi] (as seen in the acclimatisation section) and (ii) the hormonal balance inside plant roots (as seen in the regulation section). It is, however, clear that many other factors control the outcome of the symbiosis like plant and fungal genotypes, climatic conditions, and soil properties. The second level of regulation concerns all that events inside plants cells resulting in plant growth. Unluckily, this second element of regulation between the partners is still not well understood. Probably the regulation of nutrient exchange is finely regulated by the plant, under control of source-sink and feedback signalization, in order to avoid parasitic effects of the fungus. However, the growth depression observed several times in AM fungal symbiosis (Klironomos, 2003; Smith & Read, 2008), suggest that in some cases even the fungus can play a major role in regulation of nutrient trading (Johnson et al., 1997). The comprehension of the functions in both identified levels of symbiosis represents a main point of interest to elucidate and fully exploit the symbiosis for practical conditions.

IV.3.2 Markers of AM functionality are needed.

What has been stated above and what has been observed in the experiments suggest that, despite often used as marker for AM fungal functionality (Berruti et al., 2014; Lekberg & Koide, 2005), root colonization is not a good indicator of symbiosis efficacy (Ryan & Graham, 2018). In the presented experiments symbiosis efficacy was generally defined as the biomass production during early plant development, despite this term can assume different definitions for different crop type. It would be of great interest to identify a marker for the efficacy of the symbiosis. The already cited root colonization is the most widely used, but its correlation with yield, or plant growth has been recently confuted (Ryan & Graham, 2018). Some authors reported that external mycelium can be a good indicators of symbiosis efficacy (Graham et al., 1982; Mai et al., 2018; McGonigle & Fitter, 1988b; Munkvold et al., 2004; Sanders et al., 1977; Sanders & Fitter, 1992b; West et al., 1993), but other reports suggest that it is not always the case (Smith et al., 2000). Moreover, the measuring of external mycelium is based on the technical feasibility to distinguish living from death AM

fungal hyphae and to eliminate the hyphae belonging to other fungi (Gavito et al., 2003). Moreover, sampling extraradical mycelium can be prohibitive in terms of cost and time (Ryan & Graham, 2018). Therefore, molecular marker attracted the attention of scientists, since they can allow large screening in relatively short time. The identification of proper markers for mycorrhizal symbiosis efficacy is probably an essential step for the implementation of AM fungi in agriculture, and several hypothesis of candidates have been already made (Gamper et al., 2010). Development of reproducible protocols based on reliable markers to evaluate the efficacy of the symbiosis, may represent a valuable tool for plant breeders and fungal inoculum producers to select combinations that can perform better in the field, exploiting all the potential of the symbiosis. However, the identification of a proper marker implies the deep understanding at phenotypical, genetic, and metabolic level of all the processes involved in the symbiosis, and in this frame, as stressed in the previous paragraph, many knowledge-gaps still persist, making the selection quite challenging. Some authors (Campos et al., 2015) proposed AOX genes of *R. irregulare* as possible marker for mycorrhizal performance in the roots. This gene was already proposed as marker for plant yield stability (Arnholdt-Schmitt et al., 2006), however, despite it was shown that *RiAOX* expression was linked to development of the main fungal structure in the roots, no information has been given about the plant response (Campos et al., 2015). Therefore, it appears that the marker was developed only based on fungal development not associated with effects on plant growth. Respiration-related genes of *R. irregulare*, like AOX and COX have been proposed as key elements for symbiosis functionality in *S. tuberosum* (Mercy et al., 2017), but other authors excluded their role in *Lotus japonicus* (Sugimura & Saito, 2017), suggesting that the role of these genes depends on the particular plant-fungus association, therefore representing not suitable candidates as general markers. In the next years, in depth understand of the process determining the positive interactions between plant and AM fungi could be unveiled. The comprehension of these complex phenomena will surely help for the development of stable and reliable inocula for the future.

IV.3.3 Are greenhouse test valid indicators of AM performance in the field?

In absence of proper markers, the development of microbial products usually follows the path of "increasing complexity", that is from highly controlled conditions (lab, greenhouse) to uncontrolled ones (field). However, the described experiments highlighted the presence of constraints in the interpretation of greenhouse results for field application. In both proposed strategies, experiments conducted in controlled conditions confirmed the formulated hypotheses. Unluckily, the validity of the hypotheses was rejected when tested at field conditions. This fact may rise the question on whether the results observed in controlled conditions are directly transposable in the field where other factors play a role in modifying the outcomes of the symbiosis. What observed in the described experiments is not surprising for microbial application, and several authors reported shifting in the efficacy between greenhouse and field (Mehnaz et al., 2010; Ryan & Graham, 2018). This fact is linked with the huge differences occurring between these conditions where greenhouse tests usually resort to autoclaved soil/substrate, and the process alters the nutrient availability, soil structure and kill almost all the possible competitors. Moreover, the presence of containers, in which soil is included, affect root development, limiting the explorable soil volume. The limited soil volume create a different gradient of temperature compared to field soil, as well as nutrient and water dynamic that are completely altered (Bitterlich et al., 2018). Field conditions are completely different and not all the factors playing a role are totally known, and their dynamic is constantly changing during the cultivation period. Moreover, microbial inoculants applied in the field face the challenge to find their niche in the already existing microbiota and adapt to the dynamic climatic and edaphic conditions that can challenge the introduced microorganisms, exerting a selective pressure often too strong to be overcome (Veen et al., 1997).

What described clearly confirms that large limitations and knowledge gaps exist between greenhouse and field. Greenhouse tests are very useful for physiological studies that could not be performed under field conditions (Ryan and Graham, 2018; Smith and Read, 2008), but when the aim is to produce an inoculum for field application, *in vitro* and greenhouse experimental set ups may be not significant and lead to erroneous interpretations and wrong decisions. For this reason, giving the experience acquired during the described experiments, strategies and observation are proposed when the final aim of the study is to produce inocula for field applications.

IV.3.4 Strategies for successful development of inocula for field application

It was stated in the previous paragraphs that a successful development and application of AM fungal inoculum for agricultural purposes requires specific conditions and necessary elements present in the field able to modulate the symbiosis outcomes such as the climate conditions, the soil properties, the micro and macro fauna, as well as the plant cultivars and the agronomic management practices. The interactions of all these factors need to be considered when applying AM fungal inocula in the field. Otherwise, any application will result uncertain in the outcome, that can range from negative to neutral, to positive (Johnson et al., 1997; Klironomos, 2003). Generally speaking, one of the major limitations in AM fungal inocula production can be ascribed to its "generic" nature. In fact, usually, inocula, both from academia and from industry, derive from collections or from field isolation. They are usually mono-specific, tested in controlled environments and only a few parameters of interest are investigated, like the root colonization capacity and spore production. Often the tests are performed under conditions that reward the presence of the fungus, like the well-known Pi starvation. This means that the study and development of the inoculum is very limited and only standard and simplified conditions are tested. Moreover, rarely the attention is focused on all the factors that could eventually reduce the efficacy of the symbiosis or to specific stress condition (Berruti et al., 2016). The research for the "generic" inoculum is coherent from the industry point of view that strives for "silver bullet inoculum", adapted to most of the field condition and crop varieties, easy to be produced, handled, stored, transported, and applied, therefore competitive at any economical level. However, the complex web of factors influencing the efficacy of the symbiosis, ideally would require an in-depth study of each field where the inoculum should be applied, with different formulations for all the different cases, leading to dramatic increases of the price. AM fungi generally do not shows species specificity in colonization of plants (Smith and Read, 2008) and any mycorrhiza plant can be colonized by any AM fungus in a one to one situation. However, this fact may have led to the misleading simplification that the presence of the fungus in the roots will automatically generate benefits to the plants. In reality, many studies report that different AM fungal species have different outcomes in plant growth and differences in efficacy of the symbiosis have been observed even at strain level for AM fungi (Allen, 1992; Ehinger et al., 2009; Pivato et al., 2009; Scervino et al., 2005; Taylor et al., 2003). These differences are partly due to adaptations to specific environments and hosts, and, despite not completely comparable, this situation is not different from what is observed normally in agriculture. In Europe, for example, there are 4788 different corn varieties, 2024 varieties of wheat and 1665 varieties of potato registered (Community Plant Variety Office; https://cpvo.europa.eu/en/statistics). Each of these varieties respond to the need of specific environmental conditions or to different outcome of the crop, in terms of life cycle duration and product quality. Similar patterns should be expected even for AM fungi, where specific strains are adapted to specific conditions. It was shown that inocula obtained from specific plant-environment combinations are more effective in inducing plant growth rather than generic inocula (Calvente et al., 2004; Caravaca et al., 2003; Rowe et al., 2007). Therefore, different crops in different regions need their own formulations in terms of species, strains,

and application strategies to obtain the desired outcomes. Moreover, some authors raised the concern about inoculation with few exotic strains of AM fungi that may have consequences for natural ecosystems, particularly for fungal and plant communities (Hart et al., 2018), leading to biodiversity loss with consequent reduction of ecosystem resilience (Oliver et al., 2015). The proper formulation of the inocula is of vital importance for the successful implementation of these organisms in agriculture. Clearly economic limitations and lack of knowledge by the end users limit the application of the described improvements. However, an efficient use of such microorganisms in field may help to increase the interest of farmers, broadening the market and consequently reducing the cost. To do so will be of interest to determine few easy measurable factors affecting symbiosis outcome in field, paying attention to avoid oversimplification. Luckily, in the last years the technology for big data collection and analysis made impressive progress. Sensors ranging from towers to small devices able to screen for several crop parameters, like: canopy development, transpiration and photosynthesis activity (Araus & Cairns, 2014; Friedli et al., 2016; Jones et al., 2018; Naito et al., 2017; Shakoor et al., 2017; Yang et al., 2017) are well developed. Unluckily, at the moment the possibility to assess root development and their interactions are still limited (Pauli et al., 2016) even though mapping of soil properties, as well as the high throughput techniques for assessing microbial biodiversity are already well developed and could provide information useful for the understanding of the processes occurring belowground (Casa et al., 2013; Graefe & Sradnick, 2018; Thompson et al., 2017). The use of these data to generate models for crop development, with integration of AM fungi, may help to determine some key indicators promoting mycorrhizal efficacy (Bitterlich et al., 2018; Boldt et al., 2011; Romero-Munar et al., 2017). Some authors suggest an interesting strategy to tackle the complexity of microbial field inoculation. It can be described as “backwards strategy”, where identification of successful field examples is then used as basic material for further analysis and understand of phenomena connected to the symbiosis. In other words, the understanding process should follow the way from the field to the lab and not vice versa. Lab and greenhouse tests would then be fundamental in order to understand the plant-inoculum-environment interactions on model plant and control conditions (Rouphael et al., 2018). As last, other authors stressed the importance of the so called “reverse breeding” (Palmgren et al., 2015). Many studies pointed out that domestication and/or other selection process made by humans affect the microbiome associated to the plants (Reviewed by Pérez-Jaramillo et al., 2018). From the evolutionary point of view, ancestral varieties and landraces are more adapted to stress occurring in the environment, and part of this adaptation is due to the microbiome (Palmgren et al., 2015). For this reason, breeding programs should start to take into account even the genetic traits affecting plant-microbiome interactions, and insert them in the new varieties with superior production characteristics (Sellitto, 2020).

Strategies to improve mycorrhiza inoculum production are several and in any of them only positive or negative aspects can be found. However, field inoculation with AM fungi, as well as any other biological material, will always conserve a certain range of uncertainty, as consequence of the already cited complex network of interactions occurring in the field. Nevertheless, improving the quality of data and knowledge about specific conditions, as well as the formulation and selection of appropriate strains and plant varieties will lead to a significant increase of the chance to reach a successful interaction, helping to get to a sustainable intensification of agriculture. What was described regarded only the development and formulation of new AM fungal inocula. The development of a proper product, however, does not represent a guarantee of success, in fact, once the inoculum is ready, it is applied in the field, where it is inserted inside a new context, dominated by agronomic decision, that can modulate again its functionality. Formulation of improved inocula, therefore, represents only the first step for the successful application of these microorganisms. It is important to communicate better to farmers, agronomist, and all the people involved in field production the importance and the biology of AM fungi and other beneficial microorganisms and to teach them the correct way to handle and to use them. Sharing the knowledge and the objectives with

industry, scientists, farmers, agronomists, and all other people involved in food production, may lead step by step to sustainable agriculture intensification.

V. Conclusion

Two strategies to improve the inoculum quality for field application have been described and tested at experimental and practical level. None of the tested strategies showed the desired effects when applied in the field. However, despite the limited time available, some useful information has been gathered. Specifically, from what has been named the "acclimatisation strategy", experiments suggest that it is possible to acclimatise AM fungi to generally inhibitory Pi concentrations. The most promising results were obtained under controlled conditions, although the molecular investigation did not allow to determine which mechanisms were involved underlying the observed differences. The application of this technique in the field did not yield significant results. Data shows that acclimatisation may be a valuable tool able to make an inoculum more effective in the presence of high Pi levels, in terms of plant growth and colonization ability. However, the acclimatisation to Pi did not transfer all properties to the inoculum necessary to fully work at field conditions, where the occurring stressors are numerous and, sometimes, unpredictable. Therefore, acclimatisation, may be of interest in application in controlled conditions, or may represent a second step to improve inocula already tested and which efficacy has been already assessed in the field. Likewise, use of regulator molecule, showed to be able to modify the colonization patterns under controlled conditions. However, they presented limitations in their use in practice. This work may appear incomplete since it does not fulfil the main goal to develop improved inocula for field application. This is a direct consequence of the limited time and the lack of complete knowledge of the core of the problem. However, the hope is that the performed experiments managed to raise new scientific questions on the relationship between mycorrhizal symbiosis and Pi inhibition, contributing a few elements to this challenge where many questions still wait for their answer. In this work, adoption of new strategies in the formulation of commercial inocula has been proposed based on the exploiting of new technologies that could contribute substantially to the development of proper inocula for different application context, and not for unrealistic experimental conditions. We have to keep in mind, that the sustainable intensification of agriculture will be the consequence of a widespread comprehension and diffusion of novel strategies, of which AM fungi are just a little, nonetheless important, part.

VI. Bibliography

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VII. Sitography

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IX. Appendix

The paragraph “1.5.2 Regulator as modulator of AM fungal inoculum performance” and the figure I.3, I.4, I.5 has been extracted from Bedini et al. 2018, published in the Journal Frontiers of Plant Sciences. The images are published with open access under the CC-BY Creative Commons attribution license (the current version is CC-BY, version 4.0 http://creativecommons.org/licenses/by/4.0/). This means that the author(s) retain copyright, but the content is free to download, distribute and adapt for commercial or non-commercial purposes, given appropriate attribution to the original article. The ideas and images taken from the article and used for this thesis are the work of myself, matured and developed through discussion with the co-authors of the article.

Sincerely,