FRIENDS WITH BENEFITS, OR A RELUCTANT THREESOME? ARBUSCULAR MYCORRHIZAL PARTNERSHIPS AND THEIR EFFECTS ON CROP PATHOGENS

A thesis submitted by

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DEDICATION

This thesis is dedicated to my “personal PhD universe”. To all the wonderful, amazing, loving, understanding, thoughtful, patient, supportive, inspiring, uplifting people I am honoured to have in my life.

STATEMENT OF AUTHENTICATION

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution

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03. February 2023

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"And, when you want something, all the universe conspires in helping you achieve it."

Paulo Coelho

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ACRONYM OVERVIEW

Abbreviation	Meaning	Description
ABA	Abscisic acid	Plant hormones involved in mycorrhiza symbiosis
AcA	Azelaic acid	Long distance mobile signalling molecules involved in systemic acquired resistance
AGR	Absolute growth rate	Increase in plant size over time
AM fungi	Arbuscular mycorrhiza fungi	Widespread beneficial fungi from the subphylum Glomeromycotina forming a mutualistic root symbiosis land plants
Avr gene	avirulence gene	Counterpart of resistance gene derived proteins
BR	Brassinosteroid	Plant hormones involved in mycorrhiza symbiosis
CCA	Correspondance Analysis	Multivariate statistical analysis to define the relationship between/within variables
DA	Diterpenoid dehdrabietinal	Long distance mobile signalling molecules involved in systemic acquired resistance
DAP	Days after planting	Amount of days post seedling transplant
DEGs	Differentially expressed genes	Genes with significantly different expression level between treatments
EFR	EF-Tu receptor	Transmembrane receptor kinase involved in antimicrobial defence responses
ET	Ethylene	Plant hormones involved in mycorrhiza symbiosis
ETI	Effector triggered immunity	Early defence response immunity due to effector recognition and resulting suppression of the pathogen
FLS2	Flagellin insensitive 2	Transmembrane receptor kinase involved in antimicrobial defence responses
G3P	Glycerol-3-phosphate	Long distance mobile signalling molecules involved in systemic acquired resistance
GA	Gibberellic acid	and mycorrhiza induced defence response
GO-terms	Gene ontology terms	classification of genes asiigend to a defined set of functional traits
HR	Hypersensitivity response	Defence response mechanism that leads to programmed cell death to avoid proliferation of biotrophic pathogens
ISR	Induced systemic resistance	Enhanced resistance mediated by non-pathogenicmicroorganisms
JA	Jasmonic acid	Plant hormones involved in mycorrhiza symbiosis and plant defence responses
MAMPs	Microbial-associated molecular patterns	Specific structures or molecules from the attacker that can be recognized from the plant
MAPK	Mitogen activated protein kinase	Early defence response pathway triggeredfrom pathogen recognition
MIR	Mycorrhiza-induced resistance	Induced plant resistance conferred by AM fungi
NBS-LRR	Nucleotide-binding site leucine-rich repeat	Plant proteins to sense effectors of the pathogens
OTU	Operational taxonomic unit	Classification of groups of closely related individuals based on sequence similarity
PAMPs	Pathogen-associated molecular patterns	Specific structures or molecules from the attacker that can be recognized from the plant
PGPF	Plant growth-promoting fungi	Enhance nitrogen and mineral uptake and therefore
PGPR	plant growth-promoting rhizobacteria	Beneficial non-pathogenic soil microorganism that
Pip	Piperolic acid	Long distance mobile signalling molecules involved in systemic acquired resistance
PPRs	Pattern recognition receptors	Special transmembrane receptors that are able to sense signals from the attacker
PR genes	Pathogenesis related genes	Genes activated after recognition of salicylic acid signals triggered from pathogen recognition in another plant organ
PR proteins	Pathogenesis related proteins	Proteins transcribed from PR genes that possess antimicrobial effect
PSA	Projected Shoot Area	Sum of the shoot area, measured in kilopixels, from 3 camera views, comprising (obtained using RGB images)
PTI	PAMP-triggered immunity	Early defence response immunity due to pathogen recognition and resulting suppression of the pathogen
R-genes	Resistance genes	Genes encoding for nucleotide-binding site leucine-rich repeat protein that again recognise pathogen derived proteins
ROS	Reactive oxigen species	Early defence response pathway triggered from pathogen recognition
SA	Salicylic acid	Plant hormones involved in mycorrhiza symbiosis
SAR	Systemic acquired resistance	Systemic mediation of defence response mechanism
sPSA	Smoothed projected shoot area	Smoothed raw data of projected shoot area used for

ABSTRACT

High yield losses of crops due to plant pathogens represent a serious problem in agriculture. More effective and sustainable control measures, such as biological control, are essential. Most terrestrial plants, including important crop plants, benefit from a mutualistic symbiosis with arbuscular mycorrhizal (AM) fungi through enhanced nutrition and improved immune responses. Although this latter phenomenon, known as Mycorrhiza Induced Resistance (MIR), is well-reported and molecular responses to AM symbiosis have been observed, how AM fungi prime disease resistance is still poorly understood. Several factors and mechanisms have been suggested to impact the outcome of MIR, but how this phenomenon occurs and how different factors impact MIR is not known.

Evidence suggests that AM fungal species differ in their abilities to defend plants and that plant species, and even varieties, can have differing colonisation levels leading to changing outcomes of MIR. However, the underlying molecular mechanisms on a biochemical level leading to alterations of MIR are not well understood and require examination of patterns related to MIR effects and involved molecular factors in these interactions to exploit the biocontrol potential of AM fungi. To achieve this, my doctoral thesis investigates some of these fundamental MIR knowledge gaps by combining interdisciplinary research including phenotyping observations as well as molecular approaches (transcriptomics and metabolomics).

Chapter one provides an overview of the literature underlying the current state-of-the-art of MIR and provides background for the following four experimental chapters.

In **chapter two** of this thesis, a transcriptomic approach was used to assess transcriptional responses of two accessions ("KAH1" and" TR2B") of *Brachypodium distachyon* colonised (or not) with AM fungi to assess the expression of genes associated with biotic stress and connected pathways. Additionally, microbial community sequencing and colonisation data representing pathogen and AM fungal frequencies were used to reveal plant defence-related MIR-candidate genes. The findings in this chapter reveal patterns in the basic plant gene expression landscape, specifically of non-defence-related biological functional processes linked to beneficial and pathogenic microbes (abundance of AM fungi, Glomeraceae and Oomycetes). These results demonstrate the broad influence of MIR and depict the importance of investigating different stages of MIR.

Chapter three used two chickpea (*Cicer arietinum)* varieties (Sonali and Kyabra) to investigate how differential AM host receptivity may impact AM-mediated defence responses against a root rot pathogen, *Phytophthora medicaginis* isolate 7831. Inoculation with a commercial inoculum *Rhizophagus irregularis* significantly inhibited pathogen addition-related biomass losses in Kyabra, which exhibits the higher AM colonisation levels of the two varieties, while no impact on plant biomass was observed in Sonali. Using root metabolomic profiling, we detected 143 molecular features that exhibited significant variation between at least two experimental conditions. Most notably, the two chickpea varieties showed opposing metabolomic responses to microbial exposure. Furthermore, the co-occurrence of pathogenic and beneficial microbes led to a core set of differentially expressed metabolomic features in Kyabra and Sonali. These results demonstrate contrasting levels of metabolomic responses to microbial inoculation and reveal potential molecular features involved in MIR in a globally relevant crop.

Chapter four examined how species of AM fungi differ in their impact on *Solanum lycopersicum* cv. Micro-Tom influence on pathogen addition and whether this response is affected by physiological trade-offs between growth and defence in the plant. We inoculated tomato plants with different AM fungal isolates/combinations (*Glomus mosseae* WFVAM 45, *Rhizophagus irregularis* DAOM 10, WFVAM 21, *Gigaspora margarita* WFVAM 21, *Scutellospora calospora* WFVAM 35, *Rhizophagus irregularis* WFVAM 23) and exposed them (or not) to the root pathogen *Rhizoctonia solani*. Non-invasive real-time high-throughput phenotyping observations as well as a destructive end-point harvest revealed variable responses to inoculation with AM fungi and the root pathogen. I identified MIR effective and MIR non-effective isolates, which when combined in mixtures resulted in mostly non-additive effects on MIR. These results highlight the different abilities of AM fungal species/communities to improve growth and/or resistance to pathogen addition in plants.

Chapter five expands on findings from chapter four and investigated molecular interactions between plants and AM fungi using metabolomic profiling. Root metabolomic profiles to characterize defence responses of plants colonised with previously identified MIR efficient or non-efficient AM fungal species enabled insight into the varying biochemical MIR-related functional specialization of AM fungi and the involved metabolic pathways. I observed MIR-related biochemical patterns specific to AM fungal species inducing MIR and an increased number of down-regulated metabolic features. These results show the additional presence of MIR-associated AM fungal specificity on a molecular level.

In **Chapter six** I discussed the results from the previous four empirical chapters and their implications.

The results of my PhD research provide increased and novel insights into comprehensive phenotypic, transcriptional and biochemical patterns and mechanisms related to mediation of MIR. Investigation of basic plant gene expression patterns revealed the involvement of beneficial and pathogenic microbes in non-defence-related biological processes and shows that MIR is involved in a large number of processes within the transcriptomic profile. This thesis provided further evidence of the importance of AM fungal identity on the outcome of MIR and showed that the differences in metabolic profiles reflect these observations. My thesis also highlights the importance of studying MIR in natural contexts, where plants interact with diverse fungal communities, by showing the non-additive effects of AM fungal communities compared to single species observations. I also show that plant varieties with different potentials for AM fungal colonisation demonstrate opposing MIR metabolomic responses. Moreover, the potential involvement of down-regulated metabolic pathways to protect plants against pathogen addition has been demonstrated. These studies highlight the need for further investigations of the biochemical networks leading to the strength of MIR.

As many as 821 million people (or one in nine people) on the planet are currently undernourished, and the extent of this problem is expected to grow as one billion are added to the global population in the next 15 years (FAO, IFAD, UNICEF, 2018; United Nations, Department of Economic and Social Affairs, 2017). Addressing the increasing gap in food supply and demand will require substantial investment in the sustainable production of food crops and the need to balance the demand for crop production with uses in animal feed, energy production and other industrial needs (Foley et al., 2011. Plant disease can lead to substantial losses in important crops (Figure 1.1): as much as 30% of global food production losses, and up to 100% loss in specific circumstances (Savary et al., 2019; Strange and Scott 2005; Agrios, 2005). Therefore, a key aspect of enhancing sustainable crop production is preventing losses due to plant pathogens. These losses are driven by many types of pathogens including viruses, bacteria, fungi, oomycetes, nematodes, and parasitic plants (Strange & Scott, 2005).

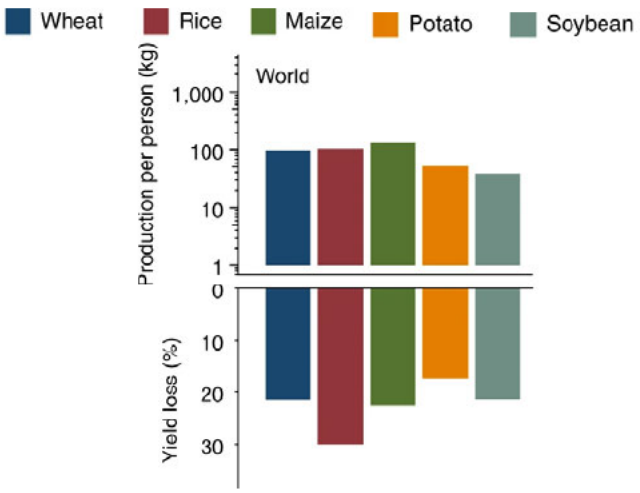


Figure 1. 1: *Global production losses in wheat, rice, maize, potato, and soybean across the globe. The kilograms of crop production per person (2010–2014 averages) are shown on a log10 scale (top panel) versus the percentage yield losses globe (lower panel). Figure adapted from* (Savary et al., 2019)

Practices to reduce losses due to pathogens include strict quarantine measures, crop rotation, pesticide use, breeding for resistance and use of genetically modified (GM) plants (Aktar et al., 2009; Christou & Twyman, 2019; FAO, IFAD, UNICEF, 2018; Oliver, 2014). However, these methods harbour potential risks, are expensive, may not be efficient, and can

lead to a dependency of farmers on big companies (Bawa & Anilakumar, 2013; Popp et al., 2013; Zhang et al., 2016). Therefore, biorational means of disease control must be sought, but these require an in-depth understanding of how the plant immune system functions.

1.1 INNATE PLANT IMMUNE SYSTEM

Plants have evolved a two-component innate immune system that can recognise a pathogen, followed by a downstream signalling cascade that leads to a variety of defence responses (Jones & Dangl, 2006;Martinez-Medina et al., 2016). The first component requires successful recognition of invading pathogens through the detection of pathogen-specific structures or molecules (Chisholm et al. 2006; Jones and Dangl 2006; Boller and Felix 2009; Marquez et al., 2019). These structures are referred to as microbial- or pathogen-associated molecular patterns (MAMPs, PAMPS) (Boller & Felix, 2009; Chisholm et al., 2006; Jones & Dangl, 2006). Examples of PAMPs are proteins like elicitins secreted from *Phytophthora* species or chitin secreted from true fungi (Ricci et al. 1989; Nurnberger et al. 2004; Ito et al., 1997). The plant can sense these elicitors via special transmembrane pattern recognition receptors (PRRs) (Table 1.1) (Chisholm et al. 2006; Jones and Dangl 2006; Boller and Felix 2009. The best-studied examples of this kind of receptor are two leucine-rich repeat receptor-like kinases, FLS2 (Flagellin insensitive 2) and EFR (EF-Tu receptor), which can sense two highly conserved structures in bacteria (Gómez-Gómez and Boller 2000; Kunze et al. 2004; Felix et al., 1999). Activation of these plant receptors induces downstream signalling and leads to several defence responses such as the activation of the Mitogen Activated Protein Kinase (MAPK)-pathway, which in turn mediates defence-related gene expression or stomatal closure (Pitzschke et al., 2009; Meng & Zhang, 2013; Asai et al., 2002; Levine et al., 1994; Shaw et al., 1999). They can also lead to bursts of reactive oxygen species (ROS) that have a direct antimicrobial effect and cause programmed cell death via the hypersensitivity response (HR) (Pitzschke et al., 2009; Meng & Zhang, 2013; Asai et al., 2002; Levine et al., 1994; Shaw et al., 1999). This latter pathway limits resources for biotrophic pathogens, which need a living host to survive (Fire et al., 1998). Callose deposition in infected cells, a strengthening of the cell wall, is another rapid defence response associated with MAMP and PAMP presence (Luna et al., 2011; Sanmartín et al., 2021). This first recognition of MAMPs/PAMPs, illustrated in Figure 1.2, and the consequences that lead to early defence responses and the suppression of the pathogen, is called PAMP-triggered immunity (PTI) (Boller & Felix, 2009; Chisholm et al., 2006; Jones & Dangl, 2006).

Pathogens have evolved novel mechanisms to overcome the initial plant immune response by generating effector molecules that modulate extra- and intracellular plant proteins to suppress PTI (Jones & Dangl, 2006; Chisholm et al., 2006; DeYoung & Innes, 2006). Plants, in turn, evolved direct or indirect sensing of effectors with the help of nucleotide binding site leucine-rich repeat (NBS-LRR) proteins that lead to the second layer of plant innate immune system: ETI (effector-triggered immunity)(Dangl & Jones, 2001; Boller & Felix, 2009; Chisholm et al. 2006; Jones and Dangl 2006; DeYoung & Innes, 2006). NBS-LRRs can encode so-called resistance genes (R-genes) and their suitable pathogen-derived recognition partners are called avirulence (Avr) genes (Figure 1.2) (Dangl & Jones, 2001; Jones & Dangl, 2006; DeYoung & Innes, 2006; Chisholm et al., 2006). An example of a pathogenic effector and its matching R-gene is the *Phytophthora infestans* protein Avr3a which counteracts cell death during the biotrophic phase, and the corresponding plant R3a gene (Bos et al., 2010). New strategies to either generate new or modify existing effectors will therefore result in enhanced plant defence (Jones & Dangl, 2006; Chisholm et al., 2006).

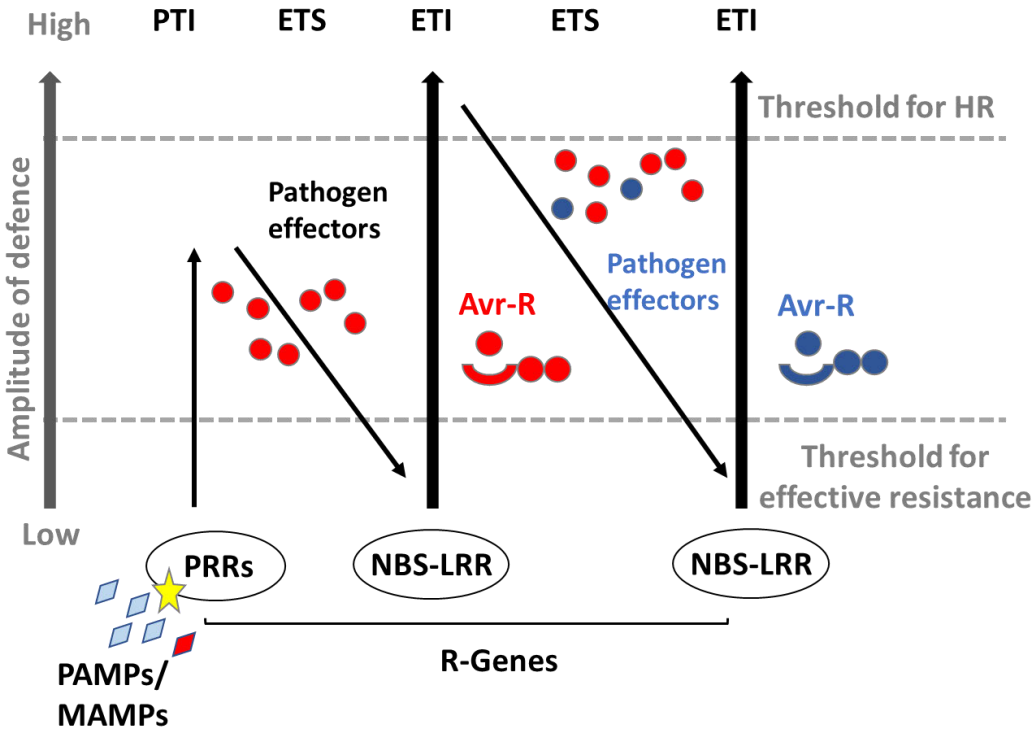


Figure 1. 2: *Zigzag model of defence-virulence response progression between plants and pathogens. PAMP-triggered immunity happens as a first result of the recognition of PAMPs of MAMPs via PPRs. As a consequence, effectors are released from the pathogen to overcome the PTI. The recognition of these effectors by the plant with nucleotide-binding site leucine rich repeat (NBS-LRR) proteins encoded from R-genes will lead to ETI. This continues with the counterattack of the pathogen with new Avr proteins and recognition from the plant in turn. PTI = PAMP-Triggered immunity, ETS = effector-triggered susceptibility, ETI = effector-triggered immunity, HR = hypersensitive cell death. Picture used with permission from (Jones & Dangl, 2006).*

1.2 SYSTEMIC ACQUIRED RESISTANCE (SAR)

Plants evolved a mechanism to systemically protect themselves and confer defence response to organs far away from the site of stress (Gozzo & Faoro, 2013). This systemic mediation of defence response mechanisms is dependent on mobile signalling molecules such as salicylic acid (SA), methyl salicylic acid (MeSA), diterpenoid dehydrabietinal (DA), azelaic acid (AcA), pipecolic acid (Pip) and glycerol-3-phosphate (G3P) (Fig. 1.3, Table 1.1) (Fu and Dong 2013; Gozzo and Faoro 2013).

SA is the best-studied long-distance signalling molecule and the recognition of special receptors to activate gene transcription is needed for activation (Fu and Dong 2013; Durrant and Dong 2004; Birkenbihl et al. 2017; Kohler et al. 2002; Cao et al. 1994; Bakshi and Oelmüller 2014; Mora-Romero et al. 2015). These signals lead to the transcription of pathogenesis-related (PR) genes and the expression of antimicrobial PR proteins (Fu and Dong 2013; Conrath 2009; van Loon et al. 2006). A link of SA alteration has been shown to the colonisation of AM fungi, but the comprehensive consequences of these modifications are still not understood (Zhang et al., 2013; Benjamin et al., 2022). Jasmonic acid (JA) is another endogenous inducer of SAR which impacts plants by cross-talking with SA (Fu and Dong 2013; Gozzo and Faoro 2013). Moreover, the recognition of necrotrophic pathogens involves JA as well as ethylene (ET) (Fu and Dong 2013; Gozzo and Faoro 2013; Mengiste 2012). Abscisic acid (ABA), another hormone that is potentially involved in the process of SAR, as it is associated with the regulation of SA signalling and has been linked to callose deposition (Gozzo and Faoro 2013; de Torres Zabala et al. 2009; Kusajima et al. 2010). Furthermore, SAR is also able to induce priming (see the later section on priming for more details)(Conrath, 2006).

SAR-involved mechanisms have been previously shown to be enhanced by AM fungi. The enhanced systemic protection of tomato plants infected with *Phytophthora nicotianae* through an AM fungus was also shown in a split root experiment depicting resistance in non-mycorrhized roots (Pozo et al., 2002). Colonization with *Glomus mosseae* conferred local but also systemic protection by stimulating plant cell wall thickening of the plants (Pozo et al., 2002). Another study showed the systemic upregulation of PR genes in leaves of potato plantlets infected with *Phytophthora infestans* after AM fungus colonization (Cameron et al., 2013)(Cameron et al., 2013). The upregulation of SA inducible PR proteins and defence-related enzymes in leaves of mycorrhized tomato plants upon *Alternaria solani* infection confirm these findings (Y. Song et al., 2015a). These results show the involvement of SAR mechanisms in MIR as well as a priming effect of AM fungi (Gallou et al., 2011). Another split-root system showed a systemic bio-protection effect correlated with AM fungi colonization rate and SA

concentration (Khaosaad et al., 2007). However, no increase in SA concentration in non-mycorrhized roots of AM fungal colonised plants was observed (Khaosaad et al., 2007). Hence, apart from mechanisms involved in the innate immune system and SAR, also SA-independent mechanisms might play a role in MIR.

1.3 Biological control through Induced Systemic Resistance (ISR)

In addition to selecting plant genotypes with improved resistance, biological control of disease is an expanding practice that may be used to reduce losses due to pathogens. This approach uses organisms to decrease the damage caused by pathogens (Cook, 1993; Heydari & Pessarakli, 2010). These organisms may control pathogenic microbes directly through the production of antibiotics or toxic metabolites (Cook, 1993; Heydari & Pessarakli, 2010; Agrios, 2005), or they may compete for nutrients and niche space thereby displacing pathogenic microorganisms (Heydari & Pessarakli, 2010). However, these biocontrol agents may impede pathogens by boosting plant immunity, a mechanism of control that needs further study so that beneficial microbes can be better leveraged in agriculture (Owen et al., 2015; Fravel, 2005; Ghorbanpour et al., 2018).

Increased plant resistance to disease-causing microbes mediated by non-pathogenic microorganisms is defined as Induced systemic resistance (ISR) (Pieterse et al., 2014; Pozo & Azcón-Aguilar, 2007; Conrath, 2009). Plant growth-promoting rhizobacteria (PGPR) such as *Pseudomonas* spp. and *Bacillus* spp. or plant growth-promoting fungi (PGPF) such as *Trichoderma* spp. and Arbuscular Mycorrhiza fungi can confer below- and aboveground bio-protection from a large number of attackers (Pieterse et al., 2014; Pozo and Azcón-Aguilar 2007; Pieterse et al. 2014; Kloepper et al. 2004; Wei 1991; Martínez-Medina et al. 2013). Unlike SAR, ISR operates predominantly in an SA-independent manner (Table 1.1) (Conrath, 2009; Van Wees et al., 2008; Shoresh et al., 2010; Pieterse et al., 1996; Ahn et al., 2007), and typically relies on the plant hormones jasmonic acid (JA) and ethylene (ET) (Conrath, 2009; Shoresh et al., 2010). However, this is not an absolute rule with some beneficial microbes triggering ISR through SA and ABA signalling pathways (Pieterse et al., 2014; Kloepper et al., 2004; Martínez-Medina et al., 2013). This illustrates the intensive cross-talk of all immune defence mechanisms, which makes them difficult, if not impossible, to consider separately (Pieterse et al., 2014; Kloepper et al., 2004; Martínez-Medina et al., 2013). Triggers of ISR are likely to be MAMPs such as lipo-chitooligosaccharides as well as microbial production of volatile compounds or antibiotics that can be recognized by the plant (Van Wees et al., 2008; Rosier et al., 2018). At the same time, many PGPRs seem to be able to suppress the local defence response to enable the

colonisation of the host roots with only comparably low transcriptomic change (Pieterse et al., 2014). Compared to the well-studied systemic response of the innate immune system (Figure 1.3) less knowledge is available regarding ISR (Table 1.1) (Pieterse et al., 2014; Yu et al., 2022; Kamle et al., 2020; Conrath et al., 2001). The outcome of ISR is the induction of JA/ET-dependent defence genes and therefore enhanced performance in case of a subsequent pathogen attack (Table 1.1) (Pieterse et al., 2014; Van Wees et al., 2008; Ahn et al., 2007; van Wees et al., 1999). This enhanced defence response at a later stage than the actual trigger is called priming (Martinez-Medina et al., 2016; Kohler et al., 2002; Conrath et al., 2015; Balmer et al., 2015; Pastor, Luna, Mauch-Mani, et al., 2013).

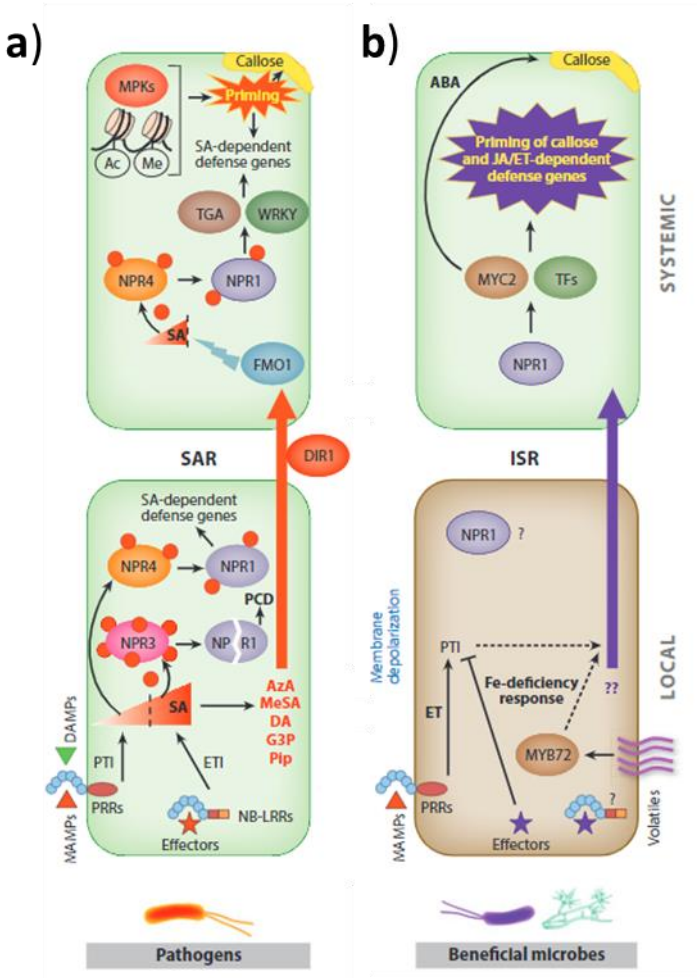


Figure 1. 3: *Known and proposed mechanisms involved in **a)** Systemic acquired resistance (SAR) and **b)** Induced systemic resistance (ISR), the solid black lines show established interactions whereas dashed lines show theoretical interactions. Abbreviations: DIR 1 = Defective Induced Resistance1, acts probably as a chaperon for the mobile SAR signals, FMO1 = Flavin-Dependent Monooxygenase 1, proposed to convert or amplifies the long-distance signals, TGA = TGA family of transcription factors interacting with NPR1 that binds together with WRKY transcription factors to promoters of SA-responsive defence genes, MYB72 = transcription factor serving as early signalling factor and intersection point in ISR, MYC2 = transcriptional regulator of JA-depended defences. Picture used with permission from* (Pieterse et al., 2014)

Table 1. 1: Summary of the underlying mechanism of the innate immune system, systemic resistances of SAR and ISR. This table shows the triggers and consequences involved in the different types of known plant immune systems.

Type of immune system		Trigger	Signal	Gene expression	Modification	Defense response	Outcome	Site of action
Innate immune System		Recognition MAMPs/PAMPs from virulent pathogens via specific receptors	Downstream signals of receptor recognition	R Genes	R proteins	local resonse - HR	PTI & ETI	local
Systemic resistance	*SAR*	Early defence responses	Accumulation and allocation of SA, MeSa, AcA, DA,AcA, Pip G3P, JA, ET, ABA	PR Genes	SA dependent antimicrobial PR Proteins	Increased survival of the cell distal from infection site	SAR	systemic
	ISR	Non pathogenic microorganism PGPR & PGPF	JA & ET	JA & ET dependent defence genes	?	JA/ET related improved systemic defence	ISR	systemic

1.4 PRIMING

The process in plants of 'memorising' environmental stimuli and thus enhancing the defence response to future attack by pathogenic microbes is called 'priming'. This imprinting is induced by a variety of different stimuli such as secreted stimuli from PGPR/PGPF including SA analogues or amino acids (Conrath, 2009; Mauch-Mani et al., 2017; Cordier et al., 1998; Conrath et al., 2006; Hilker et al., 2016). Priming shows improved disease protection with very low fitness costs for the plant compared to the direct induction of defence (Figure 1.4), which makes it a valuable tool in crop protection strategies (Martinez-Medina et al., 2016; Mauch-Mani et al., 2017; Balmer et al., 2015; Conrath, 2009; Hilker et al., 2016; Fontana et al., 2009). The stages characterizing the priming event include the priming phase, the post-challenge prime state and the duration of the primed state (Mauch-Mani et al., 2017; Balmer et al., 2015). In the priming phase, physiological changes such as rapid calcium increase in the cytosol, molecular changes such as mitogen-activated protein kinases 3/6 (MAPK3/MAPK6) and epigenetic changes such as histone modification occur in the plant (Martinez-Medina et al., 2016; Conrath, 2006; Pieterse et al., 2014; Mauch-Mani et al., 2017; Pastor, Luna, Mauch-Mani, et al., 2013). In the post-challenge primed state, the enhanced perception of the invader occurs due to increased PRRs, enhanced signal transduction of MAPK pathway genes, and/or rapid ROS or callose production (Martinez-Medina et al., 2016; Conrath, 2006; Mauch-Mani et al., 2017; Balmer et al., 2015). The final duration of this primed state is usually a long-term response and may in some cases be passed on to the next generations (Mauch-Mani et al., 2017). While a wealth of studies show that the priming effect exists, and while many different pathways are

implicated, so far, no clear pattern of gene expression and metabolomic pathways involved in this process have been identified across plant hosts or PGPR/F (Conrath et al., 2015; Balmer et al., 2015; Conrath, 2009; Goellner & Conrath, 2008).

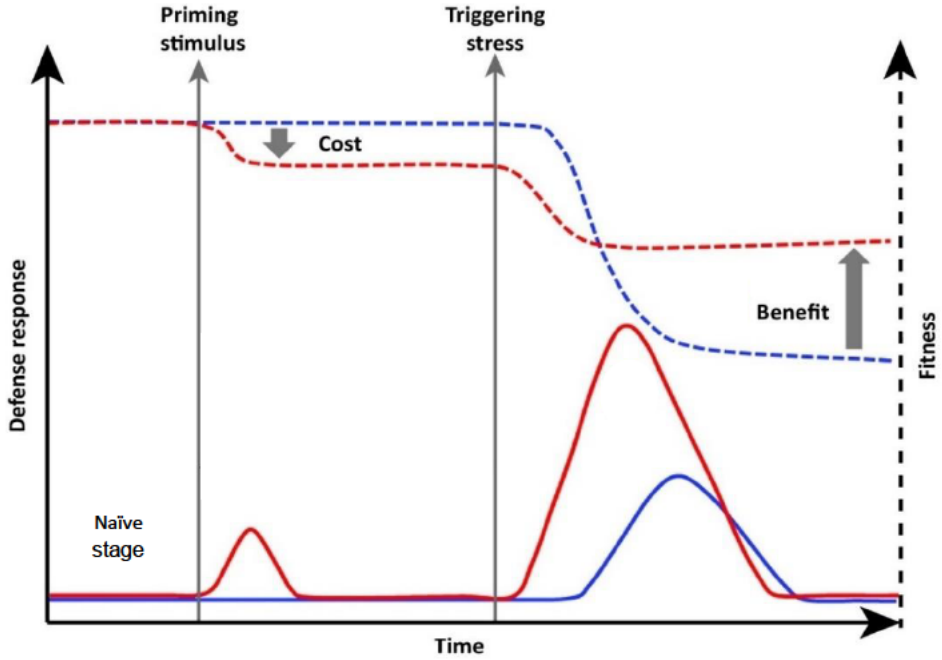


Figure 1. 4: *Trade-off between investment in defence response and fitness in primed and non-primed plants. The naïve stage depicts the time before the priming stimulus. The priming stimulus will lead to a slight decrease in fitness and a slightly increased defence response after its occurrence. However, the initial little fitness costs will pay off in case of triggered stress. In this case, the defence response will appear faster, earlier, more sensitive and stronger which on the other hand will lead to a fitness benefit. Unprimed = blue, Primed = red, defence response = solid lines, fitness = dashed lines. Picture used with permission from* (Martinez-Medina et al., 2016).

Mycorrhizal fungi, classified as PGPF, have frequently been linked to the phenomenon of priming (Pieterse et al., 2014; Pozo & Azcón-Aguilar, 2007; Conrath et al., 2015; Cameron et al., 2013; Hilker et al., 2016). However, so far there is no experimental proof concerning this link and several other factors could be involved in how PGPF confer resistance to the plant in the pathogen-plant-PGPF tripartite interaction. The lack of detailed understanding of how AM fungal interaction in plants leads to disease protection has meant that their use has resulted in unpredictable and inefficient results to date (Owen et al., 2015). This highlights the importance of investigating AM fungi and their properties in plant protection.

1.5 AM FUNGI

Arbuscular Mycorrhiza fungi form important symbiotic associations with plant roots. The advantage of this symbiotic interaction is bidirectional resource transport (Smith & Read, 2008). Mainly phosphorus and nitrogen are provided by the fungus and are traded with the plant for photosynthetic carbon (Smith & Read, 2008; Bucher, 2007; Redecker et al., 2000). The AM symbiosis is a type of mycorrhiza in which the fungi have a very wide host range, forming a mutualistic interaction with the vast majority of roots in vascular plants, including many agricultural plants (Smith & Read, 2008; Besserer et al., 2006; Brundrett, 2009). AM fungi belong to the monophyletic fungal subphylum of *Glomeromycotina* and comprise ~300 species (Spatafora et al., 2016; Schüßler & Walker, 2010; Schüβler et al., 2001). Arbuscules, tree-shaped or coiled fungal structures within the cells of plant roots, are the functional structures within plant roots enabling nutrient exchange (Smith & Read, 2008; Parniske, 2008).

1.5.1 Biology and symbiosis establishment

The life cycle of AM fungi shown in Figure 1.5, starts with the breaking of spore dormancy followed by germination of spores in the soil Figure 1.7 a-c (Giovannetti et al., 2010; Giovannetti, 2000). Hyphal extension from spores is triggered by various operating environmental conditions like pH, temperature, moisture, minerals, organic nutrients, and microorganisms, though independent of the host presence (Giovannetti et al., 2010). However, subsequent hyphal growth and branching are dependent on the presence of the host (Akiyama et al., 2005; Besserer et al., 2006; Giovannetti, 2000). Culturing these fungi in the absence of a host plant was thought to be impossible (Giovannetti et al., 2010; Mosse, 1962), although recent evidence shows host independent growth of AM fungi on a medium supplied with plant-derived fatty acids that could allow asymbiotic growth (Sugiura et al., 2020).

To initiate this symbiosis, a signalling exchange using proteins, metabolites, and nucleic acids between the plant and fungal partner is required (Figure 1.6 a). The plant releases strigolactone and cutin oligomers and the fungus releases chemical signals referred to as Myc-factors (Besserer et al., 2006; Maillet et al., 2011; Bonfante & Genre, 2015; Zipfel & Oldroyd, 2017). Strigolactones are carotenoid pathway-derived plant hormones and are signalling molecules inducing the pre-symbiotic growth of AM fungi, mainly hyphal branching (Besserer et al., 2006; Parniske, 2008; Zipfel & Oldroyd, 2017; Akiyama et al., 2005; Matusova et al., 2005; Maldonado-Mendoza et al., 2001). Short chitooligosaccharides and lipochitooligosaccharides are Myc factors released from the fungus and

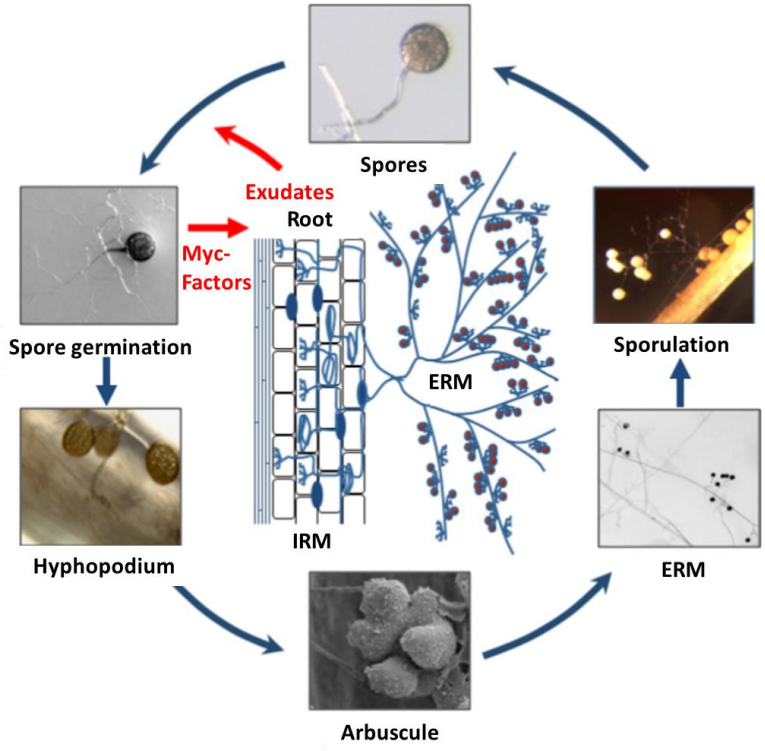


Figure 1. 5: *Life cycle of AM fungi. Spores in the soil will germinate and branch after the release of specific signals from the plant and fungus. Subsequent spore germination leads to hyphal growth including ERM (extraradical mycelium), root colonisation with IRM (intraradical mycelium) and arbuscule formation. The ERM will finally produce new spores during sporulation. Figure adopted from* (Bücking et al., 2012; *2018; Kelly et al., 2017).*

sensed by plant-derived Lysin motif receptor-like kinases as part of the symbiosis recognition system (Maillet et al., 2011; Zipfel & Oldroyd, 2017; Kelly et al. 2017; Buendia et al. 2018). The pre-symbiotic phase ends through hyphopodium formation by the AM fungus and turns into the first physical contact between both partners (Bonfante & Genre, 2015; Schmitz & Harrison, 2014; Murray et al., 2013). Regulated nuclear calcium spiking in the plant host is triggered by fungal exudates and hyphopodium contact, leading to downstream activation of the symbiosis pathway (SYM-pathway) (Matusova et al., 2005; Oldroyd et al., 2009; Kosuta et al., 2008). In advance of penetration, after hyphopodium contact, a subcellular pre-penetration apparatus is formed in the epidermal cells of the host plant (Parniske, 2008; Zipfel & Oldroyd, 2017). This pre-penetration apparatus builds the foundation for harbouring the fungus and is the first step in generating an apoplastic interface (Figure 1.6a) (Zipfel & Oldroyd, 2017; Genre et al., 2005).

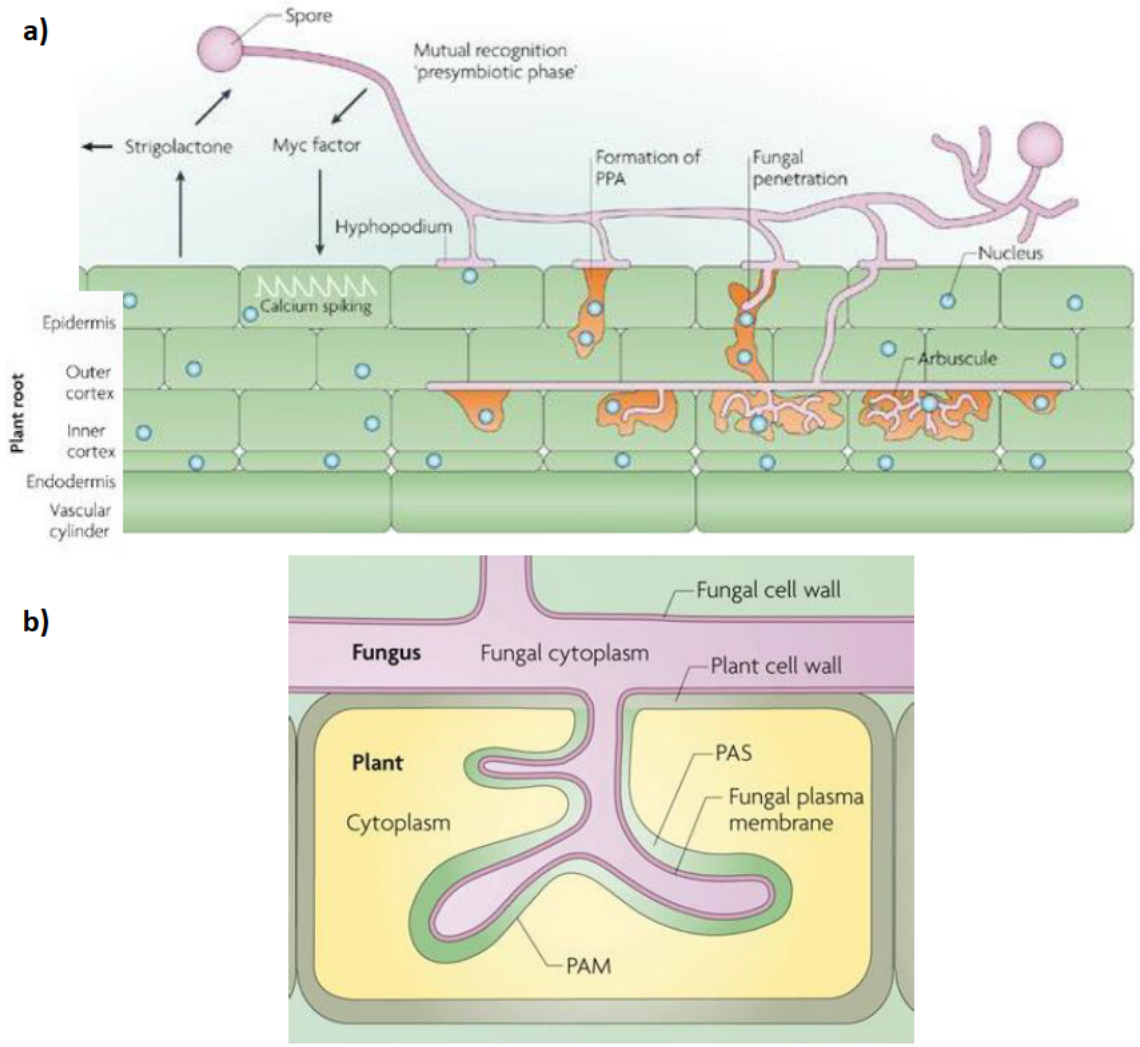


Figure 1. 6: a) *Development of the symbiosis between AM fungi and plant roots. Strigolactone elicitation from the root will lead to spore branching and Myc factors elicited from the plant will trigger the SYM pathway that leads to transcriptional reprogramming and preparation for the symbiosis in the plant. After hyphopodium development, the plant will form the pre-penetration apparatus (PPA) to enable further fungal penetration, finally leading to arbuscules forming in cortical cells.* ***b)*** *Structure of an arbuscule within a plant root cell. The fungus is surrounded by a periarbuscular membrane (PAM), a continuation of the plant plasma membrane responsible for the segregation of the fungus and the plant cytoplasm. The area between PAM and the fungal plasma membrane is called periarbuscular space (PAS). It contains compounds from plant and fungal partners* (Parniske, 2008). *Figure adapted from* (Parniske, 2008).

Finally, the nutrient exchange stage starts when the fungus reaches the cortical cells and the arbuscules are formed (Figure 1.6 b) (Parniske, 2008; Bonfante & Genre, 2010). Arbuscules are tree-like structures with a lifespan of 4-5 days and are formed within the cell surrounded by a periarbuscular membrane derived from the plant´s plasma membrane, which represents the separation between the plant cytoplasm and the fungus (Parniske, 2008; Bonfante & Genre, 2010). It is at this interface where nutrients are transferred by the activity of specialised transporters (Parniske, 2008; Maldonado-Mendoza et al. 2001; Nagy et al. 2005; Javot et al. 2007; Ezawa et al., 2004).

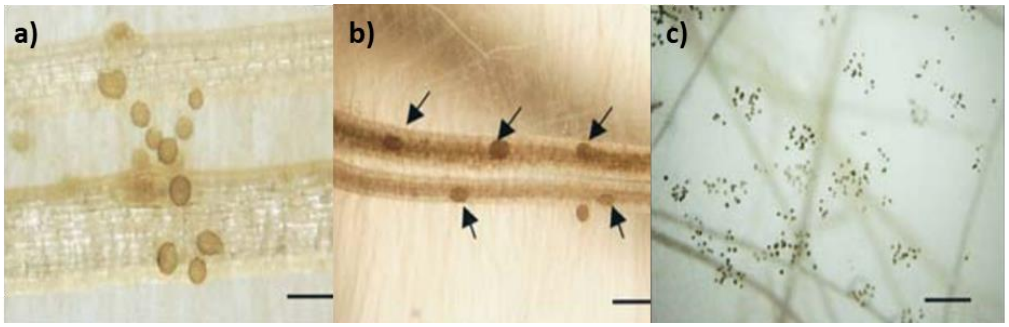


Figure 1. 7*: Microscopic pictures of the root colonisation of the tomato cv. Micro-Tom from* Rhizophagus irregularis*.* ***a-c)*** *In vitro Micro-Tom hairy root cultures with* R. irregularis *colonisation.* ***a)*** *Spores formed after successful colonisation, scale bar = 140 µm.* ***b)*** *intraradical spores, scale bar = 105 µm.* ***c)*** *spore formation after 3 months, scale bar = 0.84 mm. Pictures were adopted from* (David-Schwartz et al., 2003).

1.5.2 Signalling

The signalling pathway that allows the plant to establish this symbiosis is the SYM-pathway (Parniske, 2008; Zipfel & Oldroyd, 2017; Oldroyd et al., 2009; Bonfante & Genre, 2010; Oldroyd, 2013). This molecular reprogramming is triggered by the recognition of Myc-factors via LysM-domain receptor kinases which will lead to the oscillation of calcium (Parniske, 2008; Zipfel & Oldroyd, 2017; Oldroyd et al., 2009; Liao et al., 2018). This spike leads to the activation of a calcium–calmodulin-dependent protein kinase that associates with a protein called CYCLOPS, which is essential for mycorrhizal colonisation Oldroyd et al., 2009; Parniske, 2008; Oldroyd et al., 2009; Oldroyd 2013; Yano et al., 2008; Pimprikar et al. 2016; Prihatna et al. 2018). This formation then binds to a transcription factor, which induces the transcription of proteins that enable arbuscules to branch within the plant cell (Parniske, 2008; Oldroyd et al., 2009; Pimprikar et al., 2016).

In the first stages of infection, colonizing AM fungi are perceived as attackers by the root: salicylic acid (SA) production is enhanced as is an accumulation of defensive compounds in the plant (Jung et al., 2012; Pozo et al., 2015). After the successful recognition, SA production is down-regulated and JA production increases (Jung et al., 2012; Pozo et al., 2015). Additionally, with increasing colonisation levels, decreasing levels of strigolactone production can be observed (Jung et al., 2012; Pozo et al., 2015). Moreover, other phytohormones, like abscisic acid (ABA) and auxin have been reported to positively influence the arbuscules whereas gibberellic acid (GA) can repress the same (Pozo et al., 2015). Furthermore, hormonal crosstalk and fine-tuning involving hormones such as brassinosteroids (BR), cytokinins and ET are involved in this symbiotic process (Pozo et al.,

2015). Interestingly, these same hormonal changes are involved in the defence responses of plants that support the multifunctional immune system of the plant (Dangl & Jones, 2001). This induced resistance is called mycorrhiza-induced resistance (MIR) (Ghorbanpour et al., 2018; Pieterse et al., 2014; Pozo & Azcón-Aguilar, 2007; Conrath et al., 2015; Kadam et al., 2020; Filho, 2022).

1.6 Mycorrhiza-induced resistance (MIR) is a form of ISR

Previous studies demonstrate enhanced plant resistance responses mediated by some AM fungal species upon root colonisation. For example, *Rhizophagus irregularis* improves resistance to bacterial wilt (Chave et al., 2017) and Fusarium wilt (Akköprü & Demir, 2005) in tomato and *Xanthomonas campestris* pv. *alfalfa* in medick plants (Liu et al., 2007). *Glomus mosseae* enhanced resistance to *Alternaria solani* (Song et al., 2015) and *Rhizoctonia solani* in tomato (Kareem & Hassan, 2014). Enhanced defence gene expression was observed after inoculation of *Medicago trunculata* in *Gigaspora gigantea* and *R. irregularis* (Liu et al., 2007). Some AM fungal species are currently available as commercial products to enhance biomass and resistance, however, their different abilities to confer plant protection against disease are understudied (Owen et al., 2015; Savary et al., 2018).

The mechanisms by which AM fungi can enhance plant resistance to pathogens are still poorly understood (Javaid 2009; Parniske 2008; Cameron et al., 2013). Identified as a class of ISR, mycorrhiza-induced resistance (MIR) is a frequently observed phenomenon (Song et al., 2015; Fritz et al., 2006; Pozo et al. 2002; Fiorilli et al. 2011; Wang et al. 2018). Many different models by which MIR operates have been suggested. Some include the priming of SA inducible defence genes as well as the priming of cell wall defence (Gallou et al. 2011; Cordier et al. 1998). Others have suggested the priming of JA-related pathways or SA-independent resistance (Cameron et al., 2013; Khaosaad et al., 2007). An overview of the large variety of proposed AM fungi-mediated resistance mechanisms is given in Figure 1.8.

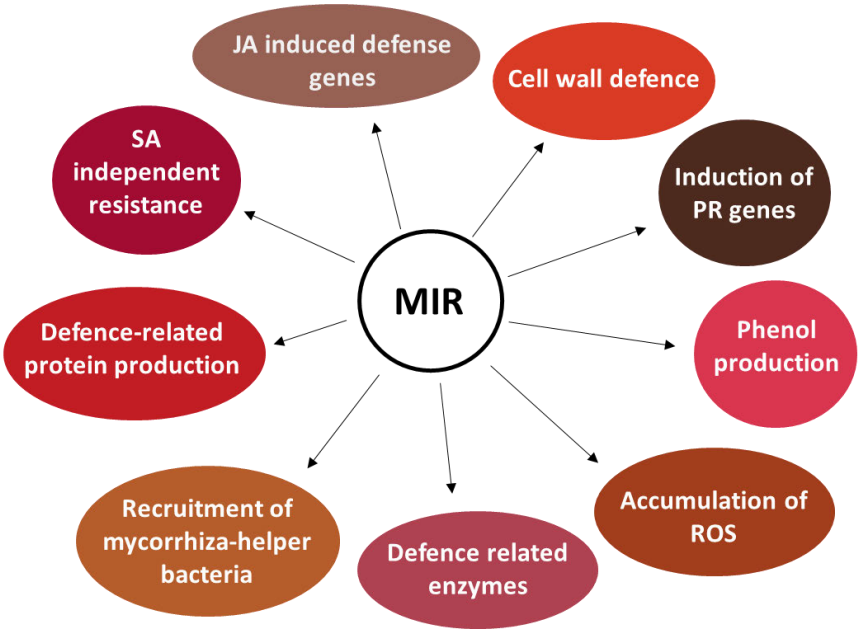


Figure 1. 8: *List of different suggested mechanisms involved in mycorrhiza-induced resistance (MIR).*

In early work, upregulation of enzymes connected to ISR (chitinases and β-glucanases) was observed in local and systemic root tissue of AM colonised plants upon pathogen attack (Jung et al., 2012; Pozo et al., 2002; Pozo et al., 1999). Recently the mutant of a gene involved in JA biosynthesis, called PvLOX2, was shown to lead to the loss of MIR without affecting the symbiosis itself, illustrating the role of the JA pathway in AM fungi-mediated resistance (Mora-Romero et al., 2015). Moreover, studies on tomatoes and early blight disease showed the increased resistance of plants inoculated with AM fungi and the link to the JA pathway (Song et al., 2015). The same study also showed that mycorrhiza inoculation alone did not influence the transcripts of most genes tested which would support the hypothesis of ISR and priming being involved (Song et al., 2015). In addition to the alteration of SA-, JA- and ET-dependent genes involvement of ABA-dependent genes has been reported upon AM fungal colonisation (Jung et al., 2012; Pozo et al. 2009; Gallou et al. 2011). Studies performed on tomatoes colonised with an AM fungus and infected with *Phytophthora nicoteanae* exhibited increased localized defence responses of mycorrhized plants in form of cell wall thickening also connected to systemic molecular responses (Cordier et al. 1998). The production of ROS was involved in mycorrhiza colonisation and proposed as a defence mechanism associated with MIR (Marquez et al., 2019; Balmer et al., 2015; Cordier et al. 1998. Effector proteins produced by AM fungi may also play a role in MIR (Voß et al., 2018; Kloppholz et al., 2011; Tsuzuki et al., 2016). For example, the effector protein SP7 secreted from the AM fungus *Rhizophagus irregularis* inhibits an ET-response transcription factor in the nucleus (Kloppholz et al., 2011) while another effector called RiCRN1 was found in the same AM fungus species to be relevant for arbuscle development and symbiosis formation (Voß et al., 2018).

The discovery of those effector proteins could also be connected to downstream triggered responses from the plant and therefore involvement in MIR. Therefore, AM colonisation appears to be connected to many different pathways involved in ISR that could be exploited for pathogen protection in crops.

1.7 MODEL ORGANISMS

The original plan for this thesis was to develop a controlled in-vitro tripartite study system including three suitable model organisms to investigate MIR. The initially selected model organisms were a tomato (*Solanum lycopersicum* cv. Micro-Tom), an AM fungus *(Rhizophagus irregularis*) and a pathogen (*Phytophthora nicotianae*). I was six months into the process of developing this system when the first COVID-19 lockdown occurred in Sydney and it was not possible to maintain the system due to lab access being severely restricted. Risks of further lockdowns (which did happen) and lost time required an alternative, more flexible plan that included analysis of existing datasets (Experimental chapter "*Effects of Arbuscular Mycorrhizal fungi on plant defence-related gene expression*") and opportunistic involvement with another experiment (Experimental chapter "*Contrasting metabolic responses of two chickpea varieties to root infection by Phytophthora are mediated by arbuscular mycorrhizal fungi*"). This resulted in me using a variety of model organisms and analytical approaches to address my research questions regarding MIR.

My PhD studies were to investigate a breadth of plant-pathogen-AM tripartite systems to further our understanding of the mechanisms and strategies that could be involved in MIR. The crop model organisms are stiff brome (*Brachypodium distachyon*), tomato (*Solanum lycopersicum* cv. Micro-Tom) and chickpea (*Cicer arietinum*). The AM fungi used in this study, selected from different families, included *Rhizophagus irregularis, Glomus mosseae, Gigaspora margarita* and *Scutellospora calospora.* Furthermore, the oomycete pathogen *Phytophthora medicaginis* and the fungal root pathogen *Rhizoctonia solani* were used. In addition, one study used a community of AM fungi and root pathogens derived from local field soil. The following sub-sections provide an overview of the selected model organism and describe why they are suitable for my research.

1.7.1 Crop model plants

1.7.1.1 Brachypodium distachyon

The worldwide distribution of *Brachypodium* species, combined with its small genome, short life cycle and small appearance compared to other grasses, and its susceptibility to AM fungi and a variety of pathogens makes this annual plant an attractive model organism to study MIR (The

International Brachypodium Initiative, 2010; Scholthof et al. 2018; Hong et al. 2012; Riley et al. 2019; Donn et al. 2017) (Figure 1.9). Dicot model species such as *Arabidopsis thaliana* are well developed and frequently used, however, there are prominent differences to monocots, and it is important to find representatives from this clade when studying MIR (Brkljacic et al., 2011; Draper et al., 2001). The close relation and trait similarities to many important cereals and forage grasses make *B. distachyon* a useful study system that is representative of monocots (Draper et al., 2001; Brkljacic et al., 2011; Kellogg & Danforth, 2015).

Brachypodium distachyon has also been used as a model grass for studies of plant-microbe interactions, including with viruses, bacteria and fungi (Scholthof et al., 2018). Particularly, and of high importance for this thesis, *B. distachyon* has the capability to form a mycorrhizal symbiosis with a variety of AM fungal species. As a model species for cereals, *B. distachyon* is also susceptible to many economically important pathogens and has also previously been used as a model organism for cereal pathogens (Figure 1.9) (Fitzgerald et al., 2015; Sandoya & Buanafina, 2014) Therefore, this grass is also an appropriate model organism to study MIR-related gene transcription alterations in mycorrhized plants.

Figure 1. 9: *Infection of Brachypodium and caused symptoms with the cereal pathogens* ***a)*** Puccinia graminis *f. sp.* triticiin *(leave infection at 28 dpi),* ***b)*** Magnoporthe oryzae *(leaves infected by spray with duplicate leaves at 3, 5 and 7 dpi, left to right)* ***c)*** Rhizoctonia solani *AG 8 (infected plants grown in infested soil after18 days after planting, left: root lengths of infected plants shown on the left, root lengths of uninfected plants shown on the right). Figure adapted from* (Fitzgerald et al., 2015).

1.7.1.2 Cicer arietinum

Chickpea (*Cicer arietinum*) is a highly nutritious and agricultural important member of pulse crops, grown all over the world (Merga & Haji, 2019; Hulse, 1994). A variety of pathogens can infect chickpeas and therefore cause substantial losses (Salam et al., 2011). Disease outbreaks in susceptible varieties lead to pronounced symptoms (Figure 1.10) and can lead to extensive losses of up to 70% depending on circumstances (Allen & Lenné, 1998; Dron et al., 2022). Breeding for resistant chickpea varieties currently represents the most important control mechanism (Salam et

al., 2011; Moore et al., 2011). However, the potential for enhanced resistance mediated by AM fungi in chickpeas has been reported (Singh et al., 2010). In my PhD work, two specific chickpea varieties are used, called *C. arietinum* var. Sonali and *C. arietinum* var. Kyabra, both susceptible to Phytophthora root rot (Bithell et al., 2021). Kyabra was previously reported to have higher AM fungal colonisation levels than Sonali (Plett et al., 2016). Therefore, the combination of these two varieties, AM fungal colonisation, and pathogen exposure depicts an excellent model system to investigate MIR-related differences in AM fungal colonisation.

Figure 1. 10*: Symptoms of Phytophthora root rot (right) in the moderately susceptible chickpea variety "Yorker" affected by chickpea disease) compared with a disease-free plant (left). Photo: Sean Bithell, NSW DPI* (Grains Research and Development Corporation 2022, 2019).

1.7.1.3 Solanum lycopersicum cv. Micro-Tom

Tomato (*Solanum lycopersicum)* is the second most important crop vegetable in the world. A broad variety of diseases can infect this crop plant leading to substantial losses (Kimura & Sinha, 2008; Food and Agriculture Organization of the United Nations, 2012; Arie et al., 2007; Manganiello et al., 2018). The importance of tomato as a crop plant and the fact that it is an already well-established model organism in scientific research makes it a suitable plant to investigate plant microbial interactions with pathogens (Kimura & Sinha, 2008; Arie et al., 2007; Piquerez et al., 2014). Additionally, tomato is also a popular model plant in mycorrhizal research, mainly focused on the nutrient exchange or resistance to abiotic stress (Nagy et al., 2005; Tisserant et al., 2012; Barker et al., 1998). A multitude of different approaches including transcriptomic, proteomic and metabolomic experiments have already been performed on tomatoes to study the impact of mycorrhizal colonisation (Nagy et al., 2005; Rivero et al., 2015; Tahiri-Alaoui & Antoniw, 1996; Sant'Ana & Lefsrud, 2018). Although many different varieties of

tomato are available, one cultivar originally bred for ornamental use has emerged as a very popular model plant for several reasons (Shikata & Ezura, 2016). *Solanum lycopersicum* cv. Micro-Tom has become a prominent model organism for plant genetics and a significant number of "omics" data are publicly available (Kudo et al., 2017; Fernandez-Pozo et al., 2015; Kudo et al., 2017; Aoki et al., 2010; Yano et al., 2006; Saito et al., 2011). Different AM fungal species have been used in the past to demonstrate sufficient colonisation of Micro-Tom, however with varying intensity (Song et al., 2015; Rillig et al., 2008; David-Schwartz et al., 2001). Most tested pathogens are pathogenic on Micro-Tom (Figure 1.11c,d) (Marti et al., 2006; Rillig et al., 2008; David-Schwartz et al., 2001; Takahashi et al., 2005; Manganiello et al., 2018). Even though very few studies investigated the tripartite interaction of AM fungi and pathogens in tomato so far, the existing literature demonstrates the suitability of Micro-Tom as a model plant for this system (Song et al., 2015).

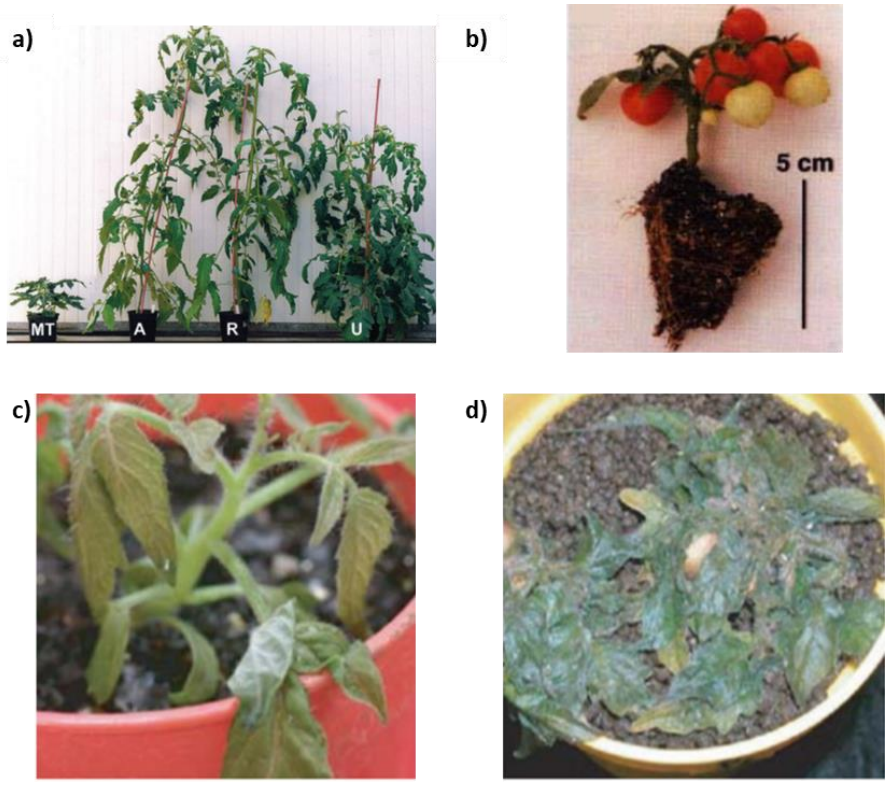


Figure 1. 11: ***a)*** *2 months old Micro-Tom (MT) plants compared with other tomato cultivars, A=Ailsa Craig, R= Rutgers, U= UC-82.* ***b)*** *Size of mature Micro-Tom plant.* ***c)*** *Symptoms on* Solanum lycopersicum *cv. Micro-Tom 7 after infection with* P. infestans. ***d)*** *Progressive symptoms on* P. infestans *infected Micro-Tom showing necrosis and dying leaves. Pictures adapted from* (Meissner et al., 1997; Marti et al., 2006; Takahashi et al., 2005).

1.7.2 AM fungal model species

The subphylum of *Glomeromycotina* includes around 300 different AM fungal species and the functional specificity of those increasingly moves into the spotlight of research (Schüßler & Walker, 2010; Schüβler et al., 2001; Smith & Read, 2008). Multiple studies based on available research showed that there are significant inter-species differences in the ability of AM fungi to induce MIR (Malik et al., 2016; Cordier et al., 1998; Lioussanne et al., 2009; Martínez-Medina et al., 2009; Malik et al., 2016). For example, a major impact on the growth of foliar pathogens by fungal identity as well as the mycorrhizal composition was shown previously (Malik et al., 2016; Sikes et al., 2009). As AM fungi evolved into a multitude of different species, species from the family of Glomeraceae are more likely to be involved in protection against pathogens while the family of Gigasporaceae are more likely to benefit host growth (Sikes et al., 2009; Sikes, 2010; Sikes et al. 2010; Maherali & Klironomos, 2007). For example, differences in levels of bioprotection in *P. nicotianae* infected tomato, *G. mosseae* exhibited MIR whereas no beneficial effect was observed in plants colonised with *R. irregularis* (Pozo et al., 2002). Studies using different isolates of *R. irregularis* led to contrasting results of pathogen inhibition, implying that even intra-specific differences could be

involved in MIR (Pozo et al., 2002; Lioussanne et al., 2009). Furthermore, varying defence-related enzyme accumulation and gene expression was observed in tomato plants depending on the AM fungus species (Gao et al., 2004; Pozo et al., 1999). Therefore, to allow the efficient use of AM fungi in bioprotection is it important to understand species-dependent changes in molecular processes.

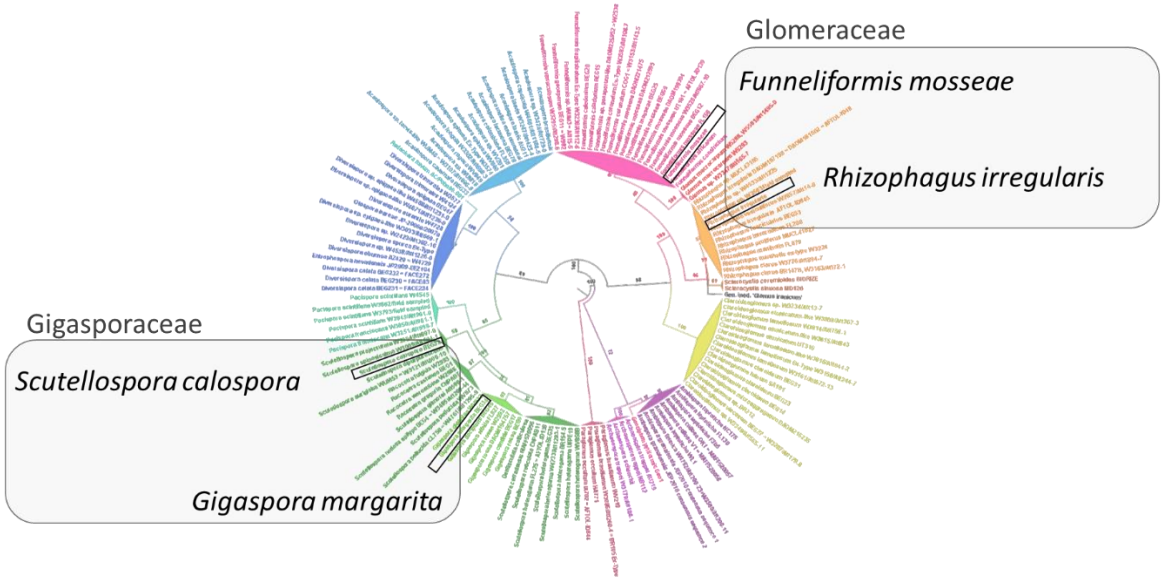


Figure 1. 12: *Phylogenetic tree of the subphylum of Glomeromycotina (New classification of the Diversisporales, 2004). The positioning of the four selected species within the families of Glomeraceae and Gigasporaceae is shown with squares and labelled. Figure adapted from: http://www.amf-phylogeny.com/ with information from* (Walker et al., 2004; *Walker & Schüßler, 2004).*

As different AM fungal species can have different potentials to mediate MIR, I included a variety of species from the subphylum of Glomeromycotina (Figure 1.12) in my thesis to investigate MIR-related species-specificity. These include *Rhizophagus irregularis* [formally known as *Glomus intraradices*] and *Glomus mosseae* [now known as *Funniliformis mosseae*] from the family of Glomeraceae, as well as *Gigaspora margarita* and *Scutellospora calospora* from the family of Gigasporaceae (Krüger et al., 2012; Redecker et al., 2013; Stockinger et al., 2009; Schüβler et al., 2001; Schüßler & Walker, 2010). Moreover, two isolates of *R. irregularis,* specifically DAOM 197198 (also known as DAOM 181602) and WFVAM 23, were included to test for isolate-specific differences (Ropars et al., 2016; Tisserant et al., 2013). The integration of "omic" approaches to investigate the alteration between species can help give a clearer picture of possible functional specificities in bio protection to soil pathogens.

1.7.3 Model pathogen organisms

1.7.3.1 Oomycetes

Besides AM fungi the soil is also the habitat of soil-borne pathogens with the potential to cause disease including bacteria, nematodes and fungi (Agrios, 2005; Raaijmakers et al., 2009). Plant diseases caused by fungi include true fungi like Ascomycota and Basidiomycota but also the fungus-like class of oomycetes (Agrios, 2005). Oomycetes are a distinct lineage of pathogens comprising some of the most devastating crop plant diseases (Strange & Scott, 2005; Agrios, 2005; Thines, 2014). In contrast to true fungi, the mycelium of oomycetes contains cellulose and glucans instead of chitin and they are more related to brown algae than to true fungi (Agrios, 2005; Baldauf et al., 2000; Latijnhouwers et al., 2003). Examples of some of the most devastating genera within the Oomycetes are *Phytophthora* and *Pythium* (Thines, 2014; Derevnina et al. 2016). Both groups can lead to significant losses in crop plants due to their broad host ranges and lack of control.

1.7.3.2 Phytophthora medicaginis

With their fitting name translating to 'plant destroyer' almost all *Phytophthora* species can cause devastating root, lower stem and fruit rot as well as blights for many crop plants (Agrios, 2005). *Phytophthora* species are hemibiotrophic organisms combining an initial biotrophic phase followed by a switch to a necrotrophic phase where the pathogen kills the host (Panabieres et al., 2016; Latijnhouwers et al., 2003; le Berre et al., 2007; Park et al., 2015). Many of the 120 known species are of economical and scientific interest and became popular plant pathogen model organisms with the already known genome (Erwin & Ribeiro, 1998; Kroon et al., 2012; Martin et al., 2014; Kamoun, et al., 2015; Yuan et al., 2017). Some of them, like *P. infestans,* show a very narrow host range, while others have a vast variety of hosts (Kamoun et al., 2015). The specific Phytophthora species used in this thesis is called *Phytophthora medicaginis* (Salam et al., 2011; Moore et al., 2011; Schwinghamer et al., 2011). This pathogen infects the roots and stems of chickpea which leads to defoliation, wilting, and damping-off and subsequent significant losses in chickpea and lucerne (Figure 1.13) (Li et al., 2015; Vock et al., 1980). This pathogen has a narrow host range and can to substantial yield losses of up to 95% in conducive conditions (Bithell et al., 2019; Moore et al., 2015). Limited control mechanisms exist and no effective pesticides are available (Moore et al., 2011) with breeding for resistant plant variants being the best means of management to date (Knights et al., 2008; Bithell et al., 2022; Li et al., 2015; Moore et al., 2011; Vandemark & Barker, 2003). Therefore, in this thesis, two pathogen-susceptible chickpea varieties with varying AM

fungal colonisation abilities were used (Plett et al., 2016), allowing investigation of MIR upon varying AM fungal colonisation levels in susceptible plants to *P. medicaginis*.

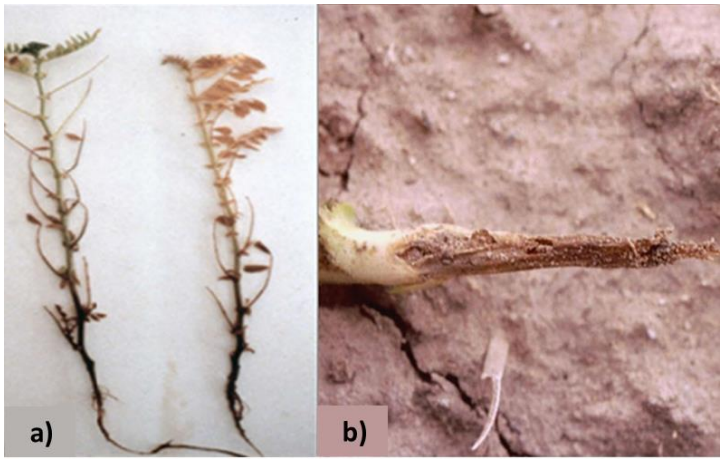


Figure 1. 13: a) *Chickpea plants infected with Phytophthora root rot displaying symptoms like discoloured roots and lack of lateral roots.* ***B)*** *root rot in dry stage. Figure adapted from* (H. Li et al., 2015).

1.7.3.3 Rhizoctonia solani

The fungus *Rhizoctonia solani* is a devastating necrotrophic root pathogen with an extremely broad host range including tomato (Adams 1988; Sneh et al. 1996; Sumalatha et al. 2018). *R. solani* is a seed and soil-borne pathogen, which leads to root rot and dumping off, with a worldwide distribution and an extremely wide host range, including many important crops (Sneh et al. 1996; Manganiello et al. 2018; Senapati et al., 2022). Moreover, long-lasting sclerotia formed that can persist for many years in the soil (Figure 1.14) (Ajayi-Oyetunde & Bradley, 2018; Senapati et al. 2022). Coupled with limited management options, *R. solani* presents a significant economic problem in agriculture for many crop plants (Ajayi-Oyetunde & Bradley, 2018).

One of the many host plants infected by *R. solani* is tomato (Manganiello et al., 2018) and the combination of tomato and *R. solani* is a frequently used study system in mycorrhizal research. MIR effects have been shown previously in different plants upon *R. solani* infection including beans (Hafez et al., 2013) and potato (Yao, 2002). Furthermore, *Glomus mosseae* enhanced the resistance of tomato plants to *Rhizoctonia solani* in glasshouse and field conditions with simultaneously enhanced growth (Kareem & Hassan, 2014). The choice of the pathogenic organism is important, as Glasshouse experiments also need to consider the spread in surroundings pots and long-term management for follow-up experiments. For the above-mentioned reasons, this pathogen presents a relevant choice to investigate MIR in tomato.

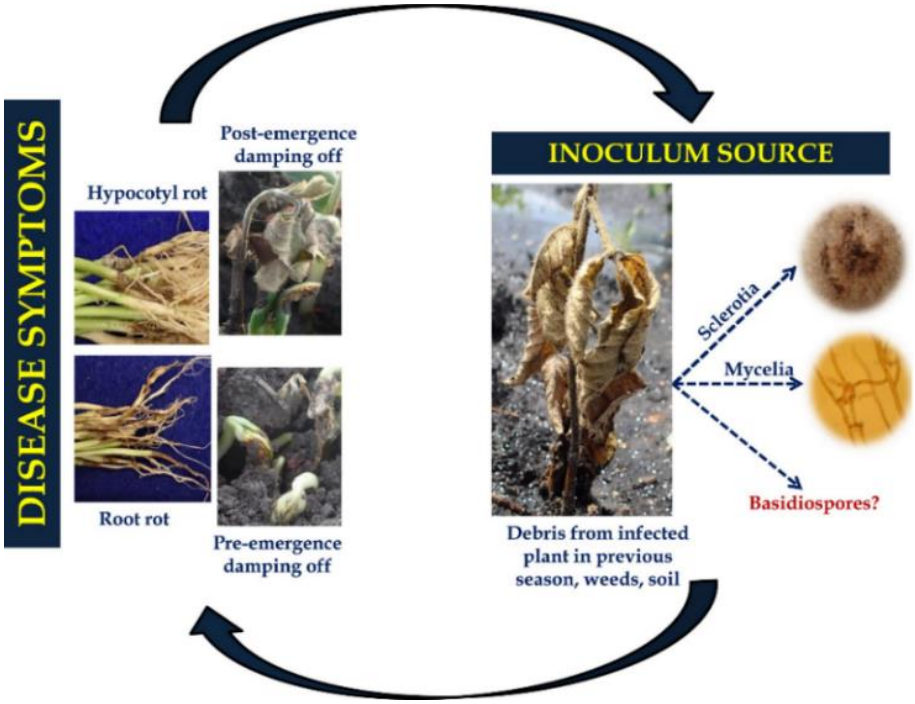


Figure 1. 14: *Disease cycle of* R. solani *on soybean showing a variety of disease symptoms and infection sources. Figure adapted from (Ajayi-Oyetunde & Bradley, 2018).*

1.8 OMICS METHODS

This thesis uses several "omics" techniques and high throughput approaches to understand the mechanisms by which MIR operates. Omics experiments represent a useful tool to enable high-throughput observation of biochemical landscapes in plants and have a great potential to investigate plant-microbe interactions (Salvioli & Bonfante, 2013; Schweiger et al., 2014; Piasecka et al., 2019). Specifically, metabolomics has the potential to help unravel immune responses including the priming mechanisms (Williams et al., 2021). Although alterations in plant metabolites in response to mycorrhization as well as the involvement of secondary metabolite were previously shown (Kadam et al., 2020), there is still very little scientific understanding of how mycorrhized plants ultimately benefit from MIR and which physiological processes are leading to enhanced resistance.

Transcriptomics is also frequently used to investigate a variety of research questions including transcriptional regulation of plant defence responses and arbuscular mycorrhiza colonisation (Birkenbihl et al., 2017; Buscaill & Rivas, 2014; Pimprikar & Gutjahr, 2018; Siciliano et al., 2007; Beaudet et al., 2018; Tisserant et al., 2012; Islam et al., 2017; Mcneil et al. 2018; Meng et al. 2021). Changes in the plant transcriptome in AM fungal colonised plants have been reported (Vangelisti et al., 2018) and research already started to investigate the transcriptomic changes related to AM fungal-mediated enhanced resistance (Marquez et al., 2019; Tian et al., 2019; Aseel et al., 2019; Shaul et al., 1999). A recent omics study proves the power of this approach by providing insight into defence-related up-/downregulated genes and proteins upon AM fungal colonisation following the response of wheat to the leaf pathogen *Xanthomonas translucens* (Fiorilli et al., 2018). More information is needed to understand the complex transcriptional changes upon AM fungal-mediated enhanced resistance across a broader range of model systems.

Phenomics is another technique, broadly used to unravel phenotypic alterations in plant genotypes (Costa et al., 2019; Pieruschka & Schurr, 2019). Image-based plant phenotyping depicts a non-invasive high throughput method to collect data on plants over time and therefore assess plant fitness, which is also in high demand for crop plant research (Pieruschka & Schurr, 2019; Zhao et al., 2019). Moreover, this quantitative non-destructive approach allows measuring differences in plant responses connected to microbial interactions and is used in plant disease-related disciplines (Mutka & Bart, 2015). This thesis takes advantage of transcriptomic data as well as metabolomic data to investigate MIR-related molecular changes. Moreover, the combination of phenomics with metabolomics provides a comprehensive in-depth examination of MIR.

1.9 MIR STATE OF THE ART

Recently, transcriptomic, proteomic and metabolomic approaches have become increasingly important to start and detangle the complicated network leading to MIR. Previous research already identified a variety of potential involved mechanisms (Figure 1.8). Studies started to investigate AM fungal-mediated defence response and looked at the transcriptional changes of fully mycorrhized and non-mycorrhized soybean plantlets including a fungal pathogen attack and found more gene alterations in the colonized plants with pathogen infection (Marquez et al., 2019). Transcriptomic and metabolomic alterations in roots but also aboveground tissues like shoots and leaves of mycorrhized plants showed major transcriptional reprogramming of defence-related compounds and therewith enhanced protection (Guadalupe Cervantes-Gámez et al. 2015; Rivero et al. 2015; Liu et al. 2007). In one study changed expression patterns related to the innate immune system responses such as secondary metabolism, stress and signalling were observed in mycorrhized plants including PR proteins, WRKY transcription factors, and receptor kinases (Marquez et al., 2019). But also alteration of hormone signalling-related genes that potentially indicate involvement of ISR were observed in one study; however, only a few genes showed primed expression (Marquez et al., 2019). Combined transcriptomic and proteomic profiling performed on wheat upon AM fungal colonization and infection with *Xanthomomas translucens* revealed first insights into the involved local and systemic pathways of MIR including primary metabolism as well as phytohormone regulation (Fiorilli et al., 2018).

As shown in previous sections, multiple proposed mechanisms and involved pathways from a variety of plant immune responses were suggested in the last decades. However, the overall network leading to the enhanced resistance mediated by AM fungi is currently still unknown (Jung et al. 2012; Pozo and Azcón-Aguilar 2007). Moreover, a recent study could provide the first proof of the detached function of AM fungal-related resistance effects and mediated nutritional benefits (Pozo de la Hoz et al., 2021). The common denominator in MIR research is the improved plant performance upon pathogen attack and local and systemic defence responses with a frequent mention of the phenomenon of priming (Pozo et al. 2002; Trotta et al. 1996; Fritz et al. 2006). So far, MIR research has been primarily focused on unravelling specific details. It is crucial to focus on investigations untangling the extensive AM fungal conferred resistance network and patterns leading to MIR mediation. Thus, the focus of this thesis was to investigate these comprehensive processes, which enable further enhancement and connection of existing knowledge pieces of the MIR puzzle.

1.10 MIR Knowledge gaps and Experimental chapter overview

As the previous sections of this thesis have argued, it is now well established that MIR involves a variety of processes interacting simultaneously. Nevertheless, there are still many knowledge gaps around how AM fungi confer enhanced resistance. Therefore, this thesis addressed some of the following areas:

1) A systematic understanding of how alterations influence the plant's ability to be more resistant to certain pathogens is still lacking and further investigation of these changes (on a transcriptional and biochemical level) is needed.
2) The link between the level of AM fungal colonisation and the level of MIR-connected molecular modifications is unknown.
3) AM fungal species-specific changes on MIR effects, if there are linked molecular changes connected variations in the strength of MIR, is unclear.

To maximize the beneficial effect of mycorrhized plants it is crucial to understand the molecular mechanisms leading to these responses. Therefore, this thesis aims to increase the knowledge regarding MIR, increase the understanding of how mycorrhized plants ultimately benefit from MIR and investigate which molecular processes support this enhanced resistance. The results are presented in the following four experimental chapters:

1. In ***"Effects of Arbuscular Mycorrhizal fungi on plant defence-related gene expression"*****,*** I focused on the influence of AM fungi of the Glomeraceae family on gene expression and the relationship to oomycete abundance in *Brachypodium distachyon*. A transcriptomic approach was used to detect patterns in defence-related molecular pathways triggered by natural variation in an AM fungal community. The findings of this chapter revealed pre-MIR-related patterns in the basic gene expression in a variety of non-defence-related biological functional processes which are determined by beneficial and pathogenic microbes.

2. "***Contrasting metabolic responses of two chickpea varieties to root infection by Phytophthora are mediated by arbuscular mycorrhizal fungi***" investigated the altered patterns of metabolomic root responses conferred by arbuscular mycorrhiza fungi in pathogen-challenged chickpea varieties. I compared the metabolomic root profiles of two pathogen-susceptible Chickpea (*Cicer arietinum*) varieties 'Sonali' and 'Kyabra' which show different AM fungal colonisation levels. By analysing the metabolomic changes in plant roots I observed variety-specific metabolomic patterns, advancing our understanding of how the degree of AM fungal colonisation mediates the strength of MIR on plant defence responses.

3. "***Arbuscular mycorrhizal fungal mixtures affect the response of tomato to Rhizoctonia solani non-additively "*** explored the ability of different AM fungal species and communities to mediate enhanced growth and resistance to a root pathogen in tomato and whether there existed trade-offs in these two functional outcomes of AM symbioses. I wanted to answer if AM fungal single-species inoculations vary in their ability to mediate MIR responses, if these effects are additive in community inoculations and if there are potentially associated trade-offs. This study used a high throughput phenotyping approach to compare long-term responses of *Solanum lycopersicum* cv Microtom inoculated with five different single AM fungal species as well as AM fungal communities, to the root pathogen *Rhizoctonia solani*. These phenotypic observations revealed AM fungal species-specific differences in growth enhancement as well as MIR functional specificity and allowed the classification into putative MIR-effective and non-effective species. Moreover, I could show the non-additive effects of AM fungal community compared to the single species treatments. The results from this chapter present novel knowledge regarding the functional specificity of AM fungi and communities.
4. The last experimental chapter "***AM fungal species-specific variation of metabolomic responses in tomato to a root pathogen reveals enhanced downregulation of metabolic compounds*****"** studied the metabolomic landscape of plants colonised by AM fungal species that had previously been defined as having variable abilities to induce MIR. This chapter aimed to examine whether the phenotypically observed species-specific MIR variations are also reflected in the metabolite profile of plants. I analysed the root metabolome of *Solanum lycopersicum* cv Microtom infected with the root pathogen *Rhizoctonia solani* and inoculated it with either two effective or two non-effective MIR AM fungal species. This analysis showed patterns specific to AM fungal species able to induce MIR and highlights a potential need to repress different metabolic pathways to protect plants against pathogen addition. These data show the importance of considering the biochemical changes in plants and the need to further investigate the biochemical networks leading to the strength of MIR.

CHAPTER TWO

Effects of Arbuscular Mycorrhizal fungi on plant defence-related gene expression

The original study design and execution were performed by Suzanne Donn, who also processed the RNAseq data. Jeff Powell processed the Miseq data. I performed all subsequent analyses, including statistical analyses, and led the interpretation of the results and writing of the chapter.

2.1 INTRODUCTION

Plants are exposed to a plethora of microbes in their surroundings, including those that are beneficial or detrimental to plant health. Therefore, plants have evolved the ability to respond to a variety of signals from different microbes to initiate defence against the latter class of microbes while facilitating symbiosis with the former. Pathogenic organisms include nematodes, viruses, bacteria, fungi, and oomycetes (Savary et al., 2019). The latter class, of the kingdom Chromista, comprises a vast group of devastating species pervasive in agricultural soils, with broad host ranges and limited control mechanisms resulting in up to 30% of yield losses globally (Kamoun et al., 2016; Bebber & Gurr, 2015; Agrios, 2005; Erwin & Ribeiro, 1998; Kamoun et al., 2015; Martin & Loper, 2010; Lamour, 2013; Nicot, 2011). Beneficial symbionts are found only within the bacterial domain of prokaryotes and within the fungal kingdom (Naik et al., 2019). Arbuscular mycorrhiza (AM) fungi from the subphylum Glomeromycotina are widespread beneficial soil microbes forming a mutualistic symbiosis with 80% of land plants, comprising an estimate of almost 300 species, and can be found in soil communities all over the world including natural and agricultural settings (Schüβler et al. 2001; Bruns et al. 2018; Spatafora et al. 2016; Schüßler & Walker 2010). This symbiosis is well known for improving nutrient uptake in plants resulting in enhanced biomass and yield (Smith & Read, 2008; Schüβler et al., 2001). Moreover, AM fungi can alter plant defence responses, a phenomenon called mycorrhiza-induced resistance (MIR) (Smith & Read, 2008; Pozo et al., 2009; Harrier & Watson, 2004; Pozo & Azcón-Aguilar, 2007). Although this phenomenon is not well understood yet, the mechanism of priming is frequently proposed to contribute to MIR; during priming, plants show accelerated activation of defence responses upon exposure to a stress trigger after mycorrhizal fungi have established in the root system (Pozo & Azcón-Aguilar, 2007; Jung et al., 2012; Martinez-Medina et al., 2016). To better understand the fundamental interactions between plants and beneficial/pathogenic microbes with the goal of improving crop production, we must improve our understanding of the molecular plant responses to different microbes.

MIR responses initiated during AM symbiosis formation are complex and involve a range of molecular and biochemical modifications including alterations to hormone, protein and defence-related enzyme levels (Cameron et al., 2013). Phytohormones including auxin, ethylene, salicylic acid, jasmonic acid and abscisic acid exhibit modified accumulation after AM fungal colonisation (Fiorilli et al., 2018; Tian et al., 2021; Stumpe et al., 2005; Bari & Jones, 2009) as have defence enzymes in tomato leaves and roots colonised with AM fungi and challenged with *Verticillium dahlia*, *Phytophthora parasitica var. nicotianae* and *Alternaria solani* (Song et al., 2015; Tian et al., 2021; Pozo et al., 2002; Rahou et al., 2021; Lawrence et al., 1996). Assessment of transcriptional changes can provide insight into these molecular responses, an aspect extensively studied from the aspect of single plant-AM or plant-pathogen symbioses (Ho-Plágaro & García-Garrido, 2022; Siciliano et al., 2007; Buscaill & Rivas, 2014; Li et al., 2016). For example, enhanced gene expression of signalling pathways controlling downstream defence responses was shown in mycorrhized roots (Tian et al., 2021; Zhang et al., 2001), in shoots (Liu et al. 2007; Ebastien Bruisson et al. 2016; Song et al., 2015), as well as alteration of pathogenesis-related (PR) proteins in leaves (Shaul et al., 1999). As previous research has been performed on either pathogen or AM fungal-induced transcriptomic changes, comparatively little is known about how the tripartite interaction influences the plant transcriptional responses (Siciliano et al., 2007; Islam et al., 2017; Mcneil et al., 2018; Meng et al., 2021; Shaul et al., 1999). Furthermore, the majority of studies to date use high levels of pathogen abundance, as opposed to natural communities of AM fungi and pathogens, and they have focused on molecular alterations following the induction of MIR, as opposed to regulation of plant signalling following AM colonisation, but prior to the occurrence of MIR (termed as 'pre-MIR' responses in this paper).

To address these understudied areas, this current study aimed to provide insight into the transcriptional plant patterns in response to normal variations of natural beneficial and pathogenic soil microbe communities. By assessing the relationship between pre-MIR expressed genes and shifts in the relative abundances of different AM fungal taxa and oomycetes, we tested the hypothesis that AM fungal colonisation regulates the early expression of genes that are involved in enhanced resistance/pathways in the plant and that this influences interactions between Oomycetes and the plant. Two accessions of *Brachypodium distachyon*, chosen based on the extent to which they were colonised by AM fungi in a previous experiment (Donn et al., 2017), were used in this study to investigate the relation of root gene expression with the abundance of AM fungi, Glomeraceae and Oomycetes. Glomeraceae, a widely distributed family within the Glomeromycotina, were focused on as they have been previously demonstrated to confer enhanced pathogen resistance to plants (Sikes et al., 2009; Sikes et al. 2010; Maherali & Klironomos, 2007). I show pre-MIR-related patterns in a variety of different biological processes and characterise the

expression patterns of MIR-related gene groups to identify potential candidate genes influencing the response to beneficial and pathogenic microbes in this model grass.

2.2 Materials and Methods

2.2.1 Experimental design

The design comprised a factorial experiment with two accessions of *B. distachyon* ("KAH1" and" TR2B") inoculated with AM fungi (+AM fungi) or control inoculum (-AM fungi) (Figure 2.1). Each treatment combination was replicated ten times. Colonisation data were derived from all ten replicates, fungal and oomycete community sequencing was performed on five replicates and transcriptomic data were collected from three of these five replicates. Selection of the three replicates was based on them being representative of plant growth (exclusion of replicates without germination and resown seeds) respectively, in the treatments from which they were drawn.

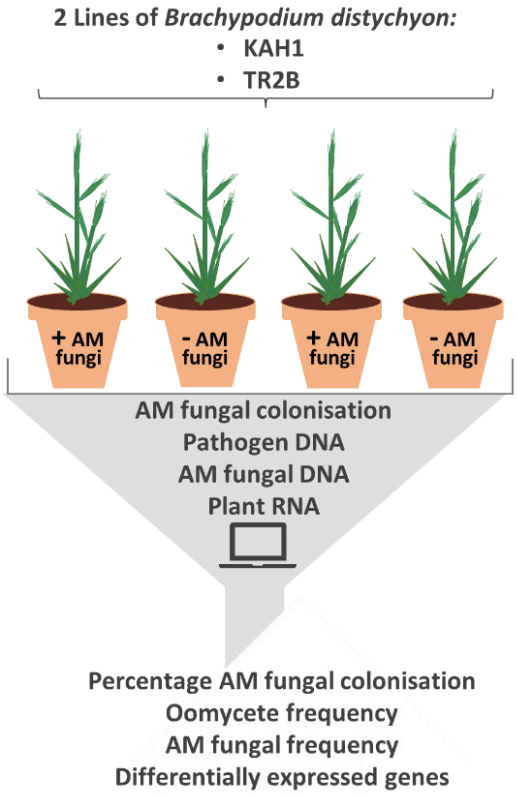


Figure 2. 1: *Experimental design including both plant accessions (KAH/TR2B), AM fungal treatments and types of collected samples and data output.*

2.2.2 Soil material and preparation of AM fungal inoculum

The soil for the experiment was collected from the top 20 cm of grassland in Richmond, NSW, Australia (33°36'41.5"S 150°44'17.6"E). This low nitrogen soil (1.7 mg/kg nitrate N,

mg/kg ammonium N) was previously found to be suitable for achieving AM fungal colonisation in Brachypodium. The soil was sterilised by gamma irradiating at a dose of 50 kGy (Steritech, NSW) and some live soil was reserved to prepare the inoculum and microbial wash. As irradiation can lead to an increase in plant available N (Mcnamara et al., 2003), in my case (1.7 mg/kg nitrate N and 6.5 mg/kg ammonium N before sterilisation, 7.4 mg/kg nitrate N and 28 mg/kg ammonium N after sterilisation), this soil was mixed 1:4 with sand (Bunnings) to keep the N concentration close to field conditions (1.48 mg/kg nitrate N, 5.6 mg/kg ammonium N). A soil microbial wash was prepared by mixing 1 part soil with 3 parts water and filtering the suspension through a sieve (20 µm; this size removes all AM spores), to incorporate microbial communities other than AM fungi and establish equal soil conditions for all treatments as closely as possible to natural conditions. This microbial filtrate was added back to the sterilised soil at a rate of 50 ml/kg and incubated for eight weeks with the wash. To generate the AM fungal inoculum, maize; a rapidly colonising host, was planted into four pots containing pre-gamma irradiated soil and grown for 3 months. The soil containing the roots from this inoculum was homogenised by combining all four pots and used for the AM fungal treatment and the AM fungal microbial wash. The final inoculum was added to experimental pots. Control pots comprised the same amount of 2 x autoclaved inoculum and the flow-through from the microbial filtrate of the equivalent volume of live inoculum (-AM fungi). Experimental pots (9cm x 25 cm, pvc pipe with bottoms made from end caps with holes drilled in them) were lined with plastic bags (to make it easier to extract the intact root system at harvest). The pots were filled by mixing enough sand and soil for one replicate at a time. The pots were filled to a bulk density of 1.2 (dry weight of soil ~1.8 kg) with 70g inoculum incorporated into the top half of the pot where growing roots would encounter it early. Control pots had 20 ml of additional microbial wash added and the AM+ pots 20 ml of water. Then 2cm of the soil-sand mix at the top (180 g). To establish the two AM fungal treatments and a diverse microbial community containing, among others, Oomycetes, we inoculated sterilised soil with (i) a microbial filtrate after removing AM fungal propagules and (ii) a mix of (live or dead) AM fungal spores, hyphae and colonised roots, with both sources of inoculum.

2.2.3 Plant material and growth conditions

Two accessions of *Brachypodium distachyon* (KAH1 and TR2B) with previously observed differing AM fungal colonisation capabilities (Donn et al., 2017) were used. Seeds were dehusked (soaked in water for several minutes followed by peeling off the thin outer husk with tweezers) and surface sterilized by soaking in 10% bleach for 5 minutes and rinsing with sterile water before they were sown

separately in pots (2 seeds/pot). Following germination plants were randomly reduced to a single plant per pot. Pots were initially watered to weight (80% field capacity) and then watered to weight once per week. Three replicates, absent of emerging seedlings, were re-sown after 5 days and allowed to grow for 28 days in a growth cabinet set to 24°C/18°C (day/night), long day conditions with 16 h light (400-500 µmol, RH = 60%) and were positionally re-randomised twice per week. 20 ml of a modified Hoagland's solution was added on days 7 and 21 after sowing: 1ml of 0.5M KH_2PO_4, 5 ml of 1M KNO_3 and $CaCl_2.2H_2O$, 2 ml of 1M $MgSO_4$, 1 ml of 0.5% Iron(III) Citrate, and 1 ml of Micronutrients (2,86 g l^{-1} H_3BO_3, 1,81 g l^{-1} MnO4S, 0,22 g l^{-1} $ZnSo_4 \cdot 7H_2O$, 0,08 g l^{-1} $CuSO_4 \cdot 5H_2O$, 0,2 g l^{-1} $H_2MoO_4 \cdot H_2O$) (Hoagland & Arnon, 1950).

2.2.4 Harvest

To harvest plant material and keep the root system intact the bags were pulled from the pots. The soil core was gently broken up and a 5 ml sample of bulk soil was taken and stored at -20°C for DNA extraction. The root system was separated from the soil and briefly washed in Phosphate buffered saline (PBS) to remove the rhizosheath (defined as the soil and microbes adhering directly to the root surface). Subsequently, for plant RNA extraction, one washed leaf nodal root was cut with a scalpel and immediately frozen in liquid nitrogen and stored at -80°C. For DNA extraction a 5 cm long section of washed root (5 cm from the top of a second leaf nodal root, including many lateral roots) was excised and stored at -20°C. The shoot was removed, and oven dried at 60°C for measurement of shoot dry weight. The remaining root system was stored in 30% ethanol at 4°C for morphology analysis. For hyphal extraction, approximately 200 g of the remaining soil was collected and stored at -20°C. Only the root extracts of RNA and DNA, and colonisation count from the morphological examination were used for further data analysis in this chapter.

2.2.5 Mycorrhizal colonisation measurement and hyphal extraction

Root segments 2 cm long were cut from the root system after scanning, mixed, and then a sub-sample was placed in a histology cassette and rehydrated in water overnight. Root staining to detect fungal structures was performed with the ink-vinegar method (Vierheilig et al., 1998). Briefly, roots were first cleared in 10 % KOH overnight at 60 °C and subsequently washed three times in acidified water. Staining was performed in 10% ink (Parker Black) in 10 % acetic acid for 10 mins at 80°C followed by destaining in lactoglycerol (1:1:1 lactic acid, glycerol and water), at least overnight. 10 root sections were mounted on microscope slides and scored (50 intersections per slide) at x200 for the presence/absence of fungal structures (hyphae, arbuscles, vesicles, spores). To extract hyphae, 25 g of soil was added to a Schott bottle containing 200 ml water and shaken by hand for 10 seconds. After settling the mixture for 1 minute the supernatant was poured through a 38 µM sieve and the residues

of the sieve were collected in a 50ml falcon tube. 40 ml of sucrose was added and centrifuged for 5 mins at 3000 rpm. The supernatant was sieved again (38 µM sieve) and residues were collected in a 15 ml tube. The 15 ml tubes were centrifuged for 5 minutes at 4000 rpm to form a loose pellet at the bottom of the tube. The supernatant was discarded and the pellet freeze-dried.

2.2.6 RNA extraction, RNA sequencing and quantification of the transcriptomic profile

Total RNA from approximately 50 mg of fresh root tissue was extracted with the Isolate II Plant RNA kit (Bioline) according to manufacturer's instructions. TruSeq RNAseq libraries (Illumina) were generated with 300 ng of total RNA and each of the 24 libraries was sequenced across two lanes by the Next Generation Sequencing Facility at Western Sydney University. RNA sequences were processed on the Galaxy Australia platform (usegalaxy.org.au). Data from biological samples sequenced across different lanes were concatenated and forward and reverse sequences were paired using the "Concatenate data sets tail-to-head" and "Build list of dataset pairs" commands in Galaxy. The data were quality checked using FastQC (Andrews, 2010), before being aligned to the *Brachypodium distachyon* genome (B.distachyon_314_v3.0.fa, (Goodstein et al., 2011)) in HISAT2 Version 2.1.0 (Kim et al., 2015) using the default paired-end options. Sequence fragments aligned to genome features were counted using featureCounts (Y. Liao et al., 2014), with "Count fragments instead of reads" enabled. Finally, the Galaxy tool "Column Join" was used to merge the feature counts for each biological sample into one table.

2.2.7 DNA extraction and operational taxonomic unit (OTU) identification

DNA was extracted from 0.25 g of soil, 10 mg root and the hyphal pellet using the PowerSoil kit (Qiagen) according to the manufacturer's instructions with some modifications. Roots and hyphae were extracted with the following modifications: The extraction buffer was first transferred to the tube with root or hyphae and bead beat (5 m/s) for 30 seconds with a steel ball. Then added the kit lysing matrix and solution C1 and beat for a further 30 seconds. Thereafter as per kit instructions. Fungal amplicon sequencing was performed at the Ramaciotti Centre for Genomics (UNSW Sydney, NSW, Australia). As the experimental setup aimed to identify additional groups besides AM fungi, the primer set fITS7 (5'-GTGARTCATCGAATCTTTG-3'; (Ihrmark et al., 2012) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; (White et al., 1990) were used to amplify the ITS2 region. Subsequently, fungal data relevant to the research question were extracted from these sequences. Oomycete amplicon sequencing was performed at the Western Sydney University Next Generation Sequencing Facility (Richmond, NSW, Australia) using the primer pairs ITS1oo (5'-GGAAGGATCATTACCACA-3'; (Riit et al., 2016) and ITS4 covering the ribosomal ITS1, 5.8S and ITS2. In both instances, the amplicons were purified using the Agencourt AMpure XP system (Beckman

Coulter) and genomic libraries were prepared using the Nextera XT Index Kit (Illumina). Paired-end (2 × 251 bases) sequencing was performed on the Illumina MiSeq platform.

Fungal DNA sequencing data processing was performed using the approach described by (Bissett et al., 2016) with modifications. Ambiguous bases and/or homopolymers greater than eight bases in length were removed from DNA sequences for initial quality filtering. Forward and reverse reads were merged (representing full-length ITS2 sequences) and filtered in mothur (Schloss et al., 2009) to obtain operational taxonomic units (OTUs). Generation of de novo OTUs was performed using numerically dominant sequences in all sequences using the '-cluster_otus' command in USEARCH (version v8.1.1803) (Edgar & Bateman, 2010) at 97% similarity. Mapping to pick OTUs was executed at 97% sequence similarity against representative sequences of these OTUSs using the '-usearch_global' command in VSEARCH (version v2.3.4) (Rognes et al., 2016). A second round of de novo OUT picking was performed as above on sequences that were not mapped but focusing solely on sequences observed at least two times. All initially unmapped sequences were aligned against the newly selected OTUs as above, and still unmapped sequences at this stage represent singleton OTUs and were omitted from subsequent analysis(Lee et al., 2019).

The Oomycete reads were processed separately without making contigs as primers produce non-overlapping forward and reverse reads. Moving from 5' to 3', we trimmed reads on the average phred score in a 15-base window, and the remainder of the read was trimmed if the average phred dropped below 30. Reads that were fewer than 150 bases in length, contained homopolymers greater than or equal to eight bases or contained any ambiguous bases were removed. Subsequently, the same approach as for the fungal DNA sequences was used for OTU picking for quality filtered forward and reverse reads. Oomycete counts were divided by all reads in the sample (primarily nontarget fungal reads) after filtering to determine the proportions of reads. Each of the treatment combinations was roughly equally represented in the two sequencing runs. However, to test whether the term "run" (differences between reads of the two sequencing processes) affected oomycete and AM fungal read counts, this factor was included in the initial model. The results of this analysis were not statistically significant; therefore, we excluded the "run" factor for the purpose of simplifying the interpretation. OTU richness per sampling effort was evaluated by plotting rarefaction curves for each sample (using the R package vegan; version 2.4-1; (Oksanen et al., 2015). BLAST (Altschul et al., 1990) comparison was used for putative taxonomic identities for OTUs. A reference database of fungal ITS sequences and taxonomic annotations was obtained from UNITE (version 7.0; (Abarenkov et al., 2010) and used to identify fungal OTUs based on the top BLAST result by e-value was assigned to the fungal kingdom (shared similarity of at least 67%). For Oomycete OTUs, we combined the UNITE database with stramenopile (including Oomycete) ITS sequences

downloaded from the NCBI nucleotide database using the NCBI Mass Sequence Downloader (Pina-Martins & Paulo, 2016) and the query '("Stramenopiles"[Organism] OR "Oomycetes"[Organism]) AND (internal[All Fields] AND transcribed[All Fields] AND spacer[All Fields])]'. OTUs showing at least 85% similarity within the top BLAST result by e-value linked to the sequence of any oomycete genus were retained. Noting that since pathogenicity cannot be confirmed, detected OTUs are considered as putative pathogens, with some potentially not capable of causing disease in this host.

The ITS1oo-ITS4 primer set selects for oomycete DNA but can also amplify fungal DNA from environmental samples, with these fungal reads often representing the majority of sequence reads (M. Lee et al., 2022). Similarly, the fITS7-ITS4 primer set can amplify AM fungal DNA from environmental samples where AM fungal biomass is present, although sequence reads from other fungal taxa make up the majority of sequence reads in samples when using this primer set (Eldridge et al., 2022). Therefore, counts of sequence reads associated with oomycete and AM fungal taxa were used, proportional to other sequence reads, as proxies for the relative abundance of these groups. For Glomeraceae abundance, the proportion of reads assigned to Glomeraceae taxa relative to all reads assigned to AM fungal taxa was calculated. BLAST comparison for AM fungi was conducted according to concurrence of at least 90% shared identity with the inquired OTU sequence. If at least 80% of the database sequences shared a common family-level annotation, they were classified at the family level.

2.2.8 Statistical analysis

AM fungal root colonisation and relative abundance of sequence reads from the AM fungi and the Oomycetes were analysed using ANOVA, calculated using marginal sums of squares and specified as variables AM fungi, Glomeraceae and Oomycetes respectively. Analyses were performed using the Anova function from the car library (Fox & Weisberg, 2019) in R (R Core Team, 2021). Relative abundances were log10-transformed after adding a small constant (0.00001) prior to ANOVA. ANOVA was not performed for relative abundances of Glomeraceae reads due to difficulty normalising the residuals by transforming the response; therefore, only descriptive results are provided.

Identification of plant-derived differentially expressed genes (DEGs) was performed using the package DESeq2 (Love et al., 2014) from the R version 4.1.1 (R Core Team, 2021), after filtering for reads <10 and with a two-fold difference in gene expression between any pairwise comparison of treatment combinations, p-value cut off 0.01. Filtering for conditions AM inoculation and accession (KAH and TR2B) revealed 118 differentially expressed genes (DEGs) out of a total of 34,310 detected

genes (see results). Therefore, functional classification of the whole gene set was carried out in October 2020 using the PANTHER classification system (Mi et al., 2013), to compare responses within functional categories to variation in AM fungal root colonisation and frequencies of reads from the Glomeraceae and Oomycetes. Genes lists classified with the Gene Ontology (GO) term response to biotic stress (GO:0009607) were identified as follows: primary metabolic compounds (GO:0044238), response to biotic stimulus (GO:0009607), hormone metabolic process (GO:0042445), signal transduction (GO 0007165), secondary metabolic compounds (GO:0019748), and cellular component organisation (GO:0016043). The GO-term classification can change and therefore results can vary when reproduced at different times (Huntley et al., 2014). To test for and visualize the variation among samples associated with microbes (AM fungi, Glomeraceae and Oomycetes) and identify patterns in genes (GO-terms and DEGs), a Correspondance Analysis (CCA) was performed using the vegan package (Oksanen et al., 2022) in R version 4.1.1 (R Core Team, 2021). Gene counts were log(x+1)-transformed and the explanatory variables were standardized by the decostand function in the vegan package (Oksanen et al., 2022). The characterization of the molecular function of the ten strongest loaded genes on each of the axis of the MIR-influential biological processes was performed in November 2022 using the PANTHER classification system (Thomas et al., 2022). As AM fungi, specifically Glomeraceae, have been frequently associated with MIR (Sikes et al., 2009; Sikes et al. 2010; Maherali & Klironomos, 2007) and this study investigates the transcriptional alterations in plants grown in soil with a frequent abundance of Glomeraceae, the term “pre-MIR” responses are used for here detected gene expression connected to beneficial microbe abundance.

2.3 RESULTS

2.3.1 Variable root colonisation and microbial composition within *B. distachyon* accessions

Root colonisation by AM fungi (see supplementary material, Figure S2.1) ranged from 2 to 35% of root length. This was significantly higher in both accessions compared with the control conditions, for which a minority of replicates also had a small level of colonisation up to 5%. Root colonisation was up to 35 % in KAH and slightly less with up to 30% in TR2B but with no significant difference between the two accessions. The number of sequence reads assigned to AM fungi ranged from 0 to 0.8% of all fungal sequence reads per sample (33,854 +/- 19,386 (SD)). On average, AM fungal reads were more frequently observed in AM fungal inoculated pots (0.2%) than in control pots (0.0003%; $P < 0.001$), and at similar frequencies between the two *B. distachyon* accessions ($P = 0.96$). Sequence reads assigned to the Glomeraceae made up between 0 and 100% of that AM fungal reads per sample, with samples tending to be either dominated by Glomeraceae reads (>80% of reads in 2/10 and 3/10 of KAH1 and TR2B samples, respectively) or other AM fungi (>90% of reads in 8/10 and 7/10 of KAH1 and TR2B samples, respectively). Oomycete read counts consisted of between 0 and 0.4% of sequence reads per sample (103,391 +/- 43755 (SD)). Average oomycete relative abundances were similar across all combinations of AM fungal inoculation and *B. distachyon* accession ($P = 0.60$).

2.3.2 Comparison of AM fungal inoculation and genotype reveals 118 differentially expressed genes

The gene expression profile using RNA-seq of *B. distachyon* revealed 118 differentially expressed genes (DEGs), with a two-fold difference in gene expression between any combination of +/- AM and accession, out of a total of 34,211 genes (p-value cut off 0.01). Figure 2.2 presents the expression of all 118 DEGs in either accession (TR2B, KAH) and/or with (AM) and without (Cont) mycorrhizal inoculation, revealing no apparent global pattern in response to AM fungal inoculation or accession line.

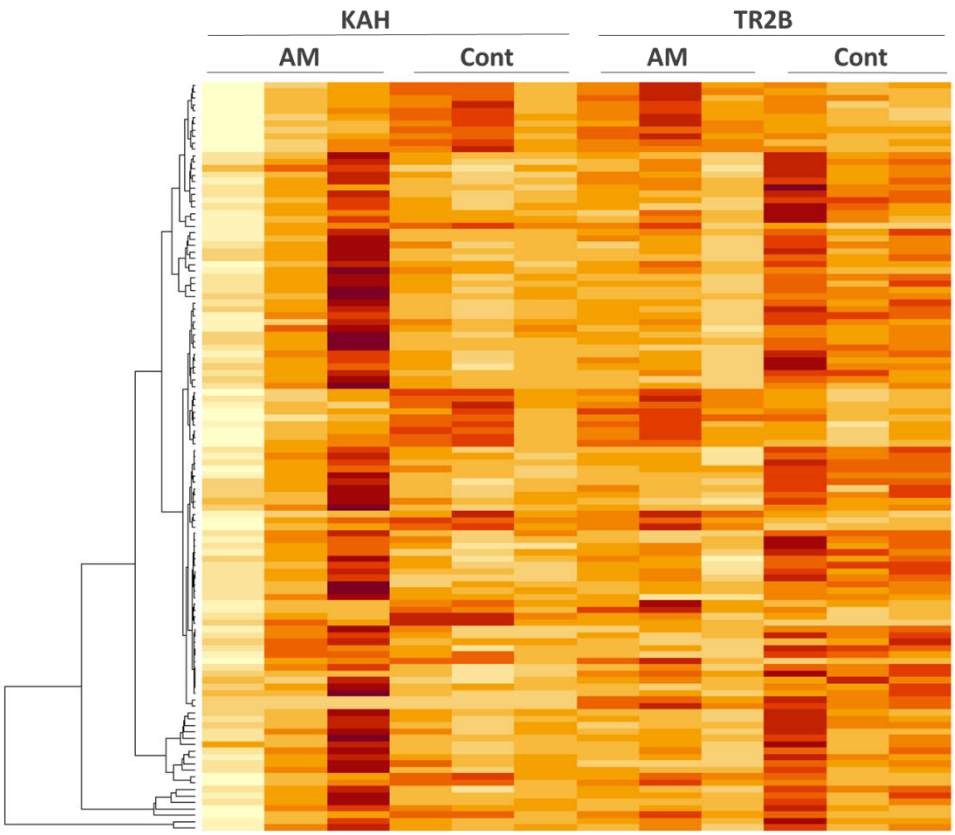


Figure 2. 2: *Hierarchical clustering of 118 DEGs identified in either accession (TR2B, KAH) and/or with (AM) or without (Cont) AM fungal inoculation, following normalisation. Dark colours represent high expression levels, while light colours represent low expression levels, dendrogram on the left represents the relation of data points.*

2.3.3 Defence-related biological processes are connected to beneficial microbe abundance

Given the high level of variation in AM fungal root colonisation and the abundance of sequence reads associated with the Glomeraceae and Oomycetes, we proceeded with analysis to estimate associations between gene expression and each of these variables (terms 'AM fungi' for root colonisation and 'Glomeraceae' and 'Oomcycetes' for sequencing reads) individually using Canonical correspondence analysis (CCA). To define subsets of genes for this analysis, a total of 34,211 genes were investigated for their association with the following Gene ontology terms (GO-terms):

primary metabolic compounds (GO:0044238) comprising 3,929 genes, response to biotic stimulus (GO:0009607) comprising 114 genes, hormone metabolic process (GO:0042445) comprising 85 genes, signal transduction (GO 0007165) comprising 338 genes, cellular component organisation (GO:0016043) comprising 1,286 genes, and secondary metabolic compounds (GO:0019748) comprising 40 genes.

The variation in gene expression for all analysed GO terms was significantly or marginally non-significantly associated with the degree of AM fungal root colonisation, with between 41 and 83% of variation explained for these terms (Table 2.1). The GO term "primary metabolic compounds" was not significantly associated with the abundance of sequence reads from the Glomeraceae or Oomycetes. Variation in genes related to "Signal transduction" also depicts marginal significant influence and explains 40% of the variation of specific Glomeraceae abundance. The GO-term "response to biotic stimulus" also showed significant relation to Oomycete abundance which is explained by ~50% of the variation. The GO-terms "cellular component organisation" and "secondary metabolic compounds" both show no significant interaction with any of the chosen GO-terms. Furthermore, "hormone metabolic process" depicts a significant level of interaction with Glomeraceae presence which depicted around 60% of the variation.

Table 2. 1: *R2-values that relate to the proportion of variation associated with the constrained component in a constrained analysis of all GO-terms connected to biotic response explaining the extent of variance for the abundance of AM fungal colonisation (AM fungi), and sequencing reads of Glomeraceae and Oomycetes. Significance of all GO-Terms connected to biotic response for the abundance of AM fungi, Glomeraceae and Oomycetes displayed in asterisks.*

GO Term	AM Fungi	Glomeraceae	Oomycetes
Primary metabolic compounds	**0.83 *** **	0.11	0.42
Signal transduction	**0.75 ** **	**0.40 .**	0.36
Response to biotic stimulus	**0.63 *** **	0.17	**0.47 .**
Cellular component organisation	**0.62 ***	0.19	0.28
Secondary metabolic compounds	**0.62 ***	0.12	0.24
Hormone metabolic process	**0.42 .**	**0.61***	0.37

Significance codes: 0.001 = '***', 0.01 = '**', 0.05 = '*', 0.1 = '.', Number of permutations: 999

2.3.4 Beneficial and pathogenic microbe abundance drive subsets of genes in three pre-MIR influential biological processes

The variables AM fungi, Glomeraceae, and Oomycete were used to explain the expression of gene sets in each of the chosen GO-terms. The proximity/distance of the genes to the direction of the variables reveals the influence on the expression of certain genes and the proximity/direction of the variables to each other reveals the relatedness of the vectors themselves. Three out of six biological processes defined in the GO terms showed associated gene expression along both CCA axes, corresponding with variation in abundance factors for beneficial and pathogenic microbes as well as correlations to each other (left panel, Figure 2.3). Therefore, the GO-terms depicting enhanced gene expression with strong loading also correlated with the variables, were characterised as pre-*MIR influential*. These patterns of response are described in more detail below. For all three biological

processes, there were several genes with strong loadings that corresponded either with or against the vectors associated with microbial abundance factors. For each of the GO-terms “Primary metabolic compounds” (Figure 2.3a), “Signal transduction” (Figure 2.3b) and “Cellular organisation” (Figure 2.3c), most of the variation in gene expression is associated with the level of colonisation by AM fungi, as indicated by the loading of that variable along the first axis of each ordination. Genes at the extremes of the second axis are associated with high or low frequencies of Glomeraceae and Oomycetes, both of which have similar loadings along that axis of each ordination, as well as along the third axis (Figure S2a-c). These gene sets might be expressed upon beneficial and pathogenic microbe colonisation and the commonly induced genes could therefore be involved in pre-MIR. Only 11 genes across these GO terms were found to be differentially expressed in response to AM fungal inoculation and/or accession, but these all were not among those that had strong loadings along either axis, indicating that they were weakly correlated with any of the three microbial abundance variables. Therefore, the presence of the Glomeraceae seems to trigger the same gene sets as the Oomycetes. The second set of biological processes assessed (“Response to biotic stimulus”, “Secondary metabolic compounds” and “Hormone metabolic process”; right panel, Figure 2.3) was observed to have much less variation along either CCA axis and very lfew genes had loadings that corresponded with variation in any of the microbial abundance factors. Therefore, the GO-terms with a comparatively low amount of gene expression and weak loading that are also not correlated with the variables were characterised as *non-pre-MIR influential*. Only four DEGs were observed in this set of GO-term genes, and none were associated with “Secondary metabolic compounds”. The proportions explained by CCA1, CCA2 and CCA3 are shown in supplementary table S2.1.

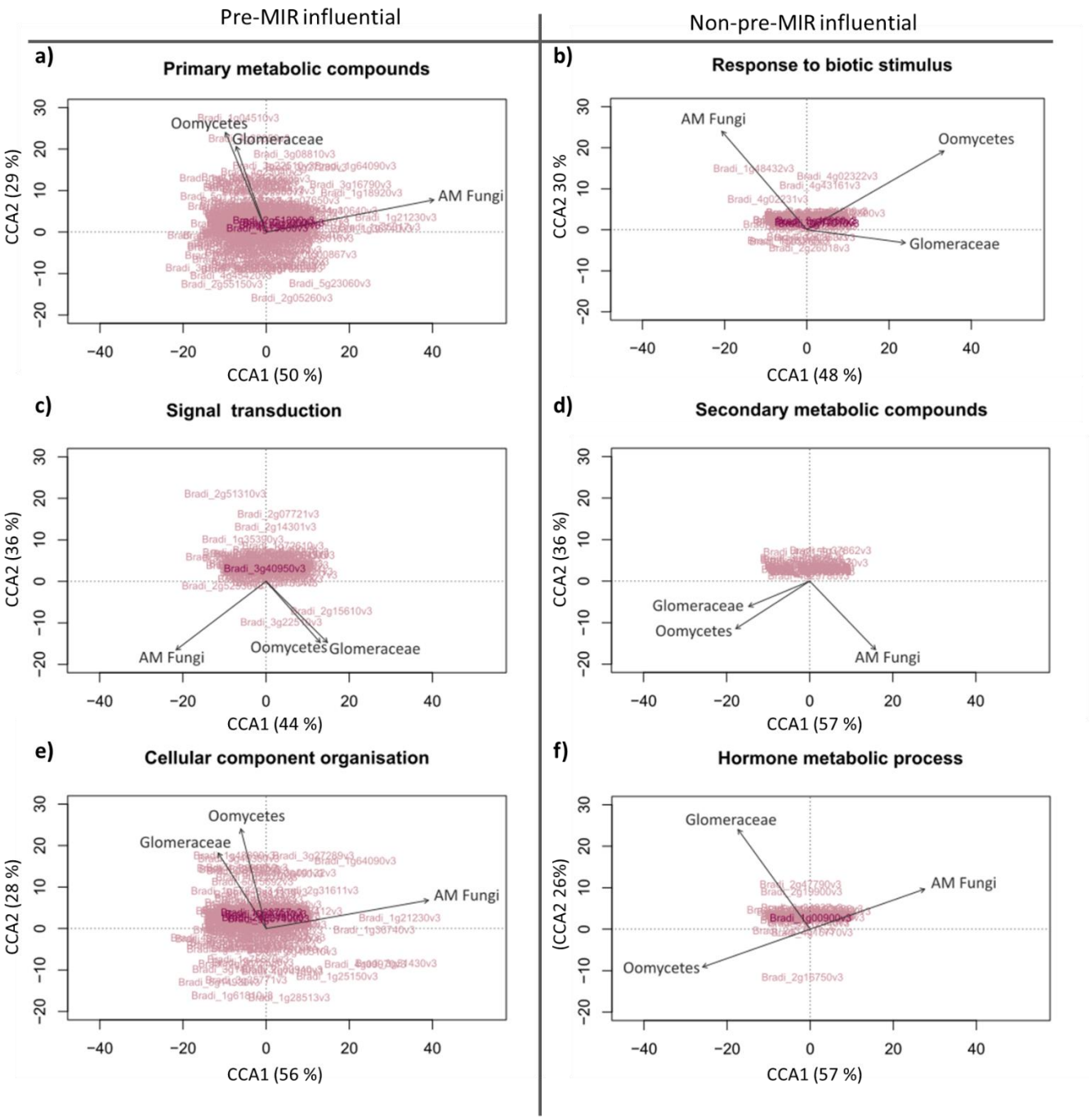


Figure 2. 3: *CCA plots of the GO-term genes, divided into panels "pre-MIR influential" (strong loading of genes) and "non-pre-MIR influential" (weak loading of genes), biological processes represented including* ***a)*** *Primary metabolic compounds* ***b)*** *Response to biotic stimulus and* ***c)*** *Signal transduction,* ***d)*** *Secondary metabolic compounds,* ***e)*** *Cellular organisation and* ***f)*** *Hormone metabolic process including the explanatory factors AM fungi (AM fungal colonisation), Glomeraceae and Oomycete abundance from sequencing reads (black arrows). Expressed genes from each GO-term are depicted in pink and genes differentially expressed amongst accession and AM fungal treatment are shown in purple. Proportions explained by CCA1 and CCA2 are shown in parenthesis.*

2.3.5 Functional biological processes reveal potential pre-MIR genes

The three GO-terms categorised as pre-MIR influential biological processes showed the strongest expression of genes and positive relation of the three microbial abundance variables. Therefore, those three GO-terms ("Primary metabolic compounds", "Signal transduction", "Cellular component organisation") may represent categories of functions that provide potential genes involved in pre-MIR. Figure 2.4a depicts 10 genes with the strongest loading along each of the four cardinal directions in the CCA plots in Figure 2.3, resulting in 40 potential pre-MIR genes associated positively or negatively with microbial abundances during these biological processes. The characterisation of these gene lists with the PANTHER classification tool revealed involved molecular functions with the GO-terms: Molecular transducer activity (GO:0060089), Binding (GO:0005488), Molecular function regulator (GO:0098772), Catalytic activity (GO:0003824), Transporter activity (GO:0005215), transcription regulator activity (GO:0140110), structural molecule activity (GO:0005198) (Figure 2.4b). Detailed information regarding the molecular functions of the genes is shown in supplementary tables S2.2, S2.3 and S 2.4.

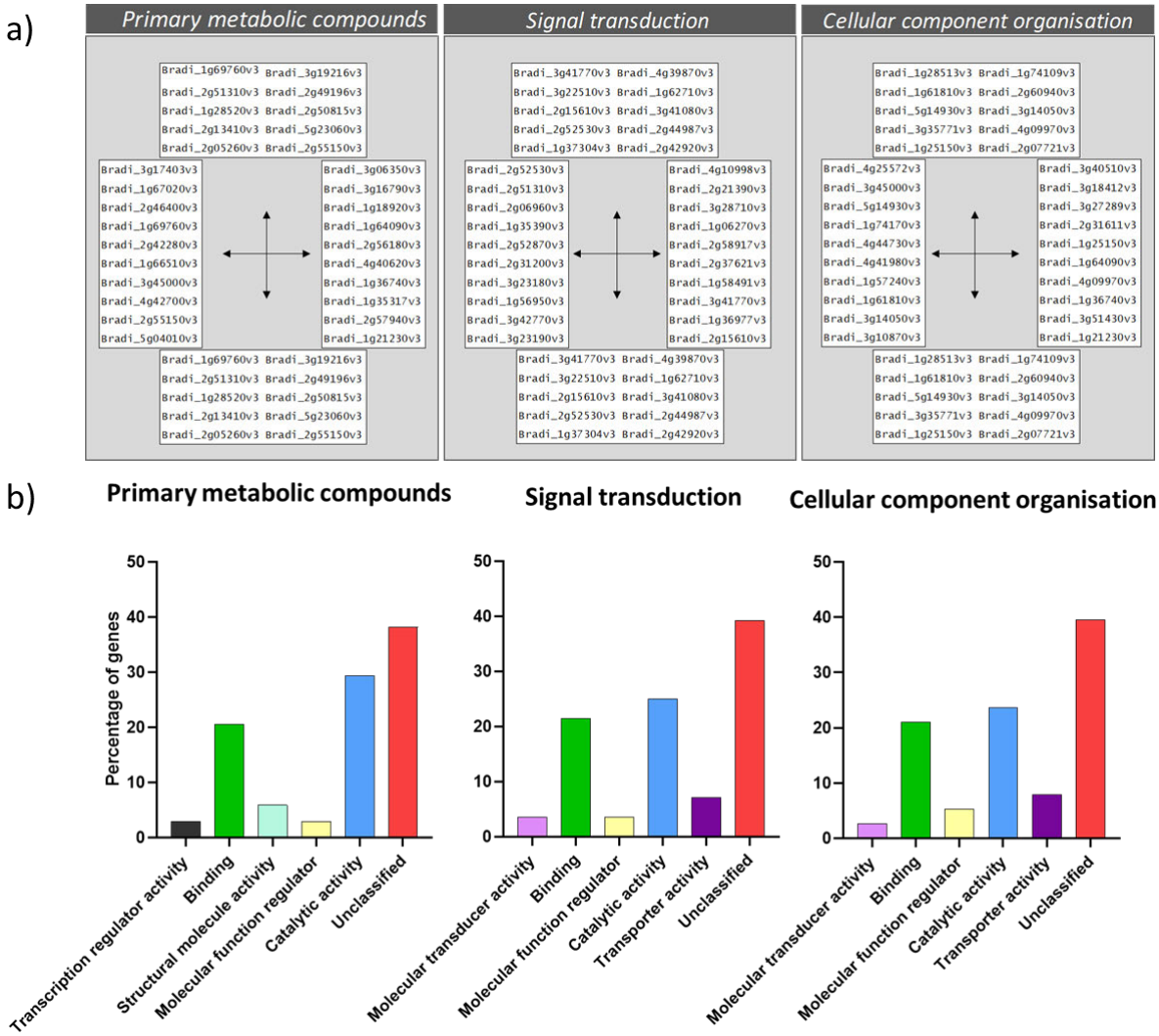


Figure 2. 4: *Information of strongest loaded potential pre-MIR genes on each axis of the CCA plots in each of the three pre-MIR influential biological processes showing* ***a)*** *Gene ID list of all detected* B. distachyon *genes (40 genes in "Primary metabolic compounds" and "Cellular component organisation" respectively and 20 genes in "Signal transduction") and* ***b)*** *Percentage of genes associated with molecular functions according to PANTHER classification.*

2.4 DISCUSSION

The overall aim of this study was to assess the influence of AM fungi on pre-MIR-related gene expression via both experimental manipulations of AM fungal presence/absence and assessing natural variation in AM fungal abundance and using an RNA-seq approach. Surprisingly, my analysis showed that biological processes not directly connected to plant defence mechanisms (i.e. "Primary metabolic compounds", "Signal transduction", "Cellular component organisation") showed pre-MIR influence in *B. distachyon* transcriptomic profiles but not biological processes previously connected to MIR (i.e. "response to biotic stimuli",' secondary metabolic compounds", "hormone metabolic compounds"). Expression analysis of individual genes identified a subset of DEGs associated with experimental treatments (genotype and AM fungal inoculation), but these were not significantly linked to variation in the abundance of beneficial or pathogenic microbial groups. These results imply that pre-MIR-related molecular processes in experimental systems reproducing microbial complexity found in nature operate differently from sets of genes activated by AM fungal presence alone. These rather surprising results reflect the importance of precisely elucidating the process of MIR under conditions with natural microbial abundance and compositions.

2.4.1 No pre-MIR influence on defence-related gene expression

The main processes involved in plant defence responses to pathogens characterised to date involve pattern recognition receptors with subsequent signalling cascades that lead to increased defence responses like increased physical barriers, production of secondary metabolite and antimicrobial proteins, secretion of defence-related enzymes as well as programmed cell death (Y. Wang et al., 2019). Transcriptional regulation of these defence strategies to pathogens, including Oomycetes, has been extensively studied in different plant species and transcripts from categories related to PR-gene expression, protein synthesis, cell structure, cell death associated with a metabolic and cellular process, as well as response to stimulus (Chen et al., 2014; Schlink, 2009; Marcel et al., 2010; Larroque et al., 2013). Furthermore, previous observation of transcriptomic changes in plants upon AM fungal colonisation highlights the most frequently represented biological processes in responses to beneficial microbes, including categories like cellular processes, primary metabolism, abiotic and biotic stress, secondary metabolism, signal transduction, and hormone metabolism (Vangelisti et al., 2018; Guadalupe Cervantes-Gámez et al., 2015; Fiorilli et al., 2009). Another study observed a correlation between the mycorrhization of wild rice, enhanced resistance to the pathogenic fungus *Magnoaporthe oryza*, and transcriptional changes in biological processes related to secondary metabolic processes and biotic stress related (Tian et al., 2019). Furthermore, systemic transcriptional alterations of biotic stress-related genes in mycorrhized plants have been observed in tomato and medick correlated with resistance to foliar pathogens (Guadalupe Cervantes-Gámez et

al., 2015; Liu et al., 2007). Interestingly, overlapping expression of gene sets in response to beneficial and pathogenic microbes has also been shown previously (Paszkowski, 2006; Güimil et al., 2005). Therefore, I hypothesised that early transcription of biological processes frequently connected to defence responses are influenced by AM fungal colonisation and connected to pathogen abundance and that there is an overlap with the gene expression upon pathogenic microbe presence. However, the findings in my study are contradictory to these previous observations. In contrast to the studies mentioned above, I could not observe any strong influence on gene drive caused by AM fungi in biological processes previously connected to plant resistance. Therefore, it seems like a low AM fungal abundance paired with low pathogen pressure leads to gene responses different from those observed upon plant defence to exceeded pathogen thresholds.

2.4.2 Potential of Glomeraceae for enhancing defence responses

The biological processes identified to have pre-MIR-related covaried with the relative abundances of Glomeraceae and Oomycetes more closely than AM fungal colonisation to Oomycetes. Similarities in how the plant responds to Glomeraceae as to Oomycetes abundance might explain why colonisation of the former may result in enhanced resistance to the latter. This concept is supported by a prior study in rice that identified a core set of shared genes activated by beneficial and pathogenic microbe exposure using *Rhizophagus irregularis*, a member of the Glomeraceae (Güimil et al., 2005). Moreover, these results are consistent with studies observing that members of the family Glomeraceae were more successful in conferring MIR than other AM fungi (Sikes et al., 2009; Sikes et al. 2010; Maherali & Klironomos, 2007). Another study identified an AM fungal effector protein in *R. irregularis* (from the Glomeraceae) that alters plant defence responses (Kloppholz et al., 2011). Also, early defence gene repression through calcium/calmodulin kinase DMI3 was observed in *Gigaspora margarita*, a member of the Gigasporaceae family (Siciliano et al., 2007), which implies that this and possibly other AM fungi could show this effect. Nevertheless, taken together it seems possible that members of the Glomeraceae have a higher potential to enhance defence responses including several different strategies compared to other AM fungal species. Moreover, this AM fungal-mediated suppression of plant defence responses could enable more accessible conditions for pathogenic microbes. This non-damaging coexisting situation might be condoned until exceeding a threshold and surge of plant defence responses. It is difficult to explain the variation of relationships between Oomycete presence to overall AM fungal colonisation or Glomeraceae presence.

2.4.3 Consideration of excess environmental factors to decipher MIR

Surprisingly, I could not detect any DEGs in my lists of genes associated with microbial abundance variables. This could mean that colonisation in the AM+ treatment was so variable that it made it difficult to detect DEGs or suggests that there is no universal effect of plant accession or AM fungal inoculation to DEGs, which was also reflected in the hierarchical clustering and the biomass data (see supplementary Figure S2.2). As I investigated conditions with natural variation in pathogen abundance, there was also no acute biotic stressor for the plant. Therefore, it may be that these DEGs are more related to primary growth-related responses or growth-related changes. Moreover, it has been shown that AM fungal conferred growth responses and defence responses to the plant function separately from each other (Pozo de la Hoz et al., 2021). Thus, it seems possible that the weak associations between microbial abundance variables and DEGs connected to plant genotype and AM fungal colonisation in biological processes connected to plant defences are another sign that MIR works independently of growth-related AM fungal benefits.

All three MIR influential processes showed that most detected molecular functions relate to binding and catalytic activity. The genes involved in processes of “Signal transduction” and “Cellular component organisation” showed the same categories of molecular functions with a similar distribution. Compared to the other two MIR-influential processes “Primary metabolic compounds” revealed the involvement of transcription regulation activity and structural molecular activity. While I was able to observe gene expression and identify potential gene candidates using transcriptomics under conditions connected to beneficial and pathogenic microbe abundance, the findings in this study may be limited by the lack of observations on the posttranscriptional level. Transcriptomics is a powerful tool to explore alteration in actively transcribed genes (Diwan et al., 2022), however, a noteworthy number of genes involved in post-transcriptional protein modification have been detected in plants with AM fungal symbiosis before (Guadalupe Cervantes-Gámez et al., 2015). Moreover, posttranslational modification has been suggested to be an important regulating mechanism in AM fungal-mediated defence responses (Pozo & Azcón-Aguilar, 2007). There are further methodological limits that need consideration in how far the concept suggested here can be taken. For example, I observed variation in inoculation treatments across the replicates (supplementary Figure S2.1) influencing the results of differentially expressed genes and the possible variation in other microbes present could have masked some of the inoculation effects. However, these limitations on the other hand also reflect the possible overestimated response of inoculation in very controlled conditions. Therefore, to identify the molecular mechanisms involved in MIR resistance, it will be important to make observations on different levels. The investigation of early gene expression but also subsequent processes including enzyme activity, protein levels, and

phenotypical responses including cell physiological responses comparing AM fungal inoculated and/or pathogen inoculated plants to control plants and in different symbiosis stages. It will be important to conduct these further studies under natural conditions harbouring beneficial and pathogenic microbes, yet also compare them to results obtained from controlled conditions.

CHAPTER THREE

Contrasting metabolic responses of two chickpea varieties to root infection by *Phytophthora* are mediated by arbuscular mycorrhizal fungi

I led all aspects of the research including study design, execution, analysis and writing. This chapter is in preparation for submission to the journal "Mycorrhiza" shortly after thesis submission: Natascha Weinberger, Krista L. Plett, Jeff R. Powell, Jonathan M. Plett "Contrasting metabolic responses of two chickpea varieties to root infection by *Phytophthora* are mediated by arbuscular mycorrhizal fungi". *Mycorrhiza*

3.1 INTRODUCTION

Crop plants are constantly threatened by a plethora of biotic stressors including viruses, bacteria, nematodes, fungi, and insects, all of which lead to significant yield losses (Savary et al., 2019). Global losses due to fungal and oomycete pathogens, alone, are estimated to account for up to 30% of productivity annually (Bebber & Gurr, 2015). Plants, in contrast to many other living organisms, are sessile and cannot escape such challenging situations. Therefore, a robust immune system that detects and responds to biotic aggressors is key to their continued health and productivity (Dodds & Rathjen, 2010; Jones & Dangl, 2006). The highly complex plant innate immune system recognises external signals and uses a variety of chemical compounds (e.g. secondary metabolites) that operate in diverse ways to protect plants (Moffitt et al., 2022; Piasecka et al., 2015). Additionally, some non-pathogenic microorganisms establish associations with plants where they support plant health by inducing the plant immune system against harmful attackers (Pieterse et al., 2014). One group of these beneficial microbes are the arbuscular mycorrhizal fungi (AM fungi), obligate biotrophs belonging to the subphylum of Glomeromycotina, which form a symbiosis with the roots of around 80% of terrestrial plant species (Bruns et al., 2018; Schüβler et al., 2001. The formation of this mutualistic interaction, in addition to nutritional benefits, has frequently been shown to enhance plant defence responses (Govindarajulu et al., 2005; Pozo & Azcón-Aguilar, 2007). This phenomenon, called Mycorrhiza Induced Resistance (MIR), if it can be understood and harnessed in a production system, has great potential to improve sustainable biocontrol in crop production (Harrier & Watson, 2004). Although the prevalence of MIR in the plant kingdom is well-reported, one of the biggest challenges is to gain a detailed understanding of the underlying mechanisms leading to this enhanced resistance.

Evidence for MIR against a variety of biotrophic and necrotrophic pathogens spans a range of plant models including tomato (Fritz et al., 2006), orange (Tian et al., 2021), medick (Liu et al., 2007), tobacco (Shaul et al., 1999) and rice (Tian et al., 2019). Enhanced resistance against fungal soil-borne pathogens like *Alternaria solani*, as well as the airborne Botrytis cinerea, was observed in tomatoes (Fritz et al., 2006; Fiorilli et al., 2011) as were systemic resistance to the oomycete *Phytophthora nicotianae* and *Cladosporium fulvum* (Pozo et al., 2002; Song et al., 2015; Wang et al., 2018). Various molecular and biochemical modifications in mycorrhized plants have been associated with enhanced plant immunity. Alterations of phytohormones, known to play an important role in defence responses (e.g. ethylene, salicylic acid) were frequently observed after AM fungal colonisation (Fiorilli et al., 2018; Tian et al. 2005; Bari & Jones, 2009). Increased gene expression of well-known defence signalling pathways has also been shown in mycorrhizal roots as has systemic activation of expression by defence-related genes in shoots of *Medicago truncatula, Solanum lycopersicum, Poncirus trifoliata*, and *Nicotiana tabacum* (Tian et al., 2021; Zhang et al., 2001; Ebastien Bruisson et al., 2016; Shaul et al., 1999; Song et al., 2015; Liu et al., 2007). Enzymatic profiles can also be changed in AM-colonised plants as has been described in plants challenged with *Verticillium dahlia*, *Phytophthora parasitica var. nicotianae* and *A. solani* (Song et al., 2015; Tian et al., 2021; Pozo et al., 2002; Rahou et al., 2021; Lawrence et al., 1996). These past studies, together, underscore the fact that MIR involves a variety of processes interacting both independently and in concert.

Recently, researchers have shown an increased interest in the metabolic changes occurring in plants during the process of colonisation by mycorrhizal fungi (Schliemann et al., 2008; Rivero et al., 2015; Sardans et al., 2021; Laparre et al., 2014). Changes in the concentrations of several secondary plant metabolites, which are compounds that play an important role in the ultimate defence against pathogenic microorganisms, were observed in mycorrhized plants including alterations of phenols (López-Ráez et al., 2010a), alkaloids (Hill et al., 2018), terpenoids (Welling et al., 2016), flavonoids (Piasecka et al., 2015), and apocarotenoids (Schliemann et al. 2008). Despite these observed metabolic modifications in mycorrhized plants, a systematic understanding of how altered metabolism influences plant resistance to certain pathogens is still lacking and further investigation of metabolomic changes is needed.

One approach to improve our understanding in this area would be to take different genotypes of a single plant species with differing levels of AM fungal colonisation and test the impact of colonisation on MIR and associated secondary metabolic changes. A study comparing transcriptional changes in wild and cultivated rice colonised by the AM fungal species *Rhizoglomus intraradices* showed a positive correlation between colonisation and defence-related gene expression in the wild rice against the pathogen *Magnaporthe oryzae* (Tian et al., 2019). Another possible model system to

test whether this is a generalisable finding is the use of chickpea (*Cicer arietinum*). Previously, different chickpea genotypes have been shown to impact AM colonisation (Plett et al., 2016). Specifically, *C. arietinum* var. Sonali and *C. arietinum* var. Kyabra shows significantly different abilities to host AM fungal colonisation, with higher mycorrhization levels in Kyabra compared to Sonali, but similar levels of susceptibility to the root rot pathogen *Phytophthora medicaginis* (Plett et al., 2016; Bithell et al., 2021). Therefore, they may represent a new model to study the degree to which AM fungal colonisation level affects MIR in pathogen susceptible crop plants. In this study, I used an untargeted metabolomic approach to examine how these two chickpea varieties differed in metabolomic responses to AM fungal colonisation in both the presence and absence of a root pathogen. Based on previous research, I hypothesized that var. Kyabra would show distinct AM-mediated metabolomic changes compared to var. Sonali, based on the increased AM fungal receptivity and linked increased metabolic responsiveness. Hence, I also hypothesized that var. Kyabra would show a stronger shift in secondary metabolism in response to pathogen attack when colonised by AM fungi, and that defence-related compounds would be the main drivers of the separation. Altogether, my results advance our understanding of how AM fungal colonisation mediates the modification of plant defence responses via secondary metabolism.

3.2 Materials and Methods

3.2.1 Plant material and growth conditions

Cicer arietinum var. Sonali and var. Kyabra, both previously rated as very susceptible to phytophthora root rot (Bithell et al. 2021), were used in this experiment. Kyabra was shown to have higher AM fungal mycorrhization levels (~21% root length colonised) when compared to var. Sonali (~7.5% root length colonised) when inoculated with *R. irregularis* in sterilized soil (Plett et al., 2016). Surface seed sterilization was performed by soaking the seed in 4% bleach solution for 15 min followed by three 5-minute washes with sterile water. Soil, obtained from a chickpea paddock in Tamworth, NSW, Australia (31.0900° S, 150.9293° E) was mixed with sterile sand in a ratio of 1:1 after which it was sterilized by gamma-irradiation (50 kGy; Steritec NSW). The final plant-available phosphorus concentration was 15 ppm (Colwell). There was no detectable difference in %N by weight (0.1% in sterile and non-sterile conditions). Prior to planting, a microorganism filtrate that contained a living soil microbe community from unsterilised soil of the same location, but which excluded AM fungal spores, was added to the irradiated soil. This soil microbial wash, with the purpose of reinstating communities of other microorganisms into the sterile soil, was obtained by combining and mixing one part of non-irradiated soil with three parts of demineralised distilled water (100 g soil + 300 g water). After 10 minutes of resting, this mixture was washed through a series of sieves with an increasingly smaller pore size (2mm > 125 µm > 20 µm). This mycorrhiza-free filtrate was collected and added to the soil:sand mix (50 mL kg^{-1}). The seeds were germinated in the soil:sand mix and chickpea plants were cultivated in round pots (9x4 cm) in a blocked arrangement in a plant growth chamber under controlled conditions: 15 h light/ 9 h dark cycle at 18˚C, 70% relative humidity, and 3,500 µmol m^{-2} s^{-1} light intensity. Plants were watered daily with distilled water and fertilized every week starting after week two with 0.5 mL/pot of Long Ashton solution minus nitrogen and phosphorus: 2mM K_2SO_4, 1.5mM $MgSO_4 \cdot H_2O$, 3mM $CaCl_2 \cdot H_2O$, 0.1mM FeEDTA and 1 ml Micronutrients (2.86 g l^{-1} H_2BO_3, 1.81 g l^{-1} $MnCl_2 \cdot 4H_2O$, 0.22 g l^{-1} $ZnSo_4 \cdot 7H_2O$, 0.08 g l^{-1} $CuSO_4 \cdot 5H_2O$, 0.025 g l^{-1} $NaMoO_4 \cdot 2H_2O$)

3.2.2 AM fungal inoculum

A commercial inoculum (Startup Ultra, Microbesmart, Adelaide, SA, Australia) that contains four isolates of the AM fungus *R. irregularis* (previously known as *Glomus intraradices*) (Schüßler & Walker, 2010), was used in this experiment. To remove the small particles of Calcined diatomaceous earth, which is an inert carrier used in this commercial inoculum, 10 g of inoculum was mixed in

demineralised distilled water and sieved through 2 mm > 125 µm >20 µm sieves and the filtrate on top of the 20 µm sieve, containing the AM fungal spores, was collected. Half of this filtrate was used for the AM fungal treatment (+AMF) and the second half was autoclaved twice and added to the non-AM fungal treatments (-AMF). The +AMF soil mixture was prepared by mixing 300 g of sterilized soil with half of the filtrate (112.5 ml) containing the AM fungal spores (10 g of inoculum contains at least 10,000 Spores). The pot set-up was as follows: 30 g of soil (without AM fungal inoculum) was added to each pot followed by either 7.5 g of +AM fungal soil mixture (for +AMF treatments) or 7.5 g of AM-free soil (for -AMF treatments). On top of this mix, one seed of either chickpea variety Sonali or Kyabra was placed and covered with 10g of sterile soil (Figure 3.1a). The autoclaved AM fungal spore suspension with the same number of spores per plant was equally distributed and added to each pot of the -AMF treatment. Subsequently, the plants were allowed to germinate and grow for 25 days to enable the establishment of the AM fungus based on previous literature (Gutjahr et al., 2015; Renaut et al., 2020), and root colonisation after which pathogen treatments were established as outlined below (Figure 3.1a).

3.2.3 Rhizobia and Pathogen inoculation

To provide efficient N intake of the plants, another important efficient nitrogen-fixing symbiotic bacterium (rhizobia) of chickpea, *Mesorhizobium ciceri* isolate CC1192**,** was added to all plants of the experiment (Laranjo et al., 2014; Nour et al., 1994). To achieve this, 10 days after planting the seeds, one mL of bacterial suspension (10,000 CFU mL^{-1}) was added to each plant by injection into the soil near the stem. Two weeks following inoculation with *M. ciceri,* and 24 days post-seeding, the plants were inoculated with the pathogen *Phytophthora medicaginis* isolate 7831. This culture, isolated from a chickpea paddock by the New South Wales Department of Primary Industries in Mungallala, QLD, Australia (26.4466° S, 147.5436° E), was grown on V8 juice agar plates (containing 200 mL L^{-1} V8^{TM} bottled juice; 3.0 g L^{-1} $CaCO_3$, 15.0 g L^{-1} agar) at 25˚C in the dark for a minimum of 5 weeks to allow for optimal oospore production. At the time of inoculation, oospores were collected, and their concentration was counted by a haemocytometer. This solution was diluted to a low number of oospores (700 oospores mL^{-1}) and 1 mL of this solution was added to each pot following flooding with distilled water to a level of 1 cm above the soil level. As both chickpea varieties are susceptible to *P. medicaginis*, oospore concentration and flooding time were limited to prevent excessive disease pressure. After 48 hours, the water was drained and this was chosen as the starting time point of pathogen inoculation (i.e. T0. For mock inoculation, the same procedure was utilized with the exception that the oospore inoculation had been heat sterilized via autoclaving twice and cooled to room temperature prior to addition to the pots.

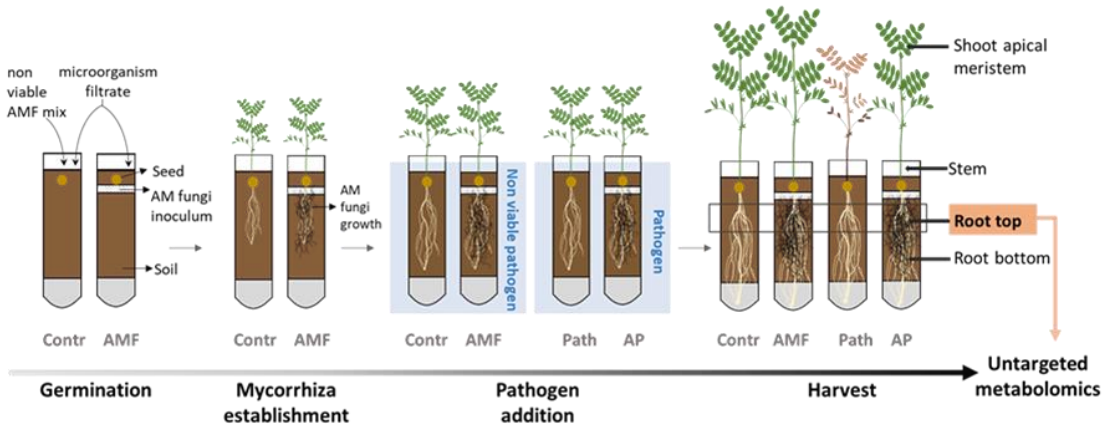
3.2.4 Experimental design

A factorial design including both chickpea varieties inoculated (or not) with one or both of AM fungi and pathogen, with one plant per pot (40 pots in total) and five biological replicates (n=5), was used in this experiment (Figure 3.1b). The treatments comprised non-inoculated control plants (Contr), plants inoculated with the *R. irregularis* AM fungal mix (AMF), pathogen-inoculated plants (Path), and plants inoculated with a combination of AM fungus and pathogen (AP). The AM fungus and pathogen-free control treatments (Contr) contained the heat-treated non-viable pathogen and autoclaved non-viable AMF inoculum (Figure 3.1a).

3.2.5 Harvest

The destructive harvest was conducted after 11 weeks of plant/AM fungal growth and 53 days post-pathogen inoculation (dpi) when mild symptoms like brown lesions on the lower stems started to be visible. The shoots were collected for fresh and dry weight measurements. For the untargeted metabolomic analysis, lateral root tissue samples without nodules from the top half of the root (Figure 3.1a) of each chickpea plant were collected, snap-frozen in liquid nitrogen and stored at -80 °C until further processing.

a)



b)

`Sonali` `Kyabra`	→	AMF Control	→	+/- Pathogen	→	*n*	=	Total amount of samples
2	x	2	x	2	x	5	=	**40**

Figure 3. 1: a) *Scheme of the study showing the different steps including treatment: Control (Contr), AM fungal inoculated plants (AMF), pathogen inoculated plants (Path), and combined AM fungus/pathogen inoculated Plants (AP).* ***b)*** *Treatments and number of levels and replicates used in this study*

3.2.6 Metabolite extraction and untargeted metabolite profiling

The frozen root samples were ground in 200 µL of cold extraction solution (4:4:2 methanol:acetonitrile:water) using an MP Biomedical bead mill for 30 seconds at a frequency of 6.0 Hz. Following the addition of a further 300 µL of extraction solution, samples were sonicated in a 4°C water bath for 25 minutes followed by 10-minute centrifugation (21,139xg, 4 °C). The supernatant was collected and stored at -80°C until analysis. Prior to analysis, the samples were diluted 4x (based on sample QC dilution series) and the mixtures were centrifuged for 5 minutes at 21,139 x g to remove particulates. Subsequently, 200 µL of supernatant was transferred into 96-well sample collection plates (Waters). The final untargeted metabolite profiling, including all biological samples as well as a method blank and a solvent blank, was performed using a nanoACQUITY UltraPerformance Liquid Chromatography coupled to a Waters Synapt G2-Si HDMS instrument (Waters, Wilmslow, United Kingdom) including positive and negative electrospray ionization mode.

3.2.7 Metabolomic data analysis and metabolite annotation

Automated data processed of acquired raw data including peak alignment, peak picking, and deconvolution, was conducted with the program Progenesis QI, version 3.0 (Nonlinear dynamics, Waters Corporation, UK). The data from the positive ion mode (determining the mass-to-charge ratio after positive ion formation) are used throughout the study and information regarding the negative ion (determining the mass-to-charge ratio after negative ion formation) mode can be found in the supplementary material (Supplementary Figure S3.1, Supplementary Table S3.3). These processing steps revealed information about peak intensities, which were further used for statistical analysis. Potential molecular feature identification and annotation of the observed peaks were obtained using the ProgenesisQI support for the web-based Chemspider structure database, including the public databases ChEBI, Phenol-Explorer, PlantCyc, KEGG and Golm Metabolome Database with a precursor tolerance 15 ppm and fragment tolerance 50 ppm. Mass error, isotope similarity and fragmentation score were used to calculate a confidence score for each potential identification. Potential compound identifications were assigned a chemical classification based on their primary structural features.

3.2.8 Statistical analysis

Statistical analysis of peak intensities was performed using MetaboAnalyst 5.0 (Pang et al., 2021; Xia et al., 2009) and R version 4.1.1 (R Core Team, 2021). The total molecular features obtained were filtered by excluding those features with low variability across samples (based on the log10 fold changes between treatments with log10FC<2 removed) and the exclusion of low-intensity peaks (molecular features with a peak intensity of <50 in all conditions). Significant differences between

remaining molecular features by treatment were determined with univariate analysis (ANOVA) followed by post-hoc analysis (Fisher's LSD, p-value 0.05) using log10 transformed and weight-specific normalised data (Supplementary Table S3.4). The treatment comparisons for subsequent analysis of significant log2 fold changes in molecular features are as follows: Pathogen contrast "Pathogen vs Control" (change in expression of molecular features between the Control and Pathogen treatment), AMF contrast "AMF vs Control" (change in expression of features between the Control and the +AMF treatment), AMF/Pathogen contrast "AMF vs Pathogen" (change in expression of features between the Control and +AMF/Pathogen treatment). The shoot dry weight data were analysed with a one-way ANOVA followed by a Fisher's Least Significant Difference (LSD) and significance (P) with standard error of the difference (SED).

3.3 RESULTS

3.3.1 AM presence rescues growth reduction linked to *P. medicaginis* in var. Kyabra

Following the final harvest of the experiment, I measured the shoot dry mass of the two chickpea varieties under all tested conditions. Biomass has been established as a reliable proxy for plant fitness and as such the measured shoot biomass was used as such to subsequently present enhanced resistance (Younginger et al., 2017). Inoculation of the chickpea variety Kyabra with *P. medicaginis* resulted in a significant reduction in shoot biomass as compared to the same variety grown without disease pressure (Figure 3.2a; $p<0.05$). However, there was no significant difference in the aboveground biomass of Kyabra plants without pathogen inoculation and those plants pre-inoculated with AM fungal spores followed by pathogen challenge (AP condition; Figure 3,2a; $p>0.05$). Despite a similar disease resistance rating, the chickpea variety Sonali did not show significantly different shoot biomass in any condition tested (Figure 3.2b).

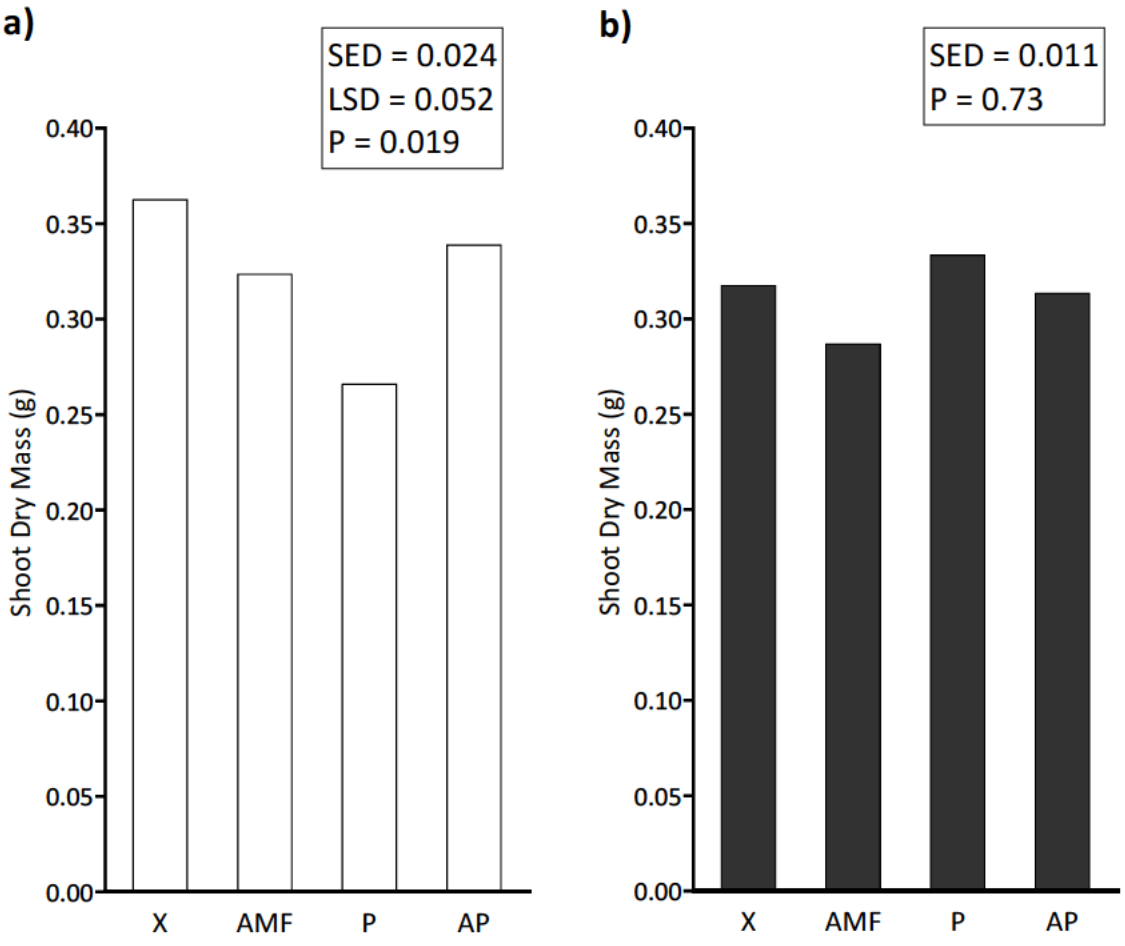


Figure 3. 2: *Plant aboveground biomass under different treatments.* ***a)*** *Chickpea var. Kyabra grown without microbial inoculation (X), with AM fungal inoculation only (AMF),* P. medicaginis *only (P), or both AM fungal inoculation and* P. medicaginis *(AP) with standard error of the difference (SED), Fisher's Least Significant Difference (LSD) and significance (P) based on a one-way ANOVA; n = 4-5 per treatment.* ***b)*** *The same treatments as in (a), but for chickpea var. Sonali.*

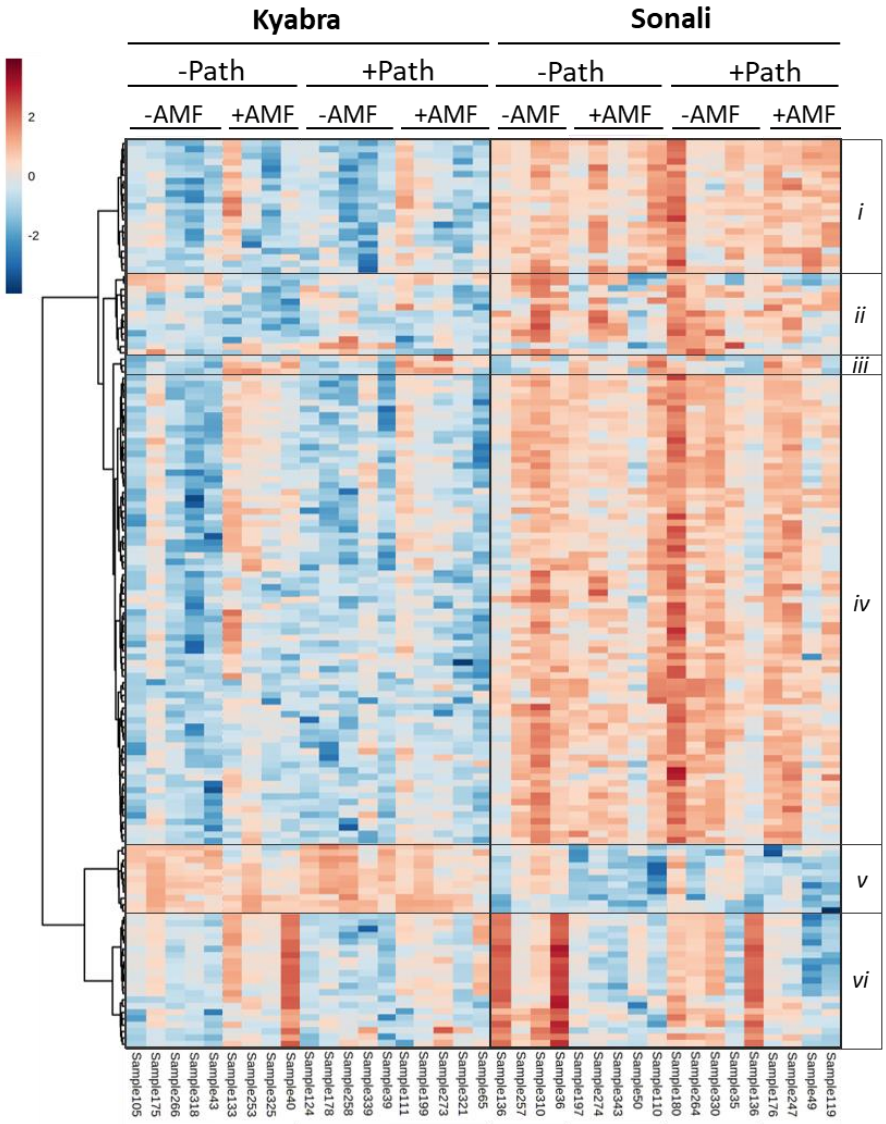


Figure 3. 3: *Hierarchical clustering of the normalised data points of all 143 significant features, detected in the two varieties Kyabra and Sonali with (+AMF) or without (-AMF) AM fungal inoculation and/or pathogen treatment with* Phytophthora medicaginis *(+/-Path) based on ANOVA (P<0.05) using log10 transformed and weight-specific normalised data. Euclidean distance measure and Ward method were used for clustering. Red values indicate high relative metabolite abundance while blue indicates low relative metabolite occurrence. Annotations on the right side (i-vi) indicate the differentiation of molecular feature groups. Blue indicates down-regulation, red up-regulation of metabolic features. Information regarding the ANOVA and post hoc results of each data point can be found in Supplementary Table S3.1*

3.3.2 Plant genotype drives metabolic differences

A total of 4,976 molecular features (hereafter called features) were detected in the root top tissue including both varieties and all treatments in positive ion mode. After filtering the entire set of detected features for those with log10 foldchanges >2 by sample treatment and with peak intensities >50, I identified a total of 1,037 features in the root top of both chickpea genotypes, with

143 of these exhibiting significant responses between at least two treatments based on one-way ANOVA with post-hoc analysis. Distinct differences can be observed in the metabolic profiles of Kyabra and Sonali with the majority of features having a higher abundance in Sonali as opposed to Kyabra. Using a hierarchically clustered heatmap presented in Figure 3.3, I classified the differentially abundant features into six groups (*i-vi*). Group *i* consists of a feature cluster exhibiting high overall normalised abundance in Sonali and overall low abundance in Kyabra. Group *ii* does not show any clear pattern while Group *iii* features showing a higher abundance of AM fungal colonised roots in both varieties. Group *iv* shows similar patterns to Group *i,* however, the differentiating feature is that Kyabra shows increased feature abundance in the +AMF treatments only – a response not observed in Sonali. In contrast, Group *v* includes highly abundant features that are not responding to any condition in Kyabra, but which are down-regulated in the +AMF conditions in Sonali. Group *vi* is characterised by AM fungal-inducible features in Kyabra that are AM fungal-repressed in Sonali. The obtained data from the negative ion mode (Supplementary Figure S3.1) revealed 300 features exhibiting significant differences among treatments with classification into three groups. This mode showed an overall higher expression of features in Sonali in group *i* and *iii* and the opposing trend in Kyabra in group *ii*.

3.3.3 Kyabra and Sonali respond in opposing manners to microbial inoculation

To further investigate the differences in how the two varieties responded to the different microbial inoculations, I undertook analyses of the activity of features in three treatment contrasts: the response to the pathogen alone (Figure 3.4a), to AM fungi alone (Figure 3.4b), and both treatments in combination (Figure 3.4c).

In response to the pathogen alone, twice as many significantly differentially expressed features were detected in Sonali as compared to Kyabra (Figure 3.4a). The relative proportion of up-/down-regulated features was similar in both varieties in the pathogen-only treatment (50% up and 50 % down, 41% up and 59% down in Kyabra and Sonali respectively, Supplementary Figure S3.3a). When the plant was only inoculated with AM fungi, opposing responses between the two varieties regarding the direction of feature responses were observed (Figure 3.4b); 82 features were found to be significantly up-regulated in Kyabra, but only two features in Sonali (Supplementary Table S3.2, Supplementary Figure S3.3b). The inverse trend was found for features that were significantly down-regulated with only 10 features showing a lower abundance in colonised Kyabra roots while there were 79 down-regulated features in Sonali (Figure 3.4b; Supplementary Table S3.1, Supplementary Figure S3.3b). When plants were exposed to both the AM fungus and the pathogen, the observed trend between the two varieties in feature abundance distribution remained consistent with that observed when inoculated with AM fungi alone. Kyabra exhibited 30% more responsive features as

compared to Sonali in the combined AMF and pathogen treatment, with most of them up-regulated in Kyabra while the majority were down-regulated in Sonali (Figure 3.4c). Combined AM fungus and pathogen inoculation resulted in 63 significantly up-regulated and 10 significantly down-regulated features in Kyabra while Sonali was found to only have five up-regulated and 46 down-regulated features (Supplementary Table S3.1, Supplementary Figure S3.3c). The negative ion mode revealed a higher number of compounds (Supplementary Table S3) and the same opposing trends (Supplementary Figure S3.2) in the treatments containing AM fungi. Taken together these results, which show that the root metabolic response to both treatments with AM fungal inoculation (+AMF, and +AMF plus pathogen) lead to similar responses that differ from the pathogen-only treatment, suggest that AM fungi play a significant role in shaping the root metabolome. By extension, the opposing responses in the two varieties under +AMF treatments along with the biomass observations, suggest that AM fungi may play a role in MIR.

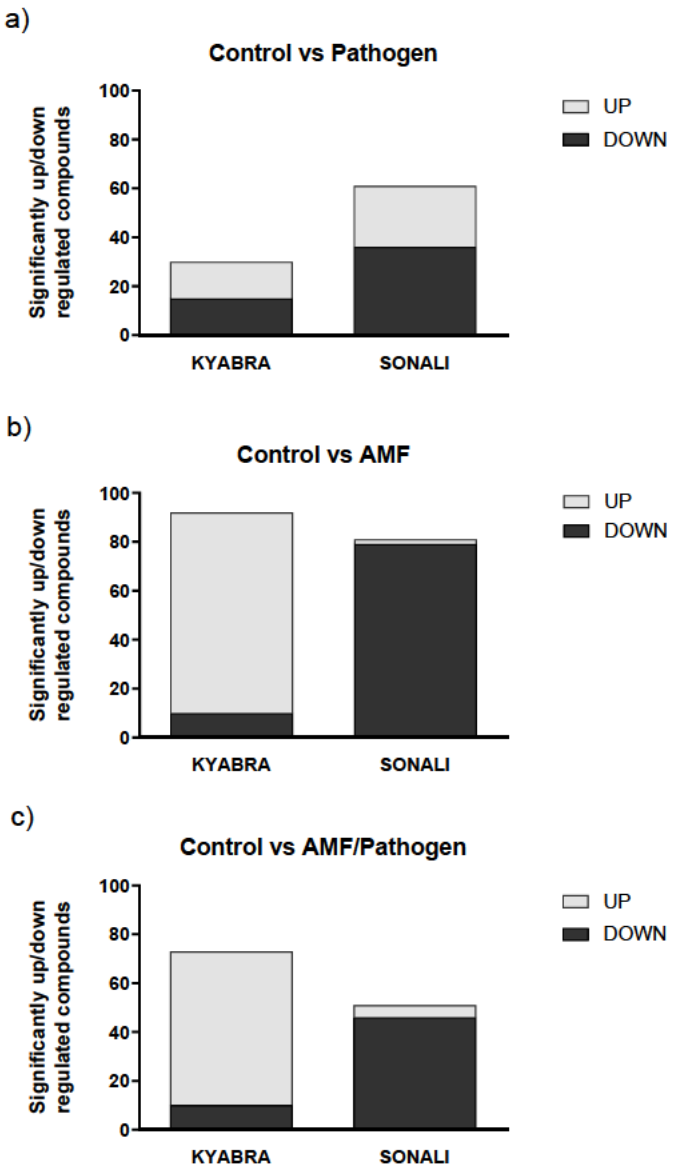


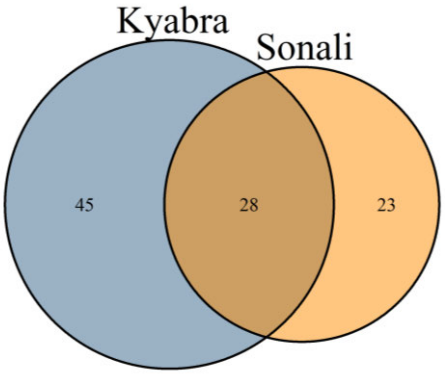
Figure 3. 4: *Analysis of the number of features in each of the three treatment contrasts.* ***A)*** *Pathogen contrast (Contr vs Path treatment)* ***b)*** *AMF contrast (Contr vs +AMF treatment)* ***c)*** *tripartite contrast of AMF/Pathogen (Control vs +AMF/Path treatment)*

3.3.4 Overlapping AM fungal-responsive features in Kyabra and Sonali suggest MIR features of interest

Further analysis of the features from the Control vs. AMF/Pathogen treatment contrast revealed that 96 features were exhibiting significant differences following microbial treatments. Of these features, 45 features were expressed in Kyabra only and 23 were specific to Sonali (Figure 3.5a). Shared between the two varieties were 28 features, many of which were opposingly expressed between the two varieties. As Kyabra exhibited improved growth when co-treated with AM fungi and *P. medicaginis,* features of interest associated with immune induction are likely to be found

within these 28 features. To gain better insight into the possible roles of these features, I attempted to classify them based on predicted structure. Group A was defined by features that were induced by AM fungal treatments in Kyabra but down-regulated in Sonali with opposite tendencies in the -AMF treatments. This group includes a wide range of features with the putative identifications of aromatic hydrocarbon, organophosphates, aromatic derivates, polyols, indole derivates, carboxyl acids, aromatic pyrimidine derivates, steroid derivates and some unknown features. Group *B* features in Kyabra showed increased abundance in AP conditions while Sonali was unresponsive. The two putative identifications in this group comprise a pyrethroid and one unknown feature. Group *C* is characterized by an overall higher expression of features in Kyabra. Both putatively identified features in this group have expression patterns that differ from all other groups. The polyol shows overall higher expression in Kyabra than Sonali and the peptide reveals increased abundance in the pathogen-only treatment for both varieties with slightly higher abundance in the +AMF treatment in Kyabra. Interestingly, high metabolite expression in all +AMF treatments could be observed in Kyabra and Sonali in Group *D*. The three features in this group consist of a carboxylic acid, a polyol and one unknown feature. Group *E* and *F* represent a set of features with overall higher expression in Sonali compared to Kyabra. The detected features include two carboxylic acids, two polyols, a 1-O-feruloyl-beta-D-glucose and three unknowns.

a)



b)

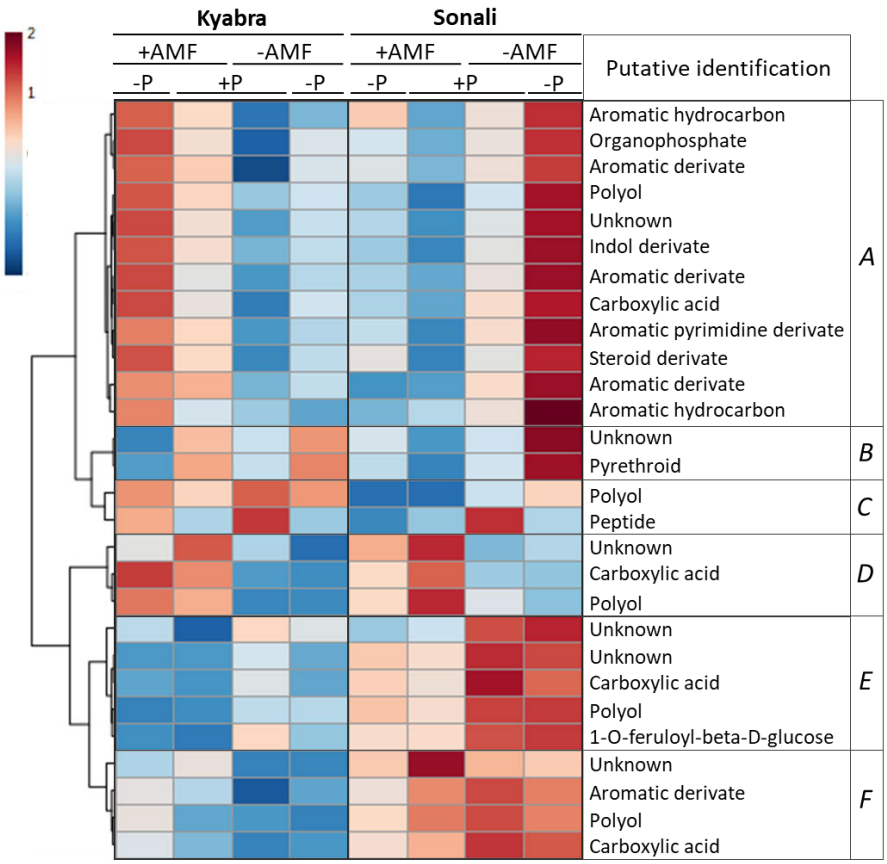


Figure 3. 5: a) *Venn diagram showing the overlapping features of Kyabra and Sonali in the +AMF vs. Pathogen treatment. ANOVA and post-hoc analysis, Fisher's LSD, (P<0.05)****. b)*** *Heatmap showing the relative abundance of each of the 28 overlapping features in both varieties in each treatment condition including the putative identification of each feature. Blue indicates down-regulation, red up-regulation of metabolic features. Information regarding the ANOVA and post-hoc results of each data point can be found in Supplementary Table S3.5.*

3.4 DISCUSSION

My study sought to investigate how the plant genotype impacts the ability of a single AM fungal species to initiate metabolic restructuring associated with MIR, by characterising the metabolomic changes in two chickpea varieties with different abilities to host the AM fungus *R. irregularis*. I observed strong opposing metabolomic responses in both chickpea varieties to microbial inoculation. While Kyabra showed elevated feature responsiveness to AM fungal colonisation, Sonali was more responsive to the pathogen treatment. Interestingly, Kyabra showed predominantly upregulated feature activity in the AM fungal treatments compared to predominantly downregulated feature activity in Sonali. Furthermore, I found several different classes of features reflecting potential compounds involved in MIR. While several excellent studies have investigated the metabolic changes in different plant species or genotypes upon pathogen infection or AM fungal colonisation (Salloum et al., 2019; Singh et al., 2004; Fernández et al., 2014), few studies combined these treatments and observations. This study provides insight into how different chickpea genotypes respond to AM fungal colonisation and/or pathogen infection and broadens the number of available metabolomic studies that investigate MIR by exploring the plant responses to the tripartite interaction with beneficial and pathogenic microbes (Salloum et al., 2019; Singh et al., 2004; Fernández et al., 2014).

Previous studies investigating root metabolomic profiles during AM fungal colonisation have found between ~30 and ~80 features responded to colonisation (Hill et al., 2018; Salloum et al., 2019; Goddard et al., 2021). I found a similar quantum of features altered by AM colonisation. It is noteworthy that previous studies on mycorrhized roots revealed relatively low metabolic responses to *Rhizophagus irregularis*, the species used in this study compared to other AM fungal species (Rivero et al., 2015; Mandal et al., 2010; Fernández et al., 2014). This may be why I observed only a slightly enhanced response to AM fungal symbiosis in the majority of features identified, a result that could change based on AM fungal species chosen as per previous research (Kaur et al., 2022). This present study also found a distinct separation of features in the roots of the two varieties tested, as hypothesized. Kyabra showed upregulated feature expression in certain sets of features in the presence of AM fungi as opposed to var. Sonali (Figure 3.2; Groups *iv, vi*). The fact that Kyabra exhibited an increased number of upregulated features compared to Sonali can have several possible explanations. First, it is likely to be related to the underlying genotypic differences between Kyabra and Sonali that may have influenced the metabolite profile patterns. Breeding for biotic stress resistance is an important and sustainable tool in chickpea production(H. Li et al., 2015). The identification of genetic resistance to nematodes and fungal pathogens in chickpea cultivars has been performed (Channale et al., 2023; Farahani et al., 2022; Li et al., 2017) and the varieties used

here have been investigated for their susceptibility to drought and microbes (Kaloki et al., 2019; Plett et al., 2021; Plett et al., 2016). Possible genetic differences between those varieties lead to changes in metabolite pattern and therefore MIR could be identified in the future. Past work has shown similar genotype-associated shifts of metabolite profiles in response to AM fungal colonisation in sorghum, soy, and pea genotypes (Salloum et al., 2019; Kaur et al., 2022; Singh et al., 2004) or in susceptible and resistant genotypes (Salloum et al., 2019; Salloum et al., 2018; Kumar et al., 2015; Fernández et al., 2014). However, my results may also partly be explained by the higher potential of Kyabra to form a symbiosis with AM fungi. Since I imposed a weak disease pressure by using a low concentration of pathogen and short exposure time, the minor/delayed observation of pathogen-related biomass losses in Sonali caused a limitation to this study. However, in line with my findings, previous studies found a correlation between decreased AM fungal colonisation and the downregulation of several metabolites (Salloum et al., 2018; Salloum et al., 2019). These observed varying changes in metabolite composition upon AM fungal colonisation in plants with different abilities to form such a symbiosis potentially reflect the link between mechanistic and biochemical change in plants that lead to MIR, although to confirm this it would be valuable to compare these responses across several pairs of varieties exhibiting differences in symbiotic formation.

Although metabolomic studies focusing on metabolic alterations in mycorrhized plants are becoming more available, most research has focused on the changes in the metabolite profile of AM fungi colonised plants versus non-colonised control plants. This study sought to give further insight into the metabolomic changes in plants during the tripartite interaction between a plant, an AM fungus, and an oomycete pathogen to identify features potentially involved in MIR. As only Kyabra showed evidence of AM fungi improving the outcome of pathogen resistance, due to decreased biomass losses, those induced/repressed by AM fungi in this variety as opposed to Sonali are likely to be the best candidates for a role in MIR. I discovered 28 features between the two varieties that were, overall, opposingly accumulated between the two varieties including features from the broader metabolite groups of polyols, carboxyl acids, and pyrethroids.

Polyols including inositol, ononitol, pinitol, sorbitol/mannitol, threitol and viburnitol have been found in AM fungal colonised plants (Goddard et al. 2021; Kaur et al. 2022; Salloum et al. 2019). Of these, certain (e.g. myo-inositol) are known to be involved in abiotic and biotic stress responses (Taji et al. 2006; Donahue et al. 2010; Chaouch & Noctor 2010; Hu et al. 2020). Combined with my results, which revealed one group of Polyols with higher expression under AM fungal treatments in Kyabra compared to Sonali (and opposing results in -AMF treatments), these findings depict the potential of AM fungi to alter the expression of polyols and thereby influence the defence responses of plants.

Several carboxylic acids have also been previously linked to plant disease resistance. Indole-3-carboxylic acid was shown to enhance disease resistance by increased callus accumulation (Gamir et al., 2018) and zelaic acid and hexanoic acid, are two well-known carboxylic acids involved in priming and enhanced defence responses in plants (Jung et al., 2009; Aranega-Bou et al., 2014; Djami-Tchatchou et al., 2017). In addition, the exogenous addition of carboxylic acid in Arabidopsis was shown to initiate transcriptomic changes related to biotic stress (Finkemeier et al., 2013). A metabolite with a putative pyrethroid-like structure was also identified as more highly induced in AP conditions; while this has a known role as an insecticide (Lybrand et al., 2020), it is possible that compounds with this structure may also have a role in defence against oomycetes. These results confirmed my second hypothesis that chickpea var. Kyabra would differentiate from var. Sonali based on defence-related features, and I can further hypothesise that some of these detected features have the potential to be involved in MIR.

I putatively identified one of the features as 1-*O*-feruloyl-β-D-glucose, which showed high overall expression in Sonali but not much difference in accumulation in Kyabra. The minimal influence on biomass loss in the pathogen inoculated +AM fungal treatment in Kyabra, which implies a stronger induction of defence response-related metabolic features in this treatment. In this treatment combination, downregulation of this feature was observed in Kyabra compared to Sonali suggesting that it was not associated with MIR in chickpea. This was surprising as this compound is a phenolic glucoside derived from ferulic acid (National Center for Biotechnology Information, 2022) and has previously been identified as a resistant related metabolite in potatoes infected with *Phytophthora infestans* (Materska & Perucka, 2005; Pushpa et al., 2014). 1-*O*-Feruloyl-β-D-glucose belongs to the class of phenylpropanoids, which is a pathway well-known to be involved in defence responses (Yadav et al., 2020). Metabolite members of this pathway, like coumarins or anthocyanins and their glycosylated form, were previously shown to be enhanced upon AM fungal colonisation (Iula et al., 2021). Moreover, 1--*O*-feruloyl-β-D-glucose is also involved in the production of lignin, which is well-known to play a role in the plant defence against pathogens (Vanholme et al., 2010; Lee et al., 2019; Bhuiyan et al., 2009). Therefore, this feature requires future investigation in the context of pathogenesis in chickpea.

Overall, my findings indicate that certain *P. medicaginis* susceptible chickpea varieties can benefit differently from an AM fungi-mediated metabolomic response; based on a two-variety comparison, my results would suggest that chickpea varieties with enhanced abilities to host AM fungi colonisation benefit from MIR. Therefore, my work indicates the importance of examining the metabolic responses of individual plant varieties related to AM fungal colonisation to identify varieties with a higher potential to benefit from MIR. I showed the influence of AM fungi on the

metabolic landscape in chickpea varieties susceptible to pathogens. Understanding the mechanisms linked to improved resistance, mediated by these beneficial symbionts, can help to effectively identify and select varieties featuring such responses. Despite the uncertainty concerning the specific identity and origin of features identified here, I found several significant metabolite classes that were previously connected to plant defences and therefore reflect MIR properties. These metabolite classes might be crucial in understanding MIR and how plants benefit from AM fungi in response to pathogens. Further in-depth studies of the detected features are needed to reveal the mechanistic role of these molecular features in plant defence response. Targeted investigations of metabolite classes in different varieties would help to understand involved pathways. These results would support future work identifying plant genotypes that benefit from soil-beneficial microbes to repress disease outbreaks more sustainably, thereby supporting future crop productivity.

CHAPTER FOUR

Arbuscular mycorrhizal fungal mixtures affect the response of tomato to *Rhizoctonia solani* non-additively

This chapter is being prepared for submission to "New Phytologist" journal. I led all aspects of the research including study design, execution and writing, except for the statistical modelling of plant phenotypes, which was performed Chris Brien (APPF) after consultation with me to determine the model structure and required outputs.

4.1 INTRODUCTION

Approximately 80 % of flowering plants form a symbiosis with ~300 species of arbuscular mycorrhizal fungi (AM fungi), which can enhance nutrient uptake and growth by plants in exchange for carbon (Smith & Read 2008; Schüßler & Walker 2010; Spatafora et al. 2016; Jung et al. 2012). In addition, this symbiosis can increase the resistance of plants to pathogens, a phenomenon named Mycorrhiza Induced Resistance (MIR) (Pozo & Azcón-Aguilar, 2007). Effects of MIR have been frequently observed in a variety of plant species (Dowarah et al., 2021) and several mechanisms have been suggested to contribute to this enhanced resistance, including a variety of physical and molecular processes (Kadam et al., 2020; Ghorbanpour et al., 2018). As a result, mycorrhized plants can therefore exhibit decreased disease symptoms and biomass losses upon pathogen infection (Pozo et al., 2002; Gernns et al., 2001; Wang et al., 2018). However, the beneficial effects on plants can vary greatly, depending on the AM fungal species (Wehner et al., 2010).

In recent years, several studies revealed the varying ability among AM fungal species to mediate enhanced growth or defence responses to plants (Malik et al., 2016; Sikes et al., 2009; Wehner et al., 2010). Most AM fungi belong to the subphylum of Glomeromycotina (Schüßler & Walker, 2010; Spatafora et al., 2016a) with species from the Glomeraceae found to be more effective in protecting plants against pathogens, while species from the Gigasporaceae were more effective in enhancing host growth (Sikes et al., 2009; Sikes et al. 2010; Maherali & Klironomos, 2007). Dowarah *et al*. reviewed the ability of AM fungi to enhance plant protection from biotic stressors including pathogenic fungi, bacteria and viruses in many different plant species, predominantly mentioning *Glomus* species (Dowarah et al., 2021). For example, several Glomus species were observed to decrease disease symptoms and enhance systemic resistance in tomato roots upon *Alternaria solani* infection (Liu et al., 2007; Song et al., 2015). Furthermore, an in-vitro study including the AM fungus *Rhizophagus irregularis*, from the Glomeraceae, delayed disease symptoms in tomato against

tomato bacterial wilt (Chave et al., 2017) and *R. irregularis* decreased disease severity against Fusarium wilt in tomato (Akköprü & Demir, 2005). *Glomus mosseae* was also observed to enhance the resistance of tomato plants to *Rhizoctonia solani* in glasshouse and field conditions while simultaneously enhancing growth (Kareem & Hassan, 2014). Therefore, there is a potential for the majority of AM fungal species, specifically members of the Glomeraceae, to support plant growth and defence.

Previous studies have highlighted the varying potential of AM fungi to protect plant hosts from disease, from no effect to systemic protection, which can be found when comparing different fungal species and even isolates (Pozo et al., 2002; Gernns et al., 2001; Peña et al., 2020). It is crucial to mention that the rhizosphere of both natural and agricultural settings typically comprises complex communities rather than individual AM fungal species (Alimi et al., 2021; Mendes et al., 2013). While single-species observations led to varying results regarding MIR, fewer studies have been performed using AM fungal communities. Of these latter studies, AM fungal communities could show beneficial effects on growth as well as defence against pathogens (Hafez et al. 2013; Al-Askar & Rashad 2010; Jaiti et al. 2007). For example, tomato plants inoculated with a mix of 26 AM fungal species were more resistant to *Verticillium dahlia* and also exhibited enhanced growth response (Rahou et al., 2021). Therefore, it is hypothesised that AM fungal communities have additive or even synergistic beneficial effects on MIR. However, previous work has focused on endpoint observations rather than taking continuous measurements that, thus, do not reflect AM-associated growth or MIR effects that might appear at earlier plant growth stages. This lack of data impedes the best use of AM fungal communities to confer disease resistance in plants.

Another topic that still lacks sufficient investigations is the potential for trade-offs between the allocation of resources to growth or defence, or even the uncoupling of these two systems, in mycorrhized plants requires further study. For instance, mycorrhizal colonisation with carbon-demanding AM fungal species or with species that triggered rapid plant growth could leave the plant immune system with fewer resources to protect against pathogen attack, or vice versa. Compromises in the nutrient allocation for growth benefits in favour of disease resistance mechanisms are possible (Jacott et al., 2017). Distinct molecular mechanisms in AM fungal species could lead to either enhanced growth or enhanced defence and potentially could lead to trade-offs between these activities. Previous studies showed that even though *G. mosseae* did not result in increased growth performance under unstressed conditions, increased protection still took place upon *P. nicotianae* infection (Trotta et al. 1996), which could be explained if slower-growing AM fungal colonised plants are more likely to be primed to resist pathogen activity. Further research targeting resource allocation is needed to elucidate the role of AM fungi in this context. To

investigate the functional diversity of AM fungi on plant growth and defence, I performed two experiments on inoculated tomato plants (*Solanum lycopersicum* cv. Micro-Tom), representing a model species for Solanaceous crop plants. For the first experiment, to characterise plant growth-related phenotypic variation among AM symbioses in the presence and absence of a root pathogen, I inoculated plants with one of four species from two families in the Glomeromycotina (*Rhizophagus irregularis* and *Glomus mosseae* [formally known as *Funniliformis mosseae*] in the Glomeraceae, *Gigaspora margarita* and *Scutellospora calospora* in the Gigasporaceae). In order to test for within-species variation in growth and defence, two isolates of *Rhizophagus irregularis* (previously known as *Glomus intraradices*) were also included. Moreover, to test the theory of additive AM fungal mixture effects reflecting more authentic natural circumstances, I also inoculated tomato plants with one of five species combinations that represent a range of levels of taxonomic and phylogenetic diversity in the presence and absence of *Rhizoctonia solani,* a devastating root pathogen that leads to root rot and damping off in a broad host range including important crop plants (Adams, 1988; Sneh et al., 1996; Sumalatha et al., 2018).

Given that previous literature already reported a large variety of plant responses to different AM fungal species, I hypothesised that *a*) the use of different single AM fungal taxa would lead to varying levels of MIR and that these varying levels would be correlated to growth-related trade-offs in the plant and *b*) combinations of different AM fungal species in the soil would lead to altered changes in defence and connected growth alterations correlated to the outcome of the single species experiment in an additive/synergistic/antagonistic manner. My results indicate that most single AM species enhanced plant growth and three out of five single AM fungal species showed MIR-effective potential during symbiosis with tomatoes. I show unexpected non-additive effects in AM fungal mixture treatments with respect to host growth and MIR. Phenotypic observations taken over the timecourse of the experiment extend the available information on AM fungal functional diversity.

4.2 Materials and Methods

4.2.1 Experimental design

A resolved, latinized row–column experimental design was conducted including 24 conditions (including all AM fungal species treatments and +/- pathogen) with six replicates for each condition for a total of 144 experimental units (Figure 4.1a). The experimental design contained five AM fungal single-species treatments (Table 4.1) and six AM fungal mixture treatments (Table 4.2) as well as the non-mycorrhizal control treatment, which is illustrated in Figure 4.1b-e to demonstrate its implementation. 6 Lanes × 26 Positions in a greenhouse, with each block occupying 2 Lanes ×13

Positions, were used for the experiment. The design was generated using the R package od (Butler, 2020) and randomized using the package dae (Brien, 2021b; R Core Team, 2021).

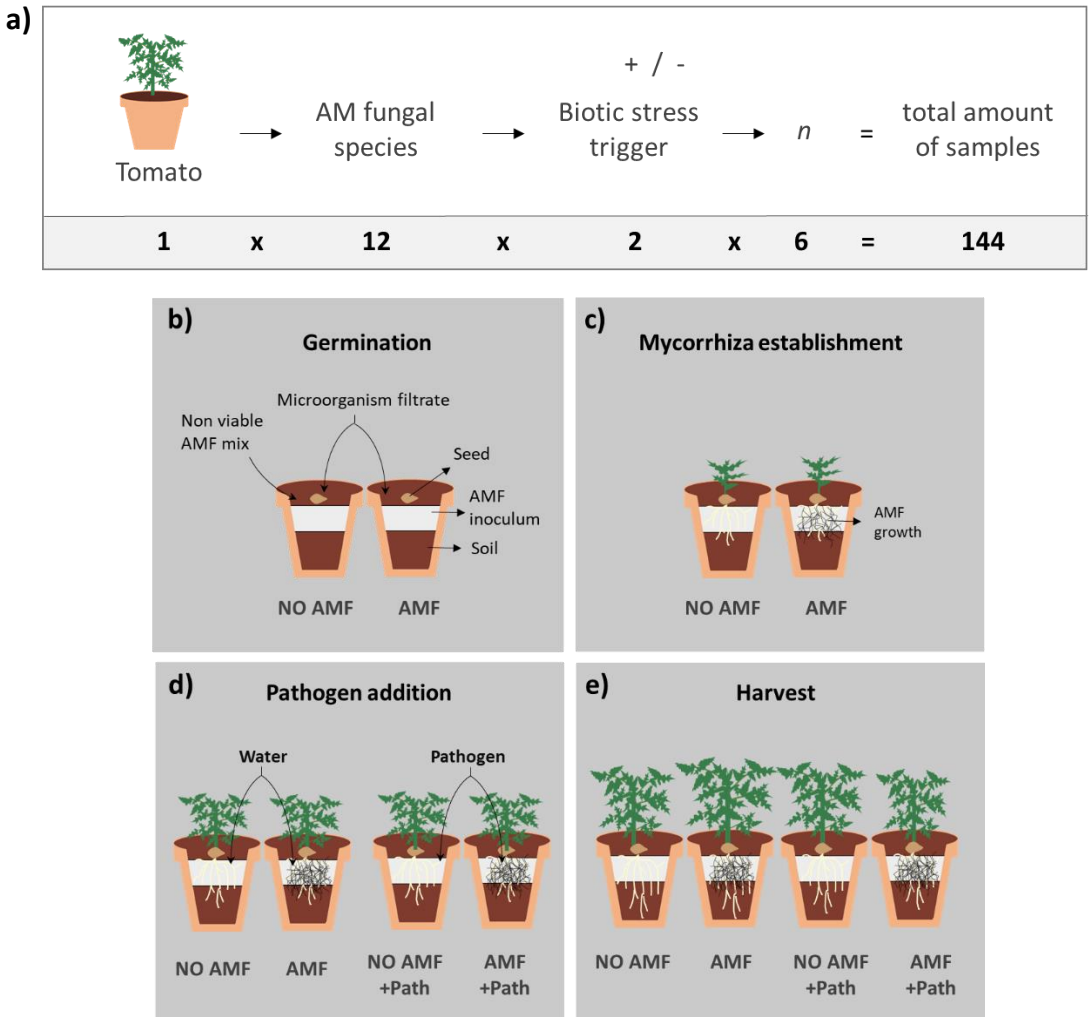


Figure 4. 1: *a) Experimental scheme. Six replicates each of tomato (*Solanum lycopersicum *cv. Micro-Tom) treated with all AM fungal species treatments with and without pathogen addition with a total number of 144 samples for the whole experiment.* **b)** Pots with soil and viable or non-viable AM fungal inoculum, **c)** mycorrhiza establishment to allow plant growth and formation of AM fungal symbiosis, **d)** Pathogen addition to create pathogen treatments and water addition for mock treatments, **e)** Harvest at the endpoint of the experiment with developed plants and AM fungal symbiosis with tissue regions harvested in all treatments,

4.2.2 AM fungal inoculum and microbial wash

AM fungal inoculum was prepared for each experiment using five isolates (*Glomus mosseae* WFVAM 45, *Rhizophagus irregularis* DAOM 10, WFVAM 21, *Gigaspora margarita* WFVAM 21, *Scutellospora calospora* WFVAM 35, *Rhizophagus irregularis* WFVAM 23) cultured in pots with *Plantago lanceolata,* a high colonising host. These bulk cultures contained sterile sand:soil (1:9), perlite and ~10% inoculum. Inoculum bulking was performed in two stages for 2.5 months each, after which root colonisation was

assessed to be at least 30% of root length to ensure colonisation in the final experiment. The whole inoculum including root fragments and spores was used for the experiment. This microbial wash, to integrate communities of other microorganisms, was obtained by mixing 1 kg of clay loam from Lewiston, South Australia (details section 4.2.4) that was filled up to 4 L with RO water. The mix was stirred and filtered through a stack of sieves (3.5mm, 125 µm, 90 µm, 38 µm) to incorporate microbes but to exclude AM fungal spores.

4.2.3 Pathogen growth

A culture of the pathogen *Rhizoctonia solani* was obtained from the NSW Plant Pathology & Mycology Herbarium culture collection (Orange, New South Wales, Australia). Originally isolated from *Lycopersicon esculentum* 'UC84' in 1988 (Supplementary Table S4.1), the isolate was sub-cultured on Potatoe dextrose Agar (Sigma-Aldrich) and its identity was verified by amplifying DNA using the primer set ITS1F forward (5'- TCCGTAGGTGAACCTGCGG -3'), ITS4 reverse (5'-TCC TCC GCT TAT TGA TAT GC-3'), followed by and sanger sequencing and alignment using BLAST (Supplementary Figure S4.2) (White et al., 1990; Brierley et al. 2016; Wallon et al. 2021). The pathogen was inoculated on plants of *Solanum lycopersicum* cv. Micro-Tom on soil and *in vitro* on full-strength Murashige and Skoog media (Caisson Laboratories) supplemented with 0.1% Gamborg's vitamin solution (Sigma-Aldrich) and 1% sucrose, to test for pathogenicity. After two weeks the plant showed symptoms of damping off, thus verifying pathogenicity. Plant material from the top region of the adventitious roots and the lower stem was collected from the *in-vitro* system and re-cultured on Potato dextrose Agar plates at room temperature. Ten pieces of 5x5 mm fungal plugs from this culture were added to each of eight 2-L flasks containing 0.8 L sterile Potato Dextrose Broth (Sigma-Aldrich). This liquid culture was incubated for 13 days at room temperature, with orbital shaking at 150 rpm. The pathogen biomass was harvested by pouring the solution on filter paper. The biomass was diluted at a rate of 2% w/v and homogenised with a stick blender in RO water (160g for 8L).

4.2.4 Soil and pot preparation

A mix of autoclaved clay loam from Lewiston, South Australia (pH 8.29, 0.34% organic carbon, 7.1 mg/kg nitrate, 1 mg/kg Ammonium, 11 mg/kg Colwell Phosphorus) and steam sterilised N40 sand in a ratio of 1:9 soil/sand (w/w) was prepared. To reintroduce a standardised mixture of microbes (without AM fungal spores) into the sterilised soil used in the experiment, a microbial wash generated as described above was added to all pots. 2550 mL of the microbial wash was mixed with 51 kg of autoclaved clay loam, mixed and stored in a clean, cool place and mixed daily for 7 days. To

promote mycorrhizal colonisation, 20 mg P kg^{-1} in the form of calcium phosphate dibasic ($CaHPO_4$)(87 mg/kg) was added to this soil/sand mix (Watts-Williams & Cavagnaro, 2012). Additionally, 100 ml of perlite/pot was added to this substrate to loosen the soil structure. The substrate for nonmycorrhizal control pots was established using a mock inoculum prepared from a mixture of inocula from the five isolates in equal parts, autoclaved twice at 121°C for 30min each and added to pots in the same amount as the live treatments. The pots were filled up to ¾ with 990 g of this substrate. The last ¼ of each pot was filled with 330 g/pot of the same substrate that contained 65 g of starting inoculum per pot (different mycorrhizal single species, combinations, or mock inoculum). Each pot contained 1320 g of total substrate including the inocula. PVC grip meshes were placed around the stem on top of the soil surface to reduce evaporation from the soil.

Table 4. 1: *Name and information of single species treatments*

Single AM fungal treatments	
Treatment Name	**Mycorrhiza isolates**
R. irr 10	*Rhizophagus irregularis* DAOM 10
G. marg	*Gigaspora margarita* WFVAM 21
G. moss	*Glomus mosseae* WFVAM 45
S. calo	*Scutellospora calospora* WFVAM 35
R. irr 23	*Rhizophagus irregularis* WFVAM 23
Contr	*No AMF*

Table 4. 2: *Name and information of mixture treatments*

Mixture treatments		
Treatment	**Mycorrhiza species**	**Rationale**
moss+irr10	*Glomus mosseae* *Rhizophagus irregularis* DAOM 10	Best MIR candidates (Maherali & Klironomos, 2007; Sikes et al., 2009)
marg+calo	*Gigaspora margarita* *Scutellospora calospora*	Least MIR candidates (Maherali & Klironomos, 2007; Sikes et al., 2009)
moss+marg	*Glomus mosseae* *Gigaspora margarita*	MIR + growth (Maherali & Klironomos, 2007; Sikes et al., 2009)
calo+moss	*Scutellospora calospora* *Glomus mosseae*	Strong colonisers (Abbott & Robson, 1985; Dickson et al., 1999)
irr10+irr23	*Rhizophagus irregularis* DAOM 10 *Rhizophagus irregularis WFVAM 23*	Two isolates of the same species
All	*Glomus mosseae* *Gigaspora margarita* *Scutellospora calospora* *Rhizophagus irregularis DAOM 10* *Rhizophagus irregularis WFVAM 23*	High diversity (Wehner et al., 2010; Frew et al., 2022)

4.2.5 Plant material growth conditions and timeline

Seeds of tomato (*Solanum lycopersicum* cv. Micro-Tom) were sterilised with 70% ethanol for two minutes, then 1% NaCl solution for twenty minutes, followed by three washes with sterile water. Two batches of seeds germinated for either one or two weeks in sterile sand. Seedlings of both batches were transferred evenly in each treatment and greenhouse conditions were kept at 26°C/18°C day/night for a daily 12h/12h day/night period, and an average humidity of 50%. LED light was provided at 350 µmole/m^2/s to ensure stable light conditions. Plants were watered-to-weight daily with 70% water holding capacity, and fertilized every week starting one week after transfer with 10 ml/pot Long Ashton solution without nitrogen and phosphorus (conducted until the pathogen inoculation), containing 2 ml K_2SO_4, 1.5 mM $MgSO_4 \cdot H_2O$, 3 mM $CaCl_2 \cdot H_2O$, 0.1 mM FeEDTA of 1 M stock solutions and 1 ml Micronutrients (2,86 g l^{-1} H_3BO_3, 1,81 g l^{-1} $MnCl_2 \cdot 4H_2O$, 0,22 g l^{-1} $ZnSo_4 \cdot 7H_2O$, 0,08 g l^{-1} $CuSO_4 \cdot 5H_2O$, 0,025 g l^{-1} $Na_2MoO_4 \cdot 2H_2O$) (Cavagnaro, Smith, et al., 2001). After germination, the plants were transplanted in the pots with drainage (height: 18 cm, width at base: 6.5 cm, width at top 0- 8.5 cm). Small pots were added into bigger pots for better stability on the conveyor belt: height – 19.5 cm, diameter at base: 12 cm, diameter at top – 14.5 cm without drainage holes) containing the different treatments. The mycorrhizal establishment was enabled for five weeks before the pathogen addition occurred five weeks after transplanting and one week after the start of imaging (see below) by adding 80 mL of the homogenised fungal mixture per pot. Control received 80 mL RO water.

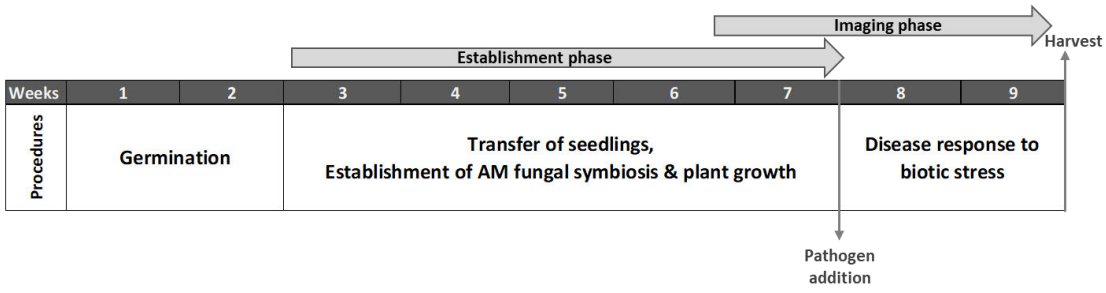


Figure 4. 2: *Experimental timeline including germination phase, AM fungal establishment and plant growth phase, plant response and imaging phase with endpoint harvest.*

4.2.6 Imaging

Non-destructive red-green-blue (RGB) images were taken daily for three weeks with Allied Vision cameras by the Scanalyzer 3D system (LemnaTec GmbH, Aachen, Germany), starting four weeks after transplanting for a total of three weeks (21 to 43 days after planting inclusive). Images were taken from a top view and two side views (Supplementary Figure S4.2) with the Scanalyzer 3D

system (LemnaTec GmbH, Aachen, Germany). These images were used to calculate the projected shoot area (sPSA) as the sum of the areas, measured in kilo pixels, and absolute growth rate (sAGR) (Al-Tamimi et al., 2016; Neilson et al., 2015).

4.2.7 Plant harvest, root staining and quantification of AM fungal colonisation

The endpoint harvest was initiated after the completion of plant growth phase observations (44d after transplanting) and carried out over two days. The plants were removed from the pots in the same order as the experimental design and separated from the soil and the roots were carefully washed with tap water. After removing the excess water the weight was recorded, and around 0.5 g of root tissue was collected for the root staining analysis. The tissue was stored in embedding cassettes (Simport Plastics M518-2) in 70% ethanol until further processing. The shoots were removed from the roots and the plant's fresh weight was recorded. The remaining plant material (roots and shoots) was snap-frozen and then freeze-dried using a Freeze-dryer Alpha 1-2 LDplus, Christ (Osterode, Germany).

To stain for AM fungal structures, cassettes with the root tissue were removed from the ethanol and rinsed with cold running tap water for 5 minutes. The cassettes were then placed in a clearing solution (10% potassium hydroxide) and incubated at room temperature for four days. The clearing solution was removed and the cassettes were washed for 10 minutes with cold tap water. Afterwards, the roots were stained in 5% v:v ink-vinegar solution (Parker Quink Ink, permanent black) at 90°C for 10 minutes, removed from the solution and rinsed with water until the water cleared. The roots were stored in lactoglycerol (1:1:1 lactic acid:glycerol:water) until further processing. The percentage of sections containing AM fungal structures (hyphae, vesicles, arbuscles) shown in Figure 4.5 c-d was calculated in per cent of root length colonisation and was evaluated by counting 50 different root sections with sections from at least three root fragments, each of length 3cm.

4.2.8 Verification of pathogen infection

Due to the experimental setting including timing of pathogen addition and timely harvest, only a subset of samples treated with *R. solani* already displayed visible disease symptoms. From pots with pathogen addition and clear visible discolouration as well as no pathogen control conditions root and/or stem tissue samples (0.5 cm) were collected and stored at 4°C. The tissue samples were then surface sterilized with 2% NaClO for two minutes, washed with sterile MiliQ water three times, dry blotted on sterile filter paper, cut into small pieces, and added on Potato Dextrose Agar (PDA) + PDA 50 ug/ml Streptomycin to test for pathogen growth. The plates were incubated at room temperature for 9 days until fungal growth with characteristics of *R. solani* was observed (Supplementary Table

S4.2, Supplementary Figure S4.3). Both pathogen-free samples displayed no growth, while three out of five samples with pathogen addition showed fungal growth on agar plates. Only one sample (All) showed no growth, possibly due to a limited number of suitable samples and a sampling error. The collected tissue area may have lacked pathogenic biomass. In the *R. irregularis* 10 + 23 treatment, growth occurred on one plate but not on the other, reinforcing the concept of randomised sample error.

4.2.9 Data preparation

The imaging data were prepared using the SET method described by (Brien et al., 2020) using the R package growthPheno (Brien, 2021c). The PSA AGR were calculated from the PSA values by differencing consecutive PSA and ln(PSA) values, respectively, and dividing by the time differences. Mild smoothing on the logarithmic scale (smoothing df set to 6) was applied to the raw PSA data. Using the smoothed PSA (sPSA), the smoothed growth rates sPSA AGR were computed analogously to PSA AGR. To investigate growth over time, the imaging period was divided into intervals with days after planting (DAP) endpoints of 21, 28, 34, 39 and 43, corresponding to the timing of pathogen inoculation (day 28) as well as general growth patterns described here. A total of 13 traits were analysed as single-day responses: sPSA for DAP 21, 28, 34, 39 and 43; and interval responses: sPSA AGR for each of the DAP intervals 21–28, 28–34, 34–39 and 39–43. Two plants were removed from the analysis (one plant died and one plant exhibited dwarf growth throughout the experiment compared to all other plants in the experiment by 80%).

4.2.10 Statistical analysis

To produce phenotypic predictions, each trait from single-day responses and interval responses (see 4.2.9) was analysed using the R packages ASReml-R (Brien, 2021a) and asremlPlus (Brien, 2021a) to fit a linear model, starting with the following maximal model: $\mathbf{y} = \mathbf{X}_t\boldsymbol{\tau} + \mathbf{X}_s\boldsymbol{\beta} + \mathbf{e}$, where y is the response vector of values for the trait being analysed; $\boldsymbol{\tau}$ is the vector of effects of interest; $\boldsymbol{\beta}$ is the vector of spatial effects; the matrices $\mathbf{X}_t$ and $\mathbf{X}_s$ are the design matrices for the corresponding effects; and e is the vector of residual effects. The vector $\boldsymbol{\tau}$ of fixed effects of interest is partitioned as [μ τMyc τPath τMyc:Path], where (i) μ is the overall mean for the experiment, (ii) τMyc allows for consistent differences between Mycorrhiza treatments, (iii) τ Path allows for consistent differences between the pathogen treatments, and (iv) τ Myc:Path allows for differential response to the pathogen (i.e. interaction). The vector $\boldsymbol{\beta}$ of spatial effects is partitioned as [μ βBlock βSide:xPosn], where (i) βBlock allows for differences between Blocks and (ii) βSide:xPosn allows for a linear east–west trend by

Position that differs between the eastern and western Sides of the Smarthouse. The residual effects are assumed to be normally distributed with variance $\sigma 2$. Residual-versus-fitted values plots and normal probability plots of the residuals were inspected to check that the assumptions underlying the analyses were met. The residual plots were satisfactory for all traits, indicating that the selected models appear to be appropriate. For each trait, a Wald F-test with $\alpha = 0.10$ is conducted for the interaction effect; if this is not statistically significant, similar tests are then performed for the main effects of Mycorrhiza and Stress. For each trait, a chosen model was identified based on statistical significance at the $\alpha = 0.10$ level. Estimated marginal means (Searle et al., 1980) were obtained for the full interaction model for all combinations of Mycorrhiza and Stress. These means were then used to calculate the estimated marginal means, for the same combinations, that conform to the chosen model. The least significant differences for $\alpha = 0.05$ [LSD(5%)] were calculated to determine the significance of pairwise differences between predicted values.

The biomass comparison and the linear regression were conducted with a type II ANOVA using the lm function from the car package in R (R Core Team, 2021). The two-way ANOVA tests for the colonisation data followed by multiple comparisons (Dunnett's for comparison of a treatment to the nonmycorrhizal control and Tukey's for comparison among multiple treatments) were conducted using GraphPad Prism 9.4.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

4.3 RESULTS

4.3.1 Projections from images accurately represent plant biomass responses

To ensure that temporal patterns in the projected shoot area are representative of the measured shoot biomass patterns and that these are comparable across treatments, the relationship between the projected shoot area on the final date of imaging and shoot dry weight (Supplementary Figure S4.4a,b) at harvest was assessed and found a this to be positively correlated (adjusted R_2= 0.73). A strong positive correlation between PSA and shoot dry weight was also observed (main effect: $F_{1,101}$=168.5, P<0.001) (Supplementary Table S4.3, Figure S4.6). This was consistent across both unstressed and stressed conditions (main effect: $F_{1,101}$=0.9, P=0.35, interaction term: $F_{1,101}$=0.1, P=0.75) and all inoculation treatments (main effect: $F_{12,101}$=1.0, P=0.46, interaction term: $F_{12,101}$=1.5, P=0.15). Therefore, PSA was used as a proxy of shoot biomass in all subsequent analyses.

4.3.2 Phenotypic responses reveal variation in enhanced growth responses within single species

Accelerating growth was observed for DAP 21–28, and then the growth rate decreased but plants continued to grow for DAP 28–34. The growth rate tended to be constant for some plants and to continue to decrease for others for DAP 34–39. The decreased growth rate was observed for most plants in DAP 39–43, although the magnitude of the decrease varied. In plants inoculated with single AM fungal species, analysis of projected shoot area and absolute growth rate revealed AM fungal-dependent and species-specific responses in tomato (Figure 4.3). Most of the single-isolate AM inoculation treatments resulted in improved growth when compared to the nonmycorrhizal control. Biomass of *G. mosseae*-inoculated plants showed the strongest growth effect with significantly greater size than control plants after 34 days of growth due to a higher growth rate in all but the last growth phase. Plants inoculated with *G. margarita* were significantly larger than controls by the end of monitoring but not earlier, due to overall increased growth rates in the later phase of the experiment. Inoculation with either of the *R. irregularis* isolates resulted in marginally nonsignificant trends toward increased biomass relative to controls due to the increases in growth rate being small and short-lived. Those inoculated with *S. calospora* exhibited growth patterns that were similar to those observed in the nonmycorrhizal controls. Therefore, these results allow the classification of the single species treatments from the most to least effective growth-enhancing species starting with *G. mosseae*, followed by *G. margarita, R. irregularis* 10, *R. irregularis* 23, and *S. calospora*.

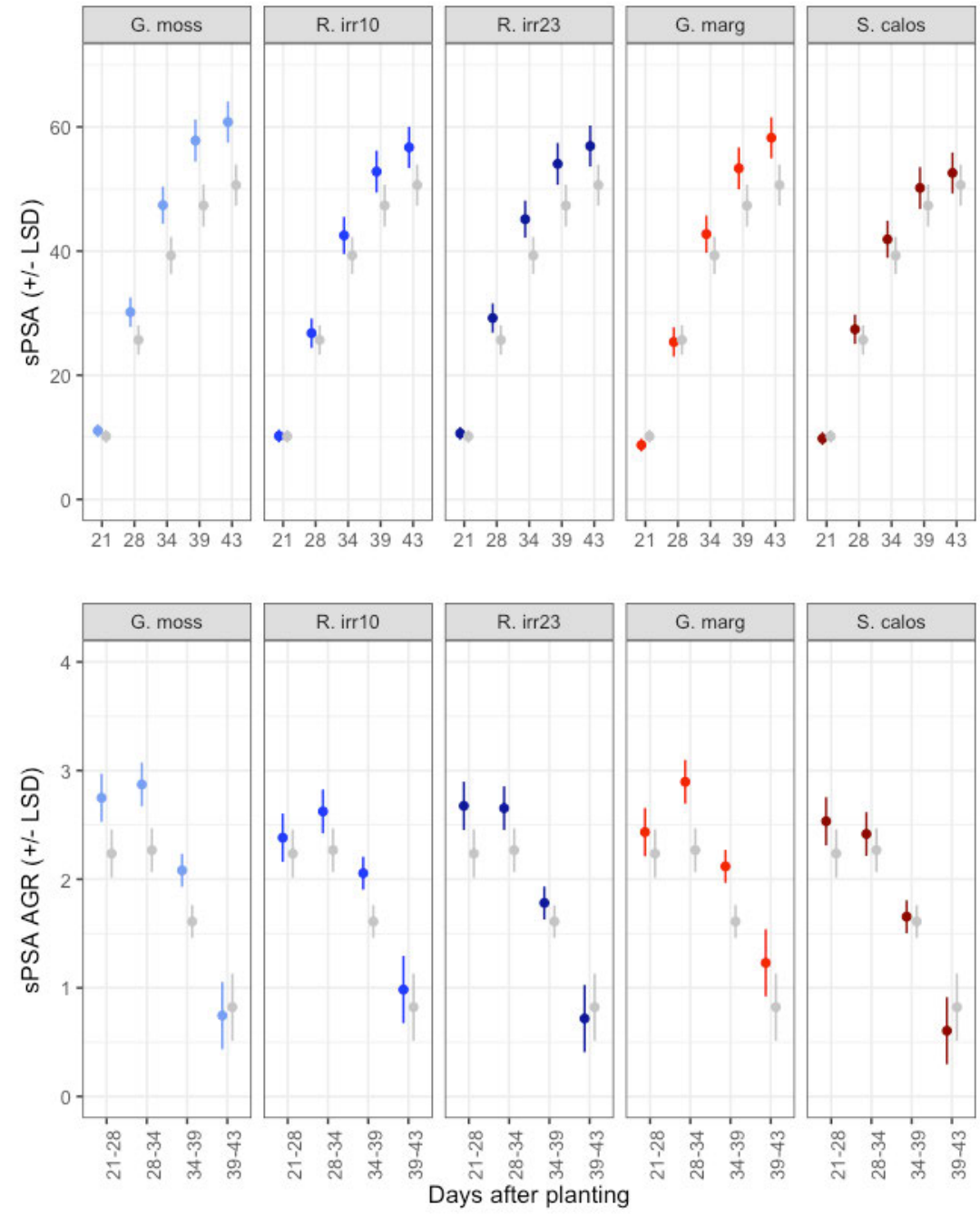


Figure 4. 3: *Temporal effects of inoculation by one of five AM fungal isolates (blue-scale = Glomeraceae, red-scale = Gigasporaceae) on plant biomass and growth rate. Points represent predictions from the mixed model for the displayed time/time window and within each inoculation treatment; error bars represent least significant differences (LSD) predictions from the model. Values for the nonmycorrhizal control treatment (grey) are repeated across each panel for ease of comparison. sPSA = Projected Shoot Area, sPSA AGR = Absolute growth rate.*

4.3.3 Non-additive growth responses predominate in AM fungal communities when compared to single-species inoculations

To answer whether AM fungal communities exhibit growth effects that are additive with respect to the single-isolate inoculations, I compared predictions from the mixed model for each mixture treatment to those of each of the compared isolates (Figure 4.4). The only AM fungal mixture treatment that exhibited greater growth than the individual isolates was the combination of *G. margarita* and *S. calospora*, which resulted in greater biomass when inoculated together compared to each individually until approximately 34 days of growth, possibly due to faster growth that occurred prior to the imaging period. Three of the combination treatments (*G. mosseae* + *G. margarita*, *R. irregularis* 10 + *R. irregularis* 23, *S. calospora* + *G. mosseae*) exhibited patterns that were generally similar or intermediate to the responses to the isolates individually. The combination of *G. mosseae* and *R. irregularis* 10 and of all five isolates, on the other hand, exhibited patterns that were generally closest to the least productive isolate individually. Therefore, the results from this experiment, with one exception, reveal mostly no added benefit and, in some cases, antagonistic outcomes for plant growth in response to inoculating with combinations of isolates.

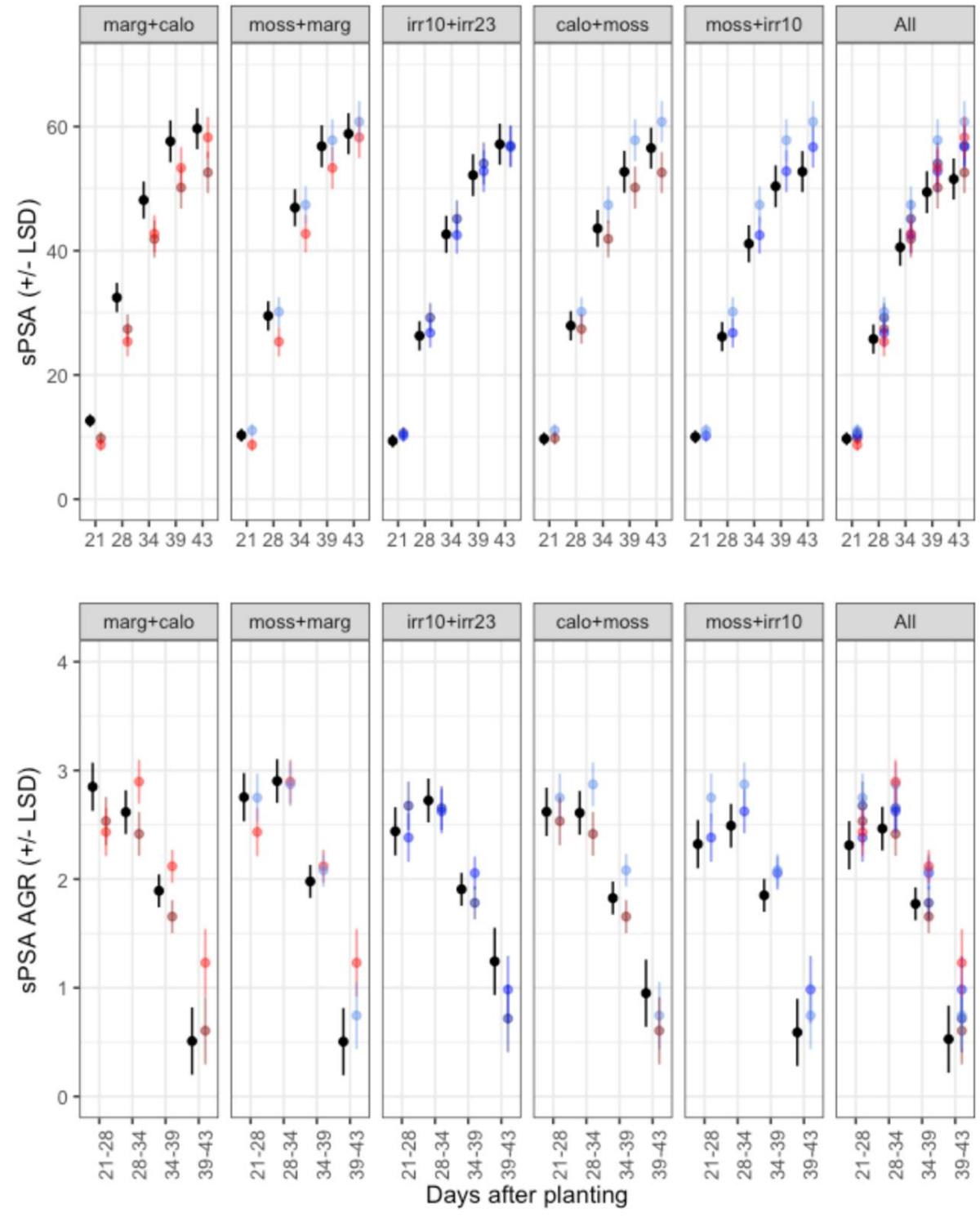


Figure 4. 4: *Temporal effects of inoculation by AM fungal mixture treatments presented (black) on plant biomass and growth rate compared to effects in each individual isolate of each combination (blue-scale = Glomeraceae, red-scale = Gigasporaceae)). Points represent predictions from the mixed model for the displayed time/time window and within each inoculation treatment; error bars represent least significant differences (LSD) predictions from the model. sPSA = Projected Shoot Area.*

4.3.4 Low levels of AM fungal colonisation in single-species treatments

The single species colonisation data in Figure 4.5a shows that no colonisation (fungal structures such as vesicles, hyphae and arbuscles; Figure 4.5c,d) was detected in the non-mycorrhizal control plants under +/- pathogen conditions. Average colonisation was observed to be greater than zero for all AM fungal inoculated treatments except dual inoculated *G. mosseae* + *R. solani* plants. The highest level of root colonisation was observed in plants inoculated with the *R. irregularis* 23 isolate, with an average of 40% of the root length colonised regardless of whether the pathogen was applied. *G. margarita*, *G. mosseae* and *S. calospora* exhibited low colonisation (around 6%) under both + pathogen and - pathogen conditions, except for *G. margarita* which had higher (around 30%) colonisation when the pathogen was present. Similar to the single species observations of the two *Rhizophagus* species, the combination of *R. irregularis* 10 *+ R. irregularis* 23 and the combination of all isolates showed relatively high colonisation in the absence of the pathogen of around 20-40% (Figure 4.5b). However, only very low colonisation was observed in *G. margarita* + *S. calospora* as well as *S. calospora* + *G. mosseae*, and no AM fungal colonisation could be detected in the *G. mosseae* + *G. margarita* combination. These latter three combinations all contain species with low colonisation characteristics in the single-species treatments. The analysis of the colonisation levels in combination treatments does not follow the same trend of increased colonisation when plants were inoculated with *R. solani* as in single-species treatments. Therefore, the colonisation data don't support the prior expectation for strong colonisers, although this may have been due to a loss of fungal structures in the roots of matured plants in single and combination treatments. Although it was highly variable, treatments for which the plants had been further developed stopped growing in the days prior to the harvest also tended to have lower levels of root colonisation (Figure 4.5e) The and increased carbon allocation in fruits of those plants and hence decreased belowground carbon allocation could explain low colonisation levels. For this reason, the colonisation data don't help assess colonisation at the time of the pathogen addition.

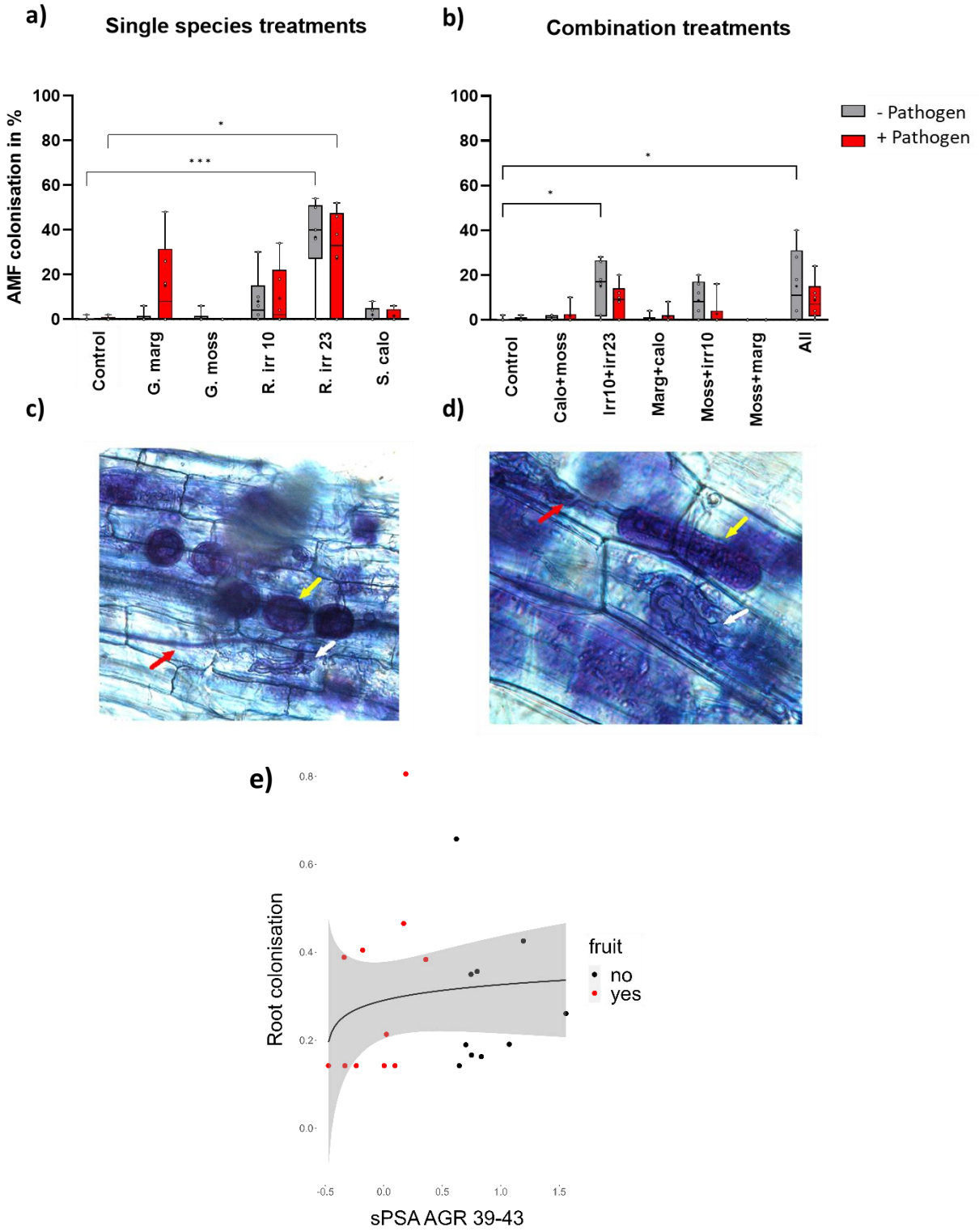


Figure 4. 5: a) *Percentage of tomato roots colonised with AM fungal structures in single AM fungal treatments and* ***b)*** *AM fungal mixture treatments. Boxplots show the min to max root colonisation, single measurements shown in white circles, median shown as line mean shown as "+",*= P ≤ 0.05, ***= P ≤ 0.001. One sample in the control treatment was found to be contaminated and therefore excluded.* ***c)*** *Tomato roots stained with the ink-vinegar method showing AM fungal structures of* R. irregularis *23 in 20x magnification,* ***d)*** *Vesicles and hyphae in R.* irregularis *23 in 40x magnification. Red arrows = hyphae, yellow arrows = vesicles, white arrows = paris-type arbuscules.* ***e)*** *Scatter plot of plants that fruited (red dots) or not fruited (black dots) with the correlation of AM fungal colonisation relative to the growth rates (sPSA AGR 39-43), trend line = black, confidence interval = grey.*

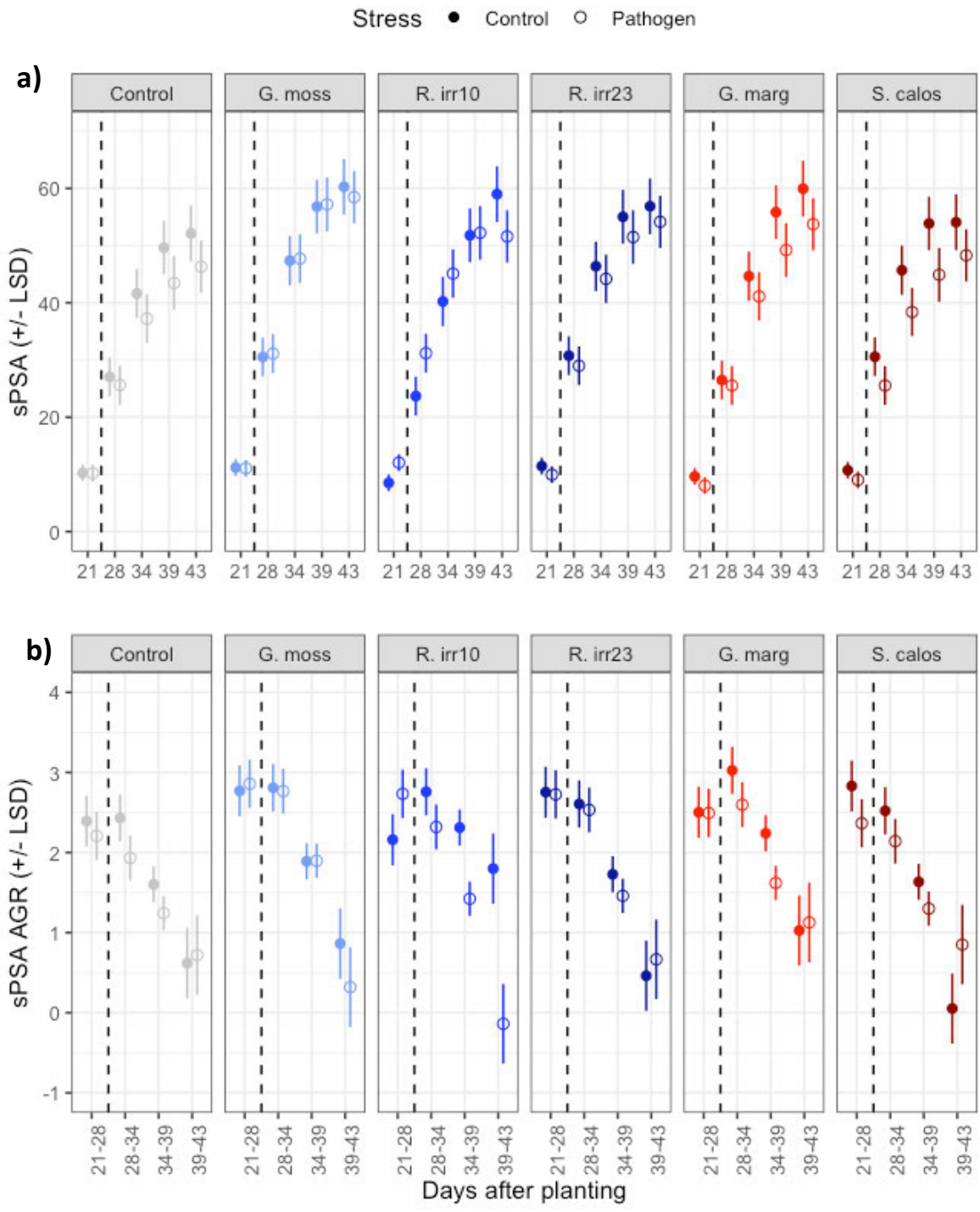


Figure 4. 6: *Temporal effects of inoculation by one of five AM fungal isolates (and an uninoculated control) on plant biomass and growth rate in pathogen-treated conditions (open symbols) and in the absence of pathogen inoculation (closed symbols) presented in* ***a)*** *projected shoot area and* ***b)*** *absolute growth rate Points represent predictions from the mixed model for the displayed time/time window and within each inoculation treatment; error bars represent least significant differences (LSD) predictions from the model. The dashed line represents the pathogen addition. PSA = Projected Shoot Area, sPSA AGR = Absolute Growth Rate.*

4.3.5 AM fungal single isolates can be classified into effective and non-effective MIR species

The projected shoot area for plants inoculated with *R. solani* revealed that pathogen addition in the absence of AM fungal colonisation resulted in a short-term reduction in growth. Although this reduction in absolute growth rate in the eleven days following pathogen inoculation in the absence of AM fungi was not large enough to result in a significant reduction in plant biomass, there was a trend in this direction (Figure 4.6a). The comparison of the growth responses obtained from the pathogen-inoculated conditions compared to pathogen-free conditions showed variation between the single AM fungal species in response to the pathogen. As shown in Figure 4.6a, three out of the five single AMF treatments (*G. mosseae* and both isolates of *R. irregularis*) could compensate for the negative plant size effects caused by the pathogen in the control plants, suggesting MIR. The growth for *G. mosseae* and *R. irregularis* 23 was similar between pathogen-inoculated and uninoculated plants, indicating that these two isolates helped plants overcome the pathogen and are suggested to be effective at MIR. Although there was a trend, the effect of *R. irregularis* 10 is difficult to interpret because there were significant differences in biomass and growth between plants assigned to the two stress treatments before the pathogen was applied. There was no apparent explanation for this incident; therefore, I don't interpret this treatment in further depth.

G. margarita and *S. calospora* each exhibited reductions in growth associated with pathogen inoculation that were similar to those plants grown in the absence of AM fungi. The effect size was larger in *S. calospora*, where there was a (marginally nonsignificant) reduction in biomass in pathogen-inoculated plants by 39 days after planting. In both treatments, the projected shoot area and absolute growth rate show similarity to the observations of the non-AMF control treatment. Based on these results, these isolates are unlikely to induce MIR, although the pathogen-inoculated *G. margarita* plants exhibited a trend towards being larger than those inoculated with pathogens in the absence of AM fungi by the end of the experiment. Hence, those two AM fungi depict similar negative responses to the pathogen-infected control and can therefore be considered non-effective MIR species. Instead of a sequential classification, the results in Figure 4.6 allow classification into effective vs. non-effective MIR single isolates. Thus, *G. mosseae, R. irregularis 23 and R. irregularis10* are categorized into effective MIR isolates and *G. margarita* and *S. calospora* into non-effective MIR isolates. I could not find support for any of the trade-off scenarios in growth reduction versus MIR (Figure 4.6, Supplementary Figure S.4.5), which would suggest growth-enhancing AM fungi leave the plant with fewer resources to protect itself in case of pathogen presence Plants that were growing

faster before inoculation did not experience significant reductions in growth in the following days after pathogen inoculation.

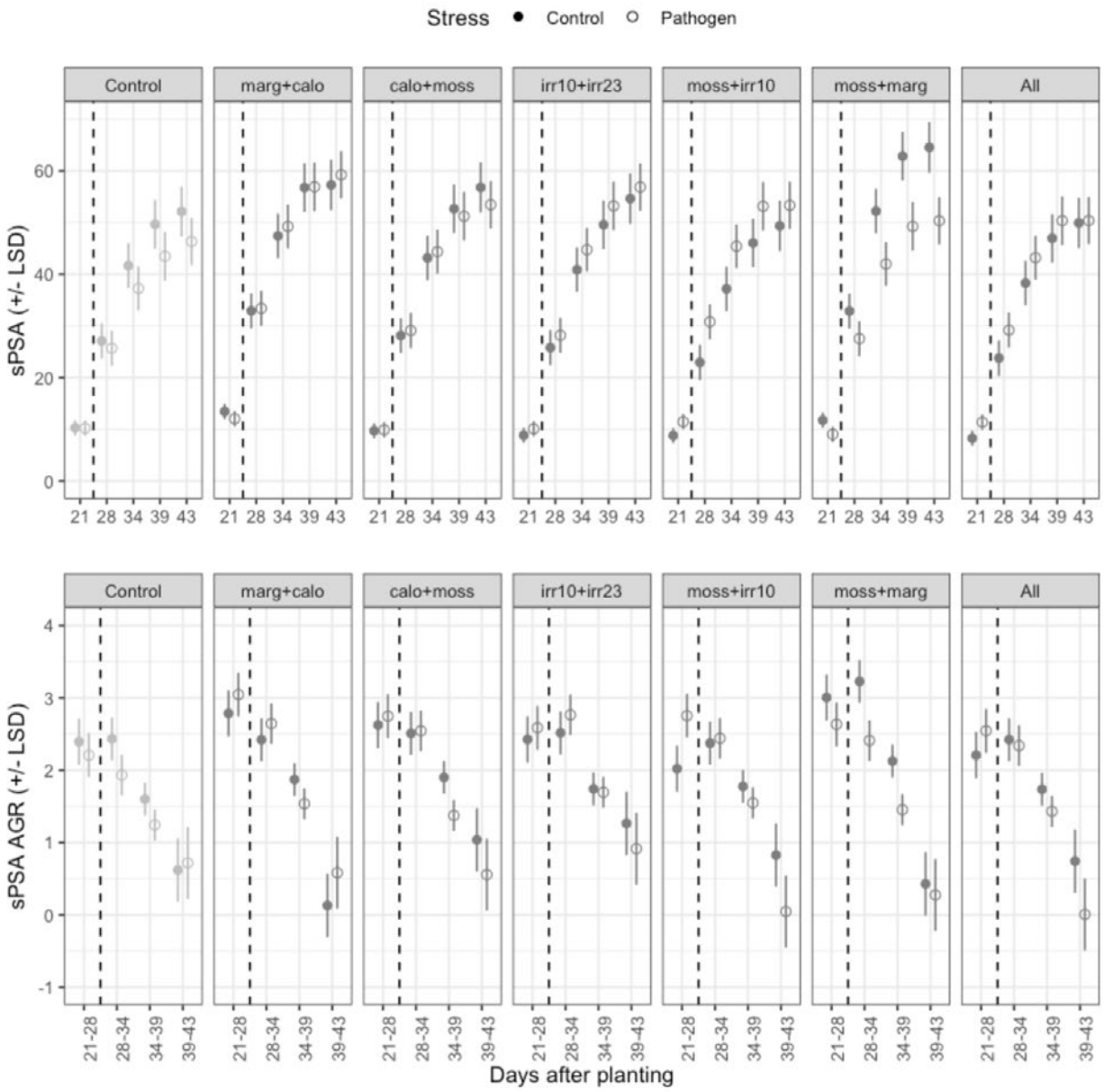


Figure 4. 7: *Temporal effects of inoculation by mixed AM fungal treatments (and an uninoculated control) on plant biomass and growth rate in pathogen-treated conditions (open symbols) and in the absence of pathogen inoculation (closed symbols). Points represent predictions from the mixed model for the displayed time/time window and within each inoculation treatment; error bars represent least significant differences (LSD) predictions from the model. The dashed line represents the pathogen addition. PSA = Projected Shoot Area, sPSA AGR = Absolute Growth Rate.*

4.3.6 Distinct MIR responses of AM fungal mixture treatments from individual AM inoculations

As shown in the first panel of Figure 4.7, inoculating with the pathogen resulted in a reversal of biomass loss in four of the dual inoculation treatments (*G. margarita* + *S. calospora*, *S. calospora* + *G. mosseae*, *R. irregularis* 10 + *R. irregularis* 23, *G. mosseae* + *R. irregularis* 10) compared to non-AMF control plants exposed to the pathogen. Thus MIR was observed for all combinations except for one (i.e. *G. mosseae* + *G. margarita*), even in cases where one or both isolates in the mixture were classified as MIR non-effective when inoculated individually. The combination of the dual inoculation with *G. margarita* and *S. calospora*, both classified as non-effective MIR isolates, enabled the growth of pathogen-infected plants to the same level as the plants without pathogen inoculation. The additive effect of the two MIR-effective single isolates *G. mosseae* and *R. irregularis* 10 could be observed when inoculated together.

4.4 DISCUSSION

Taken together the findings in this study show that AM fungal species influence growth and MIR differently and that the role of individual AM fungal species/isolates in promoting plant health cannot predict the influence of AM fungal communities on their host plant. The results provided here shed new light on the complex species-specific AM fungal effects on tomato growth and defence responses.

4.4.1 Growth promotion observations

My results broadly reflect previous literature investigating the single isolates used in this study, although there were some inconsistencies. The ability of the AM fungal species used in this study regarding the promotion of growth and defence in a host has been reported previously (Liu et al., 2007; Song et al., 2015a; Chave et al., 2017; Akköprü & Demir, 2005; Kareem & Hassan, 2014; Peña et al., 2020). In line with my findings, *G. mosseae* is regularly described as inducing enhanced growth responses in a variety of plants (Wang et al. 2018; Adeyemi et al. 2021). Beneficial effects for both species *G. mosseae* and *R. irregularis* have been demonstrated in this thesis, similar to what was observed in soybean (Adeyemi et al., 2021). However, while soybean showed a greater advantage with *R. irregularis* (Adeyemi et al., 2021), which was not observed in this study, similar to previous observations (Burleigh et al., 2002), *G. mosseae* and *S. calospora* increased growth responses in my study, however, this trend was more pronounced when inoculated as mixture. Fewer studies have been performed investigating the effect of AM fungal mixtures on plant growth, where complementarity and antagonism between fungal isolates could lead to outcomes that cannot be predicted based on responses to individual AM fungi. In line with previous observations of enhanced growth responses, dual inoculations or a combination of all five species showed primarily beneficial effects on growth. For example, a mixture of five species, including *G. mosseae*, *R. irregularis* and *G. margarita* led to enhanced growth responses alongside reduced disease occurrence against *R. solani* (Hafez et al., 2013).

4.4.2 MIR promotion observations

In this study, I found that two single isolates were effective in inducing phenotypes that would suggest MIR, *namely G. mosseae* and *R. irregularis 23,* both from the family of Glomeraceae. These findings largely support the outcomes from prior studies in this area, linking those species and families to enhanced disease resistance (Sikes et al., 2009; Maherali & Klironomos, 2007). The ability of *G. mosseae* and *R. irregularis* to compensate for the negative effects of disease on growth has been widely reported, comprising air-borne biotrophic, and necrotrophic pathogens. *G. mosseae* showed reduced *Phytophthora parasitica*, *Alternaria solani Cladosporium fulvum* and *Botrytis*

cinerea infection in tomatoes (Trotta et al. 1996; Pozo et al. 1996; Song et al. 2015; Cordier et al. 1996; Wang et al., 2018; Fiorilli et al., 2011) and largely decreased Phytophthora disease severity (Ozgonen & Erkilic, 2007; Pozo et al., 1999) While both of these species demonstrated effectiveness regarding MIR in this study, *G. mosseae* exhibited slightly greater benefit compared to *R. irregularis*. This might suggest the level of colonisation as an underlying factor; however, I observed high colonisation in *R. irregularis* and no colonisation in *G. mosseae* in pathogen-treated conditions. While this only reflects the colonisation data at the endpoint harvest, better growth enhancement with *G. mosseae* despite higher colonisation *R. irregularis* was observed previously, with increased resistance in rice to *Magnaporthe oryzae,* conferred by both species (Campo et al., 2020). This suggests that while colonisation level might not have primary influence on MIR, there could also be possible variations in colonisation over time, along with other potential influences. As for *G. mosseae* several reports have shown beneficial traits in the growth and disease defence of *R. irregularis* including in bananas against *Cylindrocladium spathiphylli* (Declerck et al., 2002), decreased mortality in potato plants upon *R. solani* infection (Yao et al., 2002), attenuated symptom extent of a foliar necrotrophic disease *Alternaria solani* in tomatoes (Fritz et al. 2006), increased resistance on soybean leaves upon *Phytophthora sojae* infection (Li et al., 2013) and decreased bacterial occurrence of *Xanthomonas campestris* in mycorrhized barrel medick plants (Liu et al., 2007). Therefore, consistent with past work, this research supports the finding that *Glomus* species are effective at promoting MIR in tomato.

Conversely, I found that *G. margarita* and *S. calospora* had no observable MIR effects. Although Gigasporaceae are better known to enhance plant growth than plant health (Sikes et al., 2009; Sikes, 2010), some studies show beneficial MIR effects of *G. margarita.* For example, colonisation by this AM fungus reduced the disease severity of *Phytophthora capsica* in pepper plants by 75% in greenhouse and field conditions (Ozgonen & Erkilic, 2007), of *Fusarium sp*. infection in succulent and cactus plants (Domenico Prisa 2020), and of *P. parasitica* in citrus (Davis & Menge 1981). Moreover, a very recent study used *G. margarita* to investigate the defence-related signalling and found increased resistance in tomatoes against the fungal pathogen *Botrytis cinerea* and the bacterial pathogen *Pseudomonas syringae* pv tomato DC300 (Fujita et al., 2022). Results such as these led authors of another study performed on *G. margarita* in tomato plants to suggest that even limited AM fungal colonisation has the potential to induce MIR (Fujita et al., 2022), which again suggests that the colonisation level might not be critical to the role of AM fungi in mediating host growth and defence. Both of the identified non-effective species in this study seemed to have similar effects, although *S. calospora* showed a slightly higher level of colonisation. There are only a limited number of studies available regarding *S. calospora, the* second non-effective MIR species reported here. The

sparse studies investigating the effects of *S. calospora* show increased plant biomass in both tomato and medick (Burleigh et al., 2002). Yet, another member of the genus of Scutellospora showed lower MIR capabilities (Sikes et al., 2009). My results regarding both of these AM fungal single species treatments do not agree with most previous literature findings regarding growth but agree with the statement that *G. margarita* has more beneficial abilities regarding growth than MIR.

4.4.3 AM communities overall beneficial to plants

I present an investigation of AM fungal mixture treatments on the growth and defence of tomato compared to single species treatment. Overall the results from the mixture treatments did not show the predicted outcome of additive single-species effects, with responses that were greater or less than expected based on their effects in isolation. Surprisingly, *G. margarita* plus *S. calospora* had beneficial MIR effects although there were no observed benefits under pathogen conditions in both single-species treatments. *G. mosseae* has been shown to rescue the negative impact of *P. parasitica* infection on biomass despite no effect on the biomass under non-stressed conditions (Pozo et al., 1999). This suggests that AM fungal species might have non-stationary capabilities to mediate growth/resistance. Hence, there is the possibility that these non-stationary properties might further transform within community contexts. So far investigations regarding AM fungal communities have mostly focused on community composition and diversity (Rengifo-Del Aguila et al., 2022; Van der Heijden et al., 1998; Vandenkoornhuyse et al., 2003). Fewer studies have investigated the effect of AM fungal communities on biotic stress conditions (Hafez et al. 2013; Al-Askar & Rashad 2010; Jaiti et al. 2007). These previously discussed studies did not pay much attention to the taxonomic diversity in the AM fungal mixtures (Hafez et al. 2013; Al-Askar & Rashad 2010; Jaiti et al. 2007). A study comparing the disease symptoms of *Phytophthora parasitica* on citrus inoculated with several single species and one combination of two Glomus species found that the latter did not lead to enhanced MIR compared to their single inoculation (Davis & Menge, 1981). In line with these findings, the combination of *G. mosseae* (effective) and *G. margarita* (non-effective) in this study could strongly enhance the growth under non-stressed conditions but did not improve the pathogen-induced growth depression in pathogen-treated plants. Negative effects due to competition between AM fungi could influence the MIR outcome in plants (Engelmoer et al., 2014; Merrild et al., 2013; Scheublin et al., 2007). Another possible factor contributing to the change from single to mixed species could be the changed composition of other microbes. Distinct functional groups of microbes have been detected in the hyphosphere of different AM fungal species (Zhou et al., 2020). Therefore, mixed species treatments could potentially alter the proportion of microbial abundance, subsequently leading to enhanced MIR. Although there was an overall growth benefit of AM fungal communities, the non-additive MIR responses in plants inoculated with some mixtures

reflect the possible changes in effects on plants in occurrence of AM fungal community settings, compared to single-species observations. Previous findings and findings from this thesis depict the complexity of plant responses to AM fungal communities and the need to further investigate and better understand the consequences.

4.4.4 Trade-offs theory observations

One purpose of this experiment was also to investigate whether a trade-off exists between AM fungal promotion of plant growth versus defence against a root pathogen. As AM fungal symbiosis is an exchange of soil nutrients with plant photosynthates, trade-offs to enhanced growth due to decreased nutrient availability are possible side effects of MIR. I did not observe such trade-off effects in single-species treatments; in fact, I found that the least effective AM fungus for promoting MIR was *S. calospora*, which was also the AM fungus that promoted plant growth the least. In line with these findings, a previous study showed that the least effective species for promoting growth was also the least effective in pathogen protection (Jaiti et al., 2007). However, another recent study could demonstrate that MIR functions separately from other beneficial mycorrhizal growth effects (Pozo de la Hoz et al., 2021). In my study, *G. mosseae* showed the strongest abilities to enhance performance in plant size under pathogen and non-pathogen conditions. Indeed, there are similarities between my results and previous observations in Solanaceous plants where *G. mosseae* was found to have growth and defence-related beneficial traits in pepper with significantly reduced disease severity of *Phytophthora capsici* in pot, greenhouse, and field conditions (Ozgonen & Erkilic, 2007). Decreased disease symptoms in mycorrhized tomato upon *Alternaria solani* infection despite unaltered biomass benefits were observed (Fritz et al., 2006). My results support these previous observations regarding MIR trade-offs being limited and show that in most scenarios plant growth is not decreased even though there are beneficial effects on growth inhibition caused by the pathogen.

Overall the plants in this experiment treated with mixtures of AM fungi seemed to have more MIR benefits regarding growth than in the single species treatments. Enhanced plant growth associated with reduced disease severity in beans to Fusarium was previously shown with an AM fungal mixture that contains three of the species also used in this study (Al-Askar & Rashad, 2010). Although no evidence of trade-offs in the mixture treatment could be observed, the dual colonisation with *G. mosseae* + *G. margarita* led to losses in growth under pathogen conditions. While most mixture treatments did not lead to growth trade-offs, the combination of ALL in this experiment led to an overall decreased growth benefit compared to the control treatments but had still beneficial effects under pathogen pressure. This suggests that the more diverse community including the here-used AM fungal isolates led to decreased growth under non-pathogen conditions, possibly due to

enhanced resource allocation from the plant to the fungi, but the successful plant MIR response suggests that this more diverse mixture still left the plant with sufficient resources to defend against the pathogen.

This study represents a novel dataset investigating both growth and MIR-related traits of different single species as well as AM fungal communities. While previous literature has observed functional specificity of AM fungi regarding colonisation, growth, nutrient uptake and gene expression (Burleigh et al., 2002; Cavagnaro et al., 2001), this study enhances the knowledge about AM fungal species-specific differences in growth-related MIR responses in tomato. The single species results in this study support previous statements that species from the Glomeraceae have better MIR abilities (Sikes et al., 2009; Sikes et al. 2010; Maherali & Klironomos, 2007). However, natural environments will always contain a combination of different AMF species (Smith & Read, 2008; Jansa et al., 2008). This leads to complex AM fungal communication with neighbouring plants about an existing infection (Babikova et al., 2013; Song et al., 2010) but also interactions with other microbes (Zhou et al., 2020), and possible competition between AM fungal species (Thonar et al., 2014; Engelmoer et al., 2014). Therefore, it is important to investigate the effect of the interrelationships in an AM fungal mixture to allow a better understanding and more efficient use of AM fungal mixtures in agriculture. The authors of a previous article discussing the current knowledge about pathogen protection and fungal diversity state the need to investigate fungal communities and the different functional mechanisms leading to these differences (Wehner et al., 2010). My study addressed this need and showed that the single isolate observations are not adequate to predict the outcome and abilities of AM fungal communities. The trade-off effects in both single and mixture treatments were negligible in this study. This is important knowledge in the process of screening for effective species and more importantly mixture combinations that might be used in commercial products to enhance plant growth and resistance. Moreover, it is important to also consider plant and fungal identity and their relationships (Campo et al., 2020; Maherali & Klironomos, 2007; Sikes et al., 2009). Therefore, further investigations will need to be performed including different plant species as well as more combinations of AM fungal species which include the effects of plant biochemistry.

CHAPTER FIVE

AM fungal species-specific variation of metabolomic responses in tomato to a root pathogen reveals enhanced downregulation of metabolic compounds

This chapter is being prepared for submission to "Planta" journal. I led all aspects of the research including study design, execution, analysis and writing.

5.1 INTRODUCTION

The relationship between plants and beneficial microbes is crucial for the continued fitness of plants. Mutualists such as Arbuscular Mycorrhiza (AM) fungi from the subphylum of Glomeromycotina, establish a root symbiosis with over 80% of land plants leading to enhanced plant growth and pathogen defence (Smith & Read, 2008; Schüßler & Walker, 2010; Spatafora et al., 2016; Jung et al., 2012). While we have a reasonable understanding of how plants and fungi control nutrient fluxes that support plant biomass and yield (Mensah et al., 2015; Wang et al., 2017), the mechanisms by which AM fungi enhanced plant resistance to pathogens (i.e., mycorrhiza induced resistance, MIR) are poorly understood (Cameron et al., 2013; Pozo et al., 2009; Harrier & Watson, 2004; Jung et al., 2012; Pozo & Azcón-Aguilar, 2007). Several processes by which MIR operates have been proposed, acting independently or in combination (Pozo & Azcón-Aguilar, 2007; Kadam et al., 2020; Filho, 2022). Hormonal regulation, the involvement of secondary metabolic compounds, as well as the involvement of AM fungal-specific signalling compounds have been reported (Schliemann et al., 2008; Hill et al., 2018; Jung et al., 2012; Wu et al., 2021; Adolfsson et al., 2017; Pastor et al., 2013; Ton & Mauch-Mani, 2004). One explanation for the observed variation among AM fungal species in their ability to induce MIR could be that they differ in their capacity to employ these different mechanisms. In support of this, the metabolic landscapes of colonised plants have been described (Laparre et al., 2014; Hill et al., 2018), and several plant metabolic compounds connected to defence mechanisms vary in their accumulation levels upon colonisation with different AM fungal species (Morandi et al., 1984; Rivero et al., 2015; Copetta et al., 2006; Kapoor et al., 2002).

Studies considering differences between AM fungal isolates have demonstrated variations in alterations of metabolic compounds including compounds previously connected to plant defence responses (Wu et al., 2021; Rivero et al., 2015). Increased disease resistance correlated to molecular changes in plants upon colonisation with *Glomus mosseae* (Glomeraceae) has been observed against the pathogens *Botrytis cinerea* (Song et al., 2015a), *Alternaria solani* (Fritz et al., 2006; Fiorilli et al., 2011) and *Rhizoctonia solani* (Kareem & Hassan, 2014) in tomato and to *Phytophthora capsica* in

pepper (Ozgonen & Erkilic, 2007). Enhanced abilities have also been frequently shown in plants inoculated with other members of the Glomeraceae (Akköprü & Demir, 2005; Chave et al., 2017). Studies frequently report pronounced beneficial growth responses in members from the Gigasporaceae, yet little association with enhanced defence responses have been shown to date (Tchameni et al., 2012; Matsubara et al., 1995; Dowarah et al., 2021). Therefore, AM fungal species differ in their abilities to enhance plant resistance (Schüßler & Walker, 2010; Spatafora et al., 2016a; Jung et al., 2012; Maherali & Klironomos, 2007), with the Glomeraceae and Gigasporaceae being the two AM fungal families that have received the most research attention concerning taxon-specific patterns related to support of plant health (Pozo et al., 2002; Yao, 2002; Sikes et al., 2009; Sikes, 2010). It is of interest, however, to also highlight that not all studies have shown qualitative species-specific expression of secondary compounds upon AM fungal colonisation (Vierheilig, Gagnon, et al., 2000). This latter observation could be due to the fact that not all AM fungal species are effective at inducing MIR, by activating metabolic plant immune responses.

To best understand MIR, it is essential to include comparisons of AM fungal species together with pathogen inoculation treatments to best identify contrasts in the mechanisms by which AM fungal species effectively induce MIR. While current studies advanced our understanding of the species-specific molecular alterations related to plant defence upon AM fungal colonisation, the majority have been performed in the absence of a pathogen. Therefore, it is not clear if these species-specific changes also impact the intensity of MIR during subsequent pathogen attacks. The tripartite interaction of crop plant, AM fungi and pathogen provides novel insight into the global alterations in gene transcripts, proteins and metabolites in the plant in the case of AM fungal conferred enhanced yield and resistance (Fiorilli et al., 2018; Marquez et al., 2019; Lu et al., 2020). By observing changing patterns in the landscape of molecular processes influenced by AM fungal colonization during biotic stress conditions, it becomes possible to categorise and establish connections between a diverse range of molecular processes and MIR. Involved pathways, such as primary or secondary compounds could be highlighted and linked to AM fungal colonisation, including defence response. The changes in metabolites play an essential role in these responses and their abundance can help to determine the missing link between gene/protein expression and phenotypic observations of plant-microbe interactions (Castro-moretti et al., 2020). Identifying alterations in metabolic regulation caused by AM fungal colonisation improves our understanding of the protective effects mediated by AM fungi. Therefore, we need more studies that investigate the tripartite interaction of plant/pathogen/AM fungal species to understand how the varying MIR potential of different AM fungal species may be connected to metabolic changes in the host.

To compare metabolomic profiles of tomato roots inoculated with different AM fungal species and challenged with a pathogen, this present study determined the extent to which variation in MIR could be explained by AM fungal species. I compared metabolomic profiles of *Solanum lycopersicum* cv MicroTom inoculated with one of four AM fungal species that varied in their effects on MIR based on analysis in Chapter 4 (MIR effective: *G. mosseae*, *R. irregularis*; MIR non-effective: *G. margarita 23*, *S. calospora*). I hypothesised that the phenotypic observations of effective and non-effective AM fungal MIR species will also affect the metabolomic patterns between these species.

5.2 Materials and Methods

5.2.1 Experimental design

The samples analysed in this study were generated in the experiment described in Chapter 4. The experimental design is explained in section 4.2.1, above (Chapter 4). The processes shown in Figure 5.1, show the steps of the experiment starting from germination (Figure 5.1a), mycorrhiza establishment (Figure 5.1b), pathogen addition (Figure 5.1c), and endpoint harvest (Figure 5.1d). Root, stem and shoot apical meristem tissues were collected and used for metabolite extraction (Figure 5.1e), but only the root metabolite data were analysed in this study. To compare the root metabolome of AM fungal effective species to AM fungal non-effective species, the two most impactful species of each category in the phenotypic observations were chosen to allow equal comparison in this study. These selected species are *G. mosseae* and *R. irregularis* 23 for MIR effective species and *G. margarita* and *S. calospora* for MIR non-effective species.

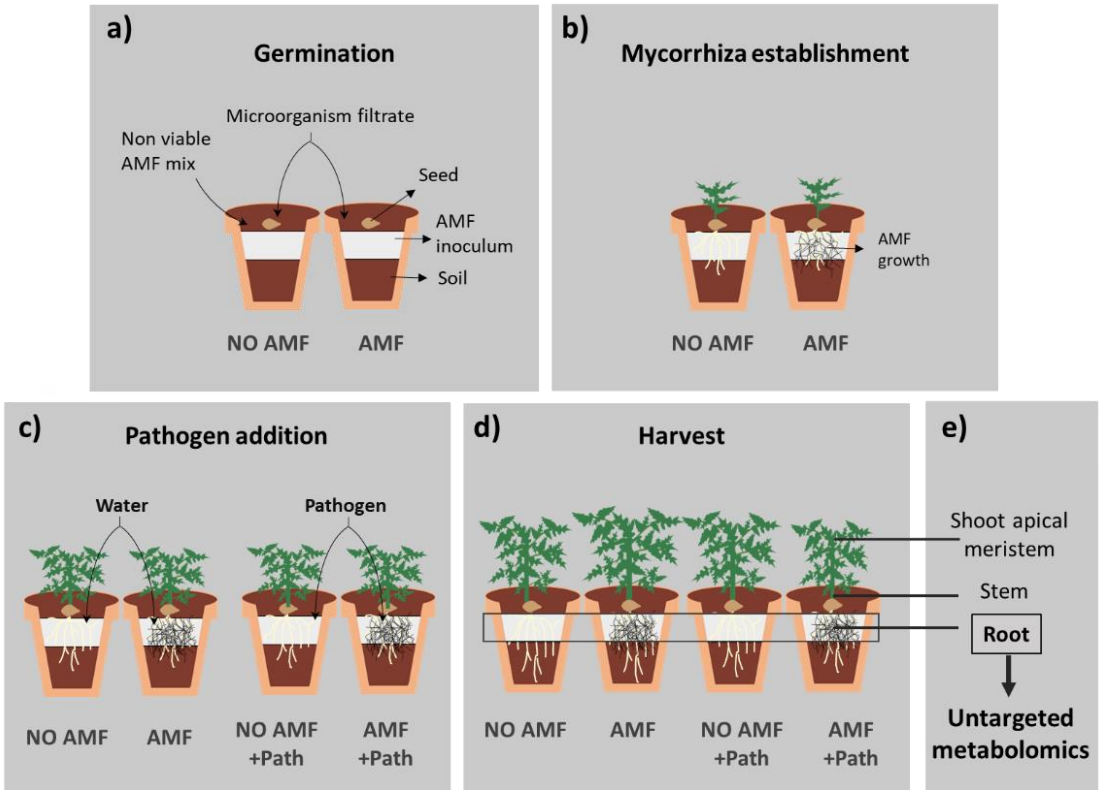


Figure 5. 1: *Diagram displaying the experimental steps including* ***a)*** *pots with soil and viable or non-viable AM fungal inoculum,* ***b)*** *mycorrhiza establishment to allow plant growth and formation of AM fungal symbiosis,* ***c)*** *Pathogen addition to create pathogen treatments and water addition for mock treatments,* ***d)*** *Harvest at the endpoint of the experiment with developed plants and AM fungal symbiosis with tissue regions harvested in all treatments,* ***e)*** *name of the tissue collected with emphasis on the root tissue; results from untargeted metabolomics are presented in this study.*

5.2.2 AM fungal inoculum and microbial wash

Single AM fungal inoculum treatment details can be found in section 4.2.2 above (Chapter 4).

5.2.3 Pathogen growth

R. solani was used as a pathogen in this study. Further details can be found in section 4.2.3 above (Chapter 4).

5.2.4 Soil and pot preparation

For details regarding soil, preparation see section 4.2.4 above (Chapter 4).

5.2.5 Plant material and growth conditions

Solanum lycopersicum cv Micro-Tom was used in this study and treated and grown under the same conditions as in section 4.2.5 above (Chapter 4).

5.2.6 Harvest

The endpoint harvest (day 44 after transplanting) took place over two days. The plants were removed from the pots and the roots were rinsed with water. Thereafter, around 30 mg of each of

the tissue types for metabolomic analysis was collected: root samples (three evenly distributed ~2cm pieces in each sample), stem samples (around 1cm taken from the lowest stem section) and apical meristem samples were collected, and the weight was recorded. The material was snap-frozen in liquid nitrogen and stored at -80°C until further processing.

5.2.7 Metabolite extraction and untargeted metabolite profiling

The metabolite extraction was performed according to section 3.2.6 above (Chapter 3). Samples were diluted 4x (based on sample QC dilution series) before analysis.

5.2.8 Metabolomic data analysis and metabolite annotation

The program Progenesis QI, version 3.0 (Nonlinear dynamics, Waters Corporation, UK) was used to perform automated data processing including peak alignment, peak picking, and deconvolution and the resulting peak intensities were used for statistical analysis. Potential molecular feature identification and annotation of the observed peaks were obtained using the ProgenesisQI support for the web-based Chemspider structure database, including the public databases ChEBI, Phenol-Explorer, PlantCyc, KEGG and Golm Metabolome Database with a precursor tolerance 15 ppm and fragment tolerance 50 ppm. Mass error, isotope similarity and fragmentation score were used to calculate a confidence score for each potential identification. Furthermore, this highest overall score or the highest fragmentation score was used to select the compound for putative identification. Chemical classification based on primary structural features was used to assign potential compound identifications.

5.2.9 Statistical analysis

Statistical analysis of peak intensities from the positive ion mode was performed using MetaboAnalyst 5.0 (Pang et al., 2021; Xia et al., 2009) and R version 4.1.1 (R Core Team, 2021). The total molecular features (hereafter named features) obtained were filtered by excluding those features with low variability across samples (based on the log10 fold changes between treatments with log10FC<2 removed) and the exclusion of low-intensity peaks (features with a peak intensity of <50 in all conditions). A total of 15 samples were removed from this analysis due to a technical glitch in the machine during the untargeted metabolomic analysis, leaving a total of 45 (with mostly five and at least three biological repeats in each treatment). These samples represented a random subset from across all treatment combinations and, when they were reanalysed separately, they exhibited significantly different metabolomic profiles compared to the rest of the samples that were analysed successfully in the previous batch. A Principal Component Analysis (PCA) with a 95 % confidence region was performed on all expressed metabolites from each AM fungal single species treatment including pathogen-treated and non-pathogen-treated conditions, to investigate patterns.

A fold change (FC) analysis with a threshold of 2.0 with a direct comparison between pathogen treatment to no pathogen treatment was executed to identify the metabolomic features that are significantly (threshold >75%) up- and downregulated in the different single AM fungal species treatments upon pathogen presence. To analyse the effect of the proportion of up-/downregulated compounds in single species treatment categories, a fitted generalised linear mixed model was performed followed (effectiveness included as fixed factor and fungal species included as random factor) by a type II Wald chi-square test using the Anova function from the car library (Fox & Weisberg, 2019) in R version 4.1.1 (R Core Team, 2021).

5.3 RESULTS

5.3.1 Roots colonised with MIR-effective AM fungal species exhibit distinct metabolomic profile

To compare metabolomic profiles between pathogen-inoculated and -uninoculated treatments within each AM fungal treatment, I performed a multivariate analysis (PLS-DA) of the detected metabolites in the control, two effective (*G. mosseae, R. irregularis 23*) and two non-effective (*G. margarita*, *S. calospora*) species, presented in Figure 5.2. The control treatment without AM fungal presence shows partial separation between the pathogen and non-pathogen conditions along each axis, with individual pathogen condition samples showing separation from all non-pathogen condition samples along one of the two axes, and with 30 % of the variance in metabolites explained by component 1 and 24% explained by component 2 (Figure 5.1a). The loadings for pathogen and non-pathogen conditions with *G. mosseae* (Figure 5.1b) were separated along component 1 (24%) but not along with component 2 (45%). A similar pattern was observed for the second effective AM fungal species treatment, *R. irregularis 23* showed similarities to *G. mosseae* with a full separation of the scores in pathogen to non-pathogen treatment along component 1 (16%) but not component 2 (41%) (Figure 5.2c). With the non-effective species, a different pattern was observed in which pathogen and non-pathogen conditions were mostly separated along the second axis but not the first. For *G. margarita* 46% of the variance in metabolites was explained by component 1 and 10% by component 2 (Fig 5.2d), while for *S. calospora* component 1 accounted for 45% and component 2 accounted for 22% (Figure 5.2e).

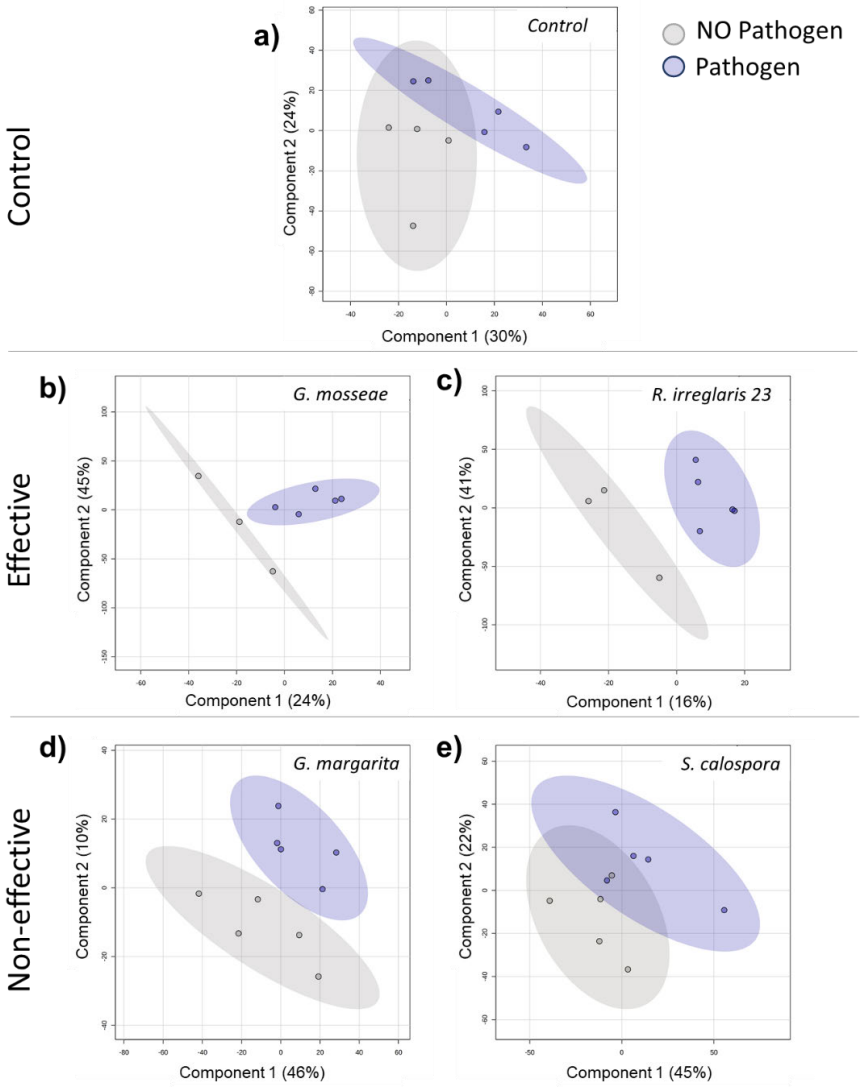


Figure 5. 2: *Score plots of Partial Least Squares Discriminant Analysis (PLS-DA**)** of **a)** control, the two effective species **b)*** G. mosseae*, **c)*** R. irregularis *23, and the two non-effective species **d)*** G. margarita *and **e)*** S. calospora*, displaying 95% confidence regions, variance explained within brackets. "No pathogen" conditions are shown in grey, and pathogen-infected conditions are shown in purple.*

5.3.2 Increased number of expressed features in AM fungal treatments

Figure 5.3 presents the total number of upregulated (Figure 5.3a) and downregulated (Figure 5.3b) features in response to pathogen inoculation for the control and each of the four selected species representing effective and non-effective MIR treatments. The control conditions showed the smallest number of significantly regulated features (155) when inoculated with the pathogen only as compared to all AM fungal inoculated conditions (ranging from 217-483). The number of upregulated features was relatively low in all AM fungal treatments, ranging from 45 to 159 when compared to the downregulated features which ranged from 113 to 324 depending on the condition (Figure 5.1a, b and Supplementary Table S5.1). The highest number of downregulated features was observed in the inoculation treatments with *G. margarita* and *G. mosseae*, whereas a similar number of up-and down-regulated features was detected in *S. calospora* and *R. irregularis 23* (Figure 5.3a, b). Within the upregulated compounds, *G. mosseae* had the highest number of differentially regulated molecular features, followed by intermediate numbers in *R. irregularis 23* and *S. calospora* and the lowest in plants treated with *G. margarita*. The results from the chi-square test showed a marginally non-significant effect (P = 0.07) on the ratio of upregulated compounds in effective species (~3%) in contrast to non-effective species (~1%), and no differences for downregulated compounds were observed. Interpretation of these results should be approached cautiously due to the limited number of species within each category (n=2), leading to low statistical power in this analysis. Therefore, overall, these results suggest that the overall number of up-/down-regulated compounds was not strongly influenced by whether AM fungi belonged to effective and non-effective AM fungal species treatments. Numbers including the control and all five different species can be found in the supplementary Table S5.1, Figure S5.1)

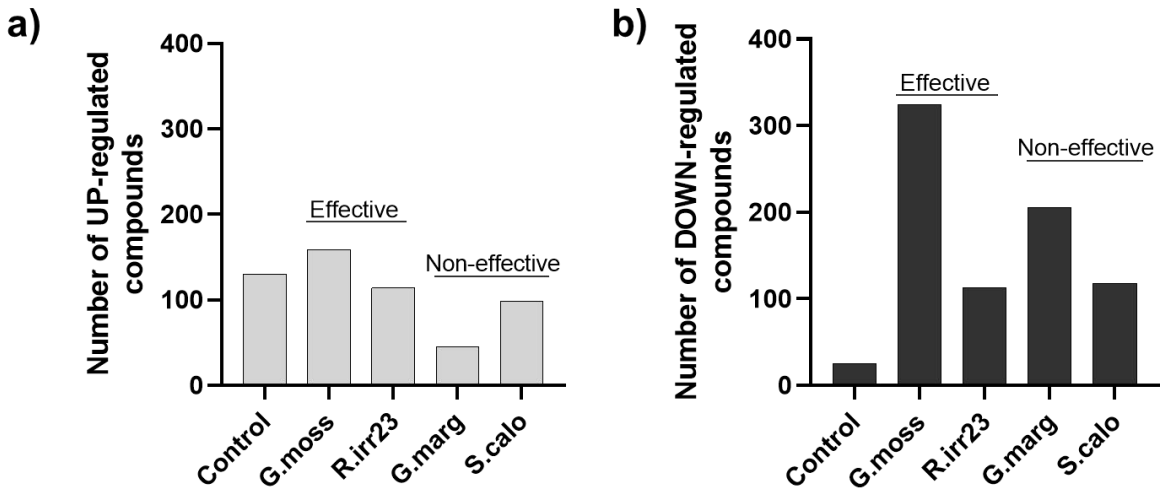


Figure 5. 3: *Number of **a)** Up-regulated and **b)** down-regulated features in response to the pathogen treatment in control and four selected single species AM fungal inoculation treatments. Effective and non-effective species are highlighted above the bars.*

5.3.3 Prevalence of down-regulated features in effective species

A closer inspection of the shared portion of significant features shown in Figure 5.4a, between AM fungal treatments within the effective or non-effective groups, revealed that 7.6% of differentially abundant features were shared between effective species (Figure 5.4 b,d). This overlap, however, is higher than in MIR non-effective pairs shown in Figure 5.4c,e (4.2%). Effective species resulted in a total of 45 common downregulated compounds while non-effective only resulted in 11, with only three of them found across effective and non-effective categories (Figure 5.4d, e, Fig 5.5a). Up-regulated compounds showed a much smaller overall number of commonly expressed features, compared to those downregulated. For these, six overlapping features were detected in the effective species (Figure 5.4b) and eight features in the non-effective species (Figure 5.4c).

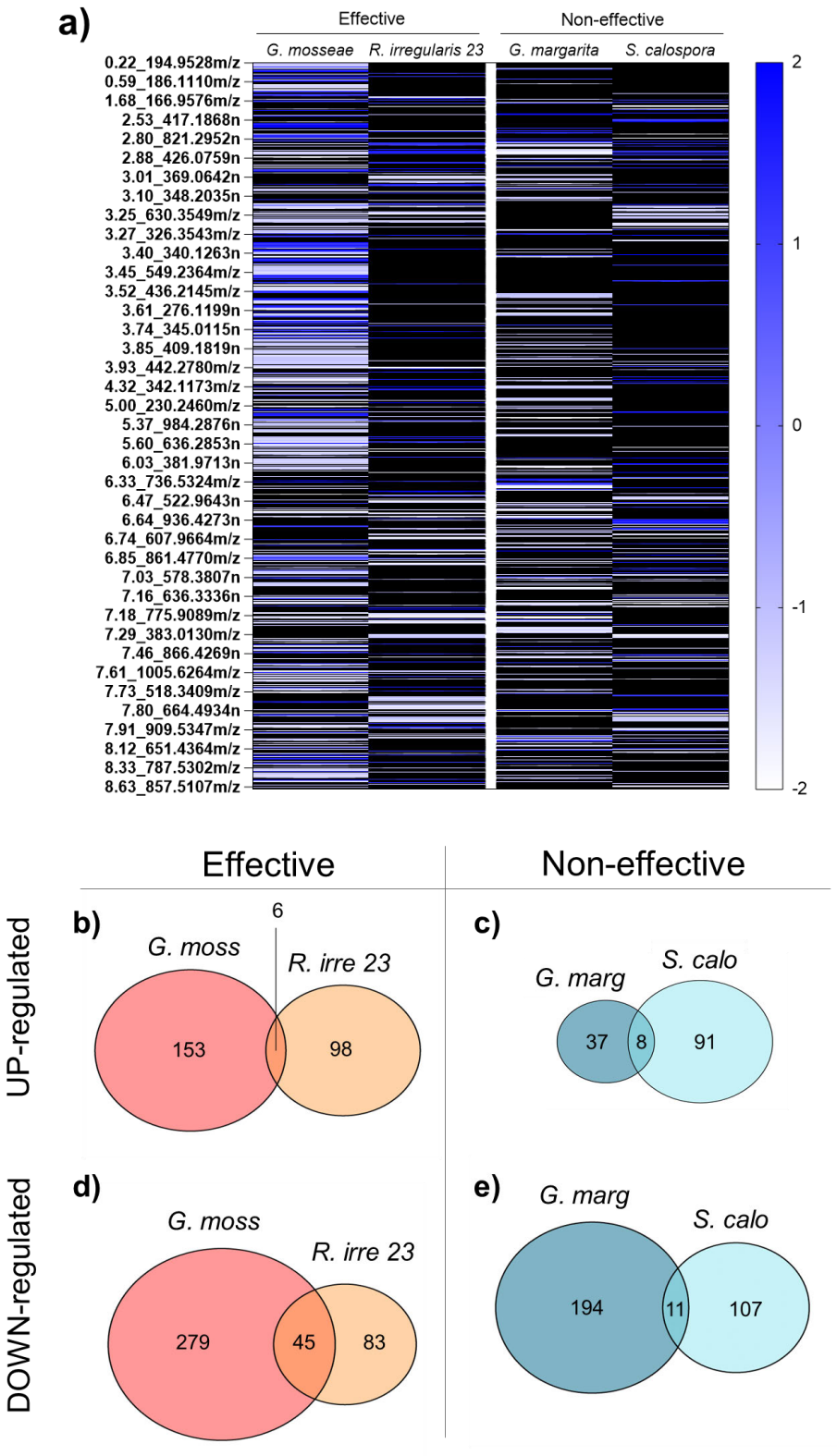


Figure 5. 4: a) *Heatmap showing the log2fold change of all 1,112 significantly expressed features in both effective and non-effective AM fungal species. blue = upregulated, light = downregulated, black = no significant fold change recorded in this treatment. Only a fraction of the molecular feature labels are shown.* ***b-e)*** *Venn diagrams displaying the amount of overlap among up-regulated (top panel) and down-regulated (bottom panel) metabolites that are shared between single species treatments in either effective (left panel) or non-effective (right panel) MIR AM fungal species.*

To understand if there was any conservation in features regulated by MIR-effective and MIR-non-effective species, I compared the shared molecular features that are up-regulated or down-regulated in MIR-effective and MIR non-effective species. Of the 53 down-regulated molecular features, 5.6% were common among the four species and of the up-regulated features I found 7.7% were common (Figure 5.5). Equally small numbers were observed in the proportion of overlapping molecular features between the four species, both in terms of upregulated and downregulated. These common features do not show any similarity to the MIR effective trends and are therefore unlikely to play a significant role in supporting MIR in tomato. Figure 5.5.b displays the total number of 42 down-regulated features in pathogen presence in effective species, while only eight of such were down-regulated in MIR non-effective species. These results show that effective species have an increased number of down-regulated metabolic compounds in response to pathogen presence, suggesting potential molecular features that may be involved in MIR.

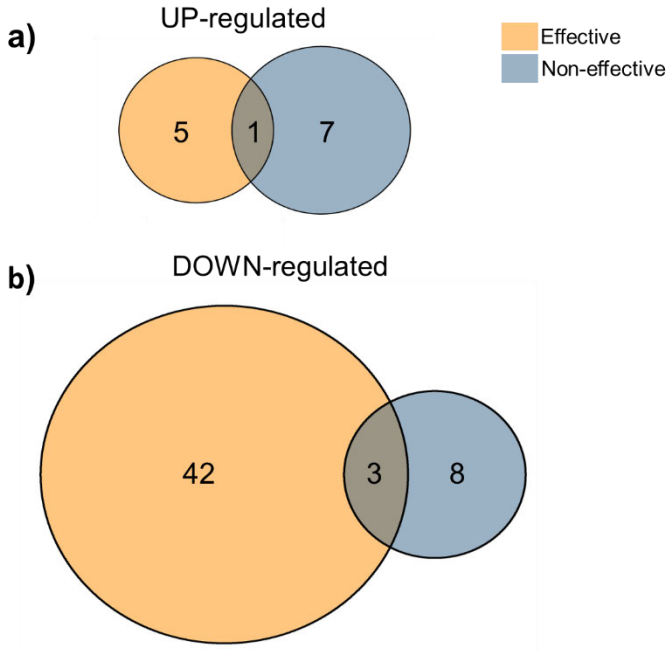


Figure 5. 5: *Venn diagram displaying the amount of overlap among* ***a)*** *up-regulated and* ***b)*** *down-regulated metabolites shared between both effective (highlighted in orange) or non-effective (highlighted in blue) MIR AM fungal species.*

5.3.4 MIR-effective features chemical classification

Further examination of the downregulated features specific to MIR-effective species revealed putative identification for 32 of the 42 features (Table 5.1). Although, the identification revealed a variety of chemical classes, derivates with similar basic structures could be identified within this list.

Three features contain sugar structures including a glycolipid, a sugar steroid derivate and a sugar-phosphate derivate. Two features contained a steroid derivate structure, while a further two contained a fatty acid. Two benzenamide derivates could be identified as well as one benamidazole derivate. Polyketide as well as polyamine derivates were found in two more pairs of downregulated compounds. Three pyranoside derivates (pyranoside, glucopyranoside, galactopyranoside) could be identified within the pool of compounds. Also, one organic sulfonate structure, as well as one organic phosphate structure, are part of the downregulated features in MIR-effective species. Besides, two features were identified as oxygen-containing heterocycle derivate and two as indole derivate. A small range of putative identifications fell into defence-related chemical classes of alkaloids, flavonoids and glucosinolates (piperidine alkaloid, rutin, allyl glucosinolate). The remaining features could be putatively identified as fucoxanthin, nitrogen and oxygen-containing heterocycle derivate, polyglycol derivate, diterpene lactone, aromatic hydrocarbon and amino acid derivate.

Table 5. 1: *List of downregulated features in MIR effective species with corresponding formula and putative identification*

Compound	Formula	Putative identification
7.59_694.4462m/z	$C_{34}H_{64}O_{13}$	Glycolipid
7.81_903.5575m/z	$C_{50}H_{88}O_7S_2$	Sugar steroid derivate
3.25_649.1071m/z	$C_{17}H_{27}N_5O_{17}P_2$	Sugar phosphate derivate
6.79_629.4534m/z	$C_{21}H_{30}O_2$	Steroid derivate
7.19_958.6450m/z	$C_{28}H_{42}O_5$	Steroid derivate
6.17_568.4245m/z	$C_{36}H_{59}O_{2-}$	Fatty acid
5.19_396.2493n	$C_{18}H_{39}NO_6P$-	Fatty acid
7.85_971.5811m/z	$C_{31}H_{38}N_4$	Benzamidazole derivate
6.61_672.4212n	$C_{40}H_{56}N_4O_5$	Benzenamide derivate
7.16_1101.6533m/z	$C_{31}H_{45}N_3O_5$	Benzenamide derivate
7.19_1163.5852m/z	$C_{56}H_{87}N_7O_{15}$	Polyketide derivate
8.14_955.5877m/z	$C_{51}H_{79}NO_{13}$	Polyketide derivate
7.61_650.4199m/z	$C_{29}H_{52}N_{10}O_6$	Polyamine derivate
4.82_428.2389n	$C_{23}H_{32}N_4O_4$	Polyamide derivate
3.89_448.2298n	$C_{21}H_{36}O_{10}$	Glucopyranoside derivate
3.93_442.2780m/z	$C_{21}H_{36}O_7$	Galactopyranoside derivate
6.52_688.4235n	$C_{35}H_{62}NO_{12}$+	Pyranoside derivate
3.25_480.0576	$C_{17}H_{22}NO_{11}S_2$-	Organic sulfonate derivate
7.19_1031.5891m/z	$C_{53}H_{84}NO_{14}P$	Organic Phosphate derivate
6.57_711.4052m/z	$C_{41}H_{62}O_{12}$	Oxygen containing heterocycle derivate
5.05_386.1712n	$C_{22}H_{26}O_6$	Oxygen containing heterocycle derivate
6.92_852.6507m/z	$C_{28}H_{39}NO$	Indole derivate
7.18_482.2938n	$C_{32}H_{38}N_2O_2$	Indole derivate
8.13_993.6470m/z	$C_{28}H_{43}N_3O_4$	Piperidine alkaloid
3.25_610.1535n	$C_{27}H_{30}O_{16}$	Rutin (flavonoid)
3.26_397.9944m/z	$C_{10}H_{17}NO_9S_2$	Allyl glucosinolate
7.60_672.4328m/z	$C_{42}H_{58}O_6$	Fucoxanthin
6.79_749.5152m/z	$C_{38}H_{72}N_2O_{12}$	Nitrogen & oxygen containing heterocycle derivate
5.37_437.2361m/z	$C_{18}H_{38}O_{10}$	Polyglycol derivate
7.19_964.4667m/z	$C_{24}H_{34}O_8$	Diterpene lactone
2.80_784.2327n	$C_{31}H_{41}N_6O_{16}P_{-2}$	Aromatic hydrocarbon
2.23_195.0884n	$C_{10}H_{13}NO_3$	Amino acid derivate

5.4 DISCUSSION

A variety of molecular mechanisms have been suggested to be involved in MIR, and studies have started to investigate the mechanisms supporting this phenomenon (Jung et al., 2012; Kadam et al., 2020; Pozo & Azcón-Aguilar, 2007). As an added complexity, studies are needed that analyse the effect of different AM fungal species on the strength of, and biochemical pathways associated with MIR. This study sought to determine patterns in the metabolomic profile of plant roots colonised with previously defined MIR-effective and non-effective AM fungal species. I could show that the process of mycorrhizal colonisation made the plants more metabolically responsive to pathogen inoculation. However, this responsiveness led to the repression of many metabolic pathways in plants colonised by MIR-effective AM fungal species as compared to MIR-non-effective species. Therefore, there are significant differences in the root metabolomic landscape of plants colonised with MIR effective and non-effective species. My data support the hypothesis that metabolomic profiles are also reflected in effective and non-effective species. These findings suggest that global changes in the plant metabolite composition might be involved in the MIR network and that the downregulation of metabolic compounds might play an important role in enhanced AM fungal-mediated resistance.

5.4.1 MIR is real and there are effective and non-effective species

A recent study demonstrated that increased defence-related gene expression was triggered by AM fungal colonisation, but not by the addition of nitrogen or phosphorus (Stratton et al. 2022). These results show that MIR is not just a side effect of enhanced growth and fitness, but rather that this is a unique response of the plant to the presence of molecular processes in their roots. The AM fungal family of Glomeraceae, including the species *G. mosseae* and *R. irregularis*, have been widely studied and been defined as more effective in eliciting MIR defence responses compared to species from the Gigasporaceae, which includes *G. margarita* and *S. calospora* (Schüßler & Walker, 2010; Spatafora et al., 2016a; Jung et al., 2012; Maherali & Klironomos, 2007). Our results do support these past results (Chapter 4). Increasing evidence reveals that differences in how AM fungal species affect plant health are due to species-specific biochemical changes induced in the plant upon colonisation. A subset of these may lead to enhanced plant resistance, requiring further investigation concerning the underlying processes leading to this phenomenon.

5.4.2 AM colonisation alters root metabolism

Secondary metabolites are a crucial support to plants under biotic stress conditions (Hartmann, 2007). The colonisation process of plant roots by AM fungi alters this secondary metabolism, including defence-related metabolic pathways (Rivero et al., 2015). This study showed that non-mycorrhized plant roots showed more similarity in MIR metabolomic landscape patterns to species from the Gigasporaceae than those from the Glomeraceae, which might explain the varying altered MIR capabilities as reported previously in this thesis and literature (Sikes et al., 2009; Sikes et al. 2010; Maherali & Klironomos, 2007). There is also evidence for the production of secondary metabolomic compounds specifically associated with AM fungal colonisation (Vierheilig, Maier, et al., 2000; Maier et al., 1995), some of which act as defence molecules (Copetta et al., 2006; Kapoor et al., 2002). Overall, in line with previous studies, the metabolomic profiles observed in this study showed AM fungal species-specific alterations (Morandi et al., 1984; Rivero et al., 2015; Copetta et al., 2006; Kapoor et al., 2002). Alterations of defence-related compounds were previously shown to be triggered by *R. irregularis* and *G. mosseae* (Vogt, 2010; Rivero et al., 2015). However, AM fungal mycorrhization in this study led to a general increase in down-regulated molecular features in these AM fungal species, which is comparably less often observed than upregulation (Kaur & Suseela, 2020). Studies have reported upregulation in secondary compounds such as carotenoids, phenolics, anthocyanins, flavonoids and cyclohexanone derivatives in mycorrhized plants (Baslam et al., 2011; Grandmaison et al., 1993; Morandi et al., 1984; Ponce et al., 2004; Toussaint et al., 2007; Adolfsson et al., 2017; Larose et al., 2002; Wang et al., 2018;)(Maier et al., 1997, 2000; Vierheilig, Gagnon, et al., 2000; Maier et al., 1995). Therefore, metabolic compounds related to defences are likely part of shaping these differing MIR metabolic landscapes. However, the current knowledge does not allow linking these findings to MIR effects and to fully understand the common MIR network responses. While variation in plant and pathogen species is undeniably involved part of this network, I showed that MIR-effective AM fungal species influence metabolic changes in a distinct way. This is crucial to recognise MIR-related metabolomic changes and consequently identify the involved key pathways, along with their direction of influence on MIR. My thesis started to reveal underlying patterns in MIR, but further explorations including a variety of plant, pathogen and AM fungal species and communities are needed to identify consistent molecular MIR patterns connected to increased enhanced resistance responses. This will allow deciphering the complex molecular interactions of MIR and contribute to a more targeted utilisation of AM fungi.

5.4.3 Possible mechanisms of metabolite-driven MIR through discussion of main molecular feature classes

A variety of features in this study were putatively identified as compounds that are associated with defence responses and microbial interactions. Chemical classes such as alkaloids, flavonoids and glucosinolates have been detected in mycorrhized plants before, however mostly in an enhanced manner (Hill et al., 2018; Piasecka et al., 2015; Cosme et al., 2014). Different from many studies mentioned, the results presented in this study show that the majority of differentially abundant features in roots colonised by MIR-effective species were down-regulated. In this category, I putatively identified two fatty acids, that play an important part in sustaining the symbiosis between the host plant and AM fungus (Jiang et al., 2017; Keymer et al., 2017; Luginbuehl et al., 2017). Therefore, it was unexpected to find these down-regulated as this may reflect decreased nutrient exchange between plants and fungus. Similarly, I found repression of polyketide derivates, compounds involved in plant defence responses to fungal pathogens (Koskela et al., 2011). Some of the downregulated features in this study were putatively identified as sugar derivates, which likewise have been associated with defence responses to pathogens, including a role as signalling molecules (Morkunas & Ratajczak, 2014). Phytosterols are another well-known class of compounds frequently mentioned with plant defence responses and have been found in the putative identification of downregulated MIR effective molecular features. Plant sterols and brassinosteroids for example have multiple roles in plants including growth, reproduction, and plant defence (Cile Vriet et al., 2012; de Bruyne et al., 2014; Sharfman et al., 2014). Although previous reports showed increased steroid levels associated with increased resistance (Ali et al., 2013; Nakashita et al., 2003; Xiong et al., 2022), increased susceptibility to root pathogens provoked by brassinosteroid application has been observed (de Vleesschauwer et al., 2012; Nahar et al., 2013). A final example of features recorded here as downregulated by MIR-effective AM fungal species are polyamines, a precursor of key secondary metabolites constantly connected to plant defence responses and mediation of such (Takahashi, 2016; Walters, 2000, 2003; Bassard et al., 2010). Majority of previous studies have indeed reported upregulation of compounds, but also decreased levels have been reported, such as some polyamines in pathogen-infected tobacco plants (Walters et al., 1985, 1986; Edreva, 1997). While I cannot determine how these changes are favourable to MIR, it is possible that they are also a reflection of a trade-off occurring whereby some pathways are down-regulated to conserve resources to fight the pathogen. In the case of sterols, it has also been shown that both increased and decreased endogenous sterols can modulate plant defence responses (Belkhadir et al., 2012). With regards to polyamines, Legaz et al. suggest that increased susceptibility could be the consequence of polyamine roles in the detoxification of phenols (Legaz et al., 1998), meaning lower levels of polyamines would increase resistance. Our results may also not be completely counter to

past work. Previous studies report lowered antioxidant compounds such as phenols and flavonoids in Sage upon mycorrhization with *G. mosseae* (Geneva et al., 2010; Maier et al., 2000) report that enhanced secondary metabolites increased disease susceptibility in some models (Shu et al. 2022). Therefore, it could be speculated that AM fungal colonisation triggers the downregulation of certain metabolites that have the potential to increase susceptibility to pathogens which could reciprocally lead to increased resistance in plants.

5.4.4 Mini Conclusion

The demonstration here that MIR-effective AM fungal species change the metabolomic landscape of plants differently compared to non-effective species provides valuable insight into why the role of AM fungi in MIR can be difficult to manage and to see reproducibly across studies. While further in-depth studies are needed to identify each of the common MIR-effective features identified here and prove their role in MIR, these results demonstrate the importance to investigate MIR on the biochemical level. Further investigation of the functional diversity of AM fungal species will then lead to a stronger understanding of the processes that lead to MIR.

CHAPTER SIX GENERAL DISCUSSION

This thesis investigated a variety of contexts for MIR responses, including inoculation with specific pathogens or using natural environmental settings, assessing responses across varying AM fungal colonisation levels, and comparing responses across different plant varieties as well as different AM fungal species in isolation and mixtures. These investigations aimed to further enhance our understanding of MIR, on both a whole-plant level and a molecular level. These findings together with their implications are discussed in the following paragraphs.

6.1 PLANT AND FUNGAL IDENTITY AS AN IMPORTANT INFLUENCE ON MIR OUTCOMES

The findings in this thesis depict the importance of variation in the plant as well as in fungal identity on the MIR outcome. Multiple potential drivers can influence the variation between both partners in this symbiotic interaction including the extent of AM fungal colonisation and/or the plant root architecture (Chen et al., 2021; Gutjahr & Paszkowski, 2013; Treseder, 2013; Sikes et al., 2009; Chen et al., 2021; Gange & Ayres, 1999). In chapters 4 and 5 I observed that AM fungal species led to varying MIR responses in tomato plants with an increase in down-regulated compounds observed in those species with the potential to confer MIR. I also showed in Chapter 3 that changes in AM fungal colonisation levels in different plant varieties led to opposing responses of metabolomic features. The involvement of plant and fungal identity in MIR responses has been suggested previously (Bell et al., 2022; Sikes et al., 2009; Ramos-Zapata et al., 2010; Johnson et al., 1997). It is important to investigate the impact of the diverse layers of influence that plant and fungal identities have on MIR. My thesis has contributed to an enhanced understanding of how the varying abilities of AM fungal partners can influence phenotypic responses, which are also reflected on a metabolomic level. Additionally, I was able to show the important role of plant genotype, as metabolomic landscapes change based on the plant's potential to form a symbiosis with AM fungi. These findings are important, considering the goal of optimising the utilization of AM fungi for enhanced biological control strategies. Previous research suggested family-specific plant responses to colonisation with AM fungal members of the Glomeraceae versus Gigasporaceae (Sikes et al., 2009; Sikes et al. 2010; Maherali & Klironomos, 2007) and these findings suggest higher MIR potential for members of the Glomeraceae including species like *Rhizophagus irregularis* and *Glomus mosseae*. My phenotypical observations in Chapter 4 support these previous observations

and identified species-specific responses to AM fungal colonisation and identified those two species as MIR-effective species. While all single species tested in this thesis had beneficial effects on plant growth, only members of the Glomeraceae showed phenotypically beneficial MIR plant responses (Chapter 4). Moreover, I detected altered metabolomic profiles in MIR effective compared to non-effective species (Chapter 5).

In addition, in Chapter 2, I demonstrated the enhanced influence of MIR-influenced gene expression in the presence of members of the family Glomeraceae in AM fungal communities. These findings further support the concept that certain AM fungal families have stronger MIR abilities and suggest contribution on a molecular level (Sikes et al., 2009; Sikes et al. 2010; Maherali & Klironomos, 2007). I showed that the AM fungal identity, also on a family level, and the type of plant variety alongside the degree of AM fungal colonisation level have an influence on MIR on a phenotypical and molecular level. To better understand the differences in MIR outcomes, future work needs to consider the implementation of different AM fungal species as well as different plant species and varieties. Apart from plant-specific physiological differences, variation in the molecular landscape of mycorrhized plants to non-mycorrhized plants is well-reported (Liu et al., 2007; Tian et al., 2019; Güimil et al., 2005; Song et al., 2015a), and should now be investigated on AM fungal species-specific levels.

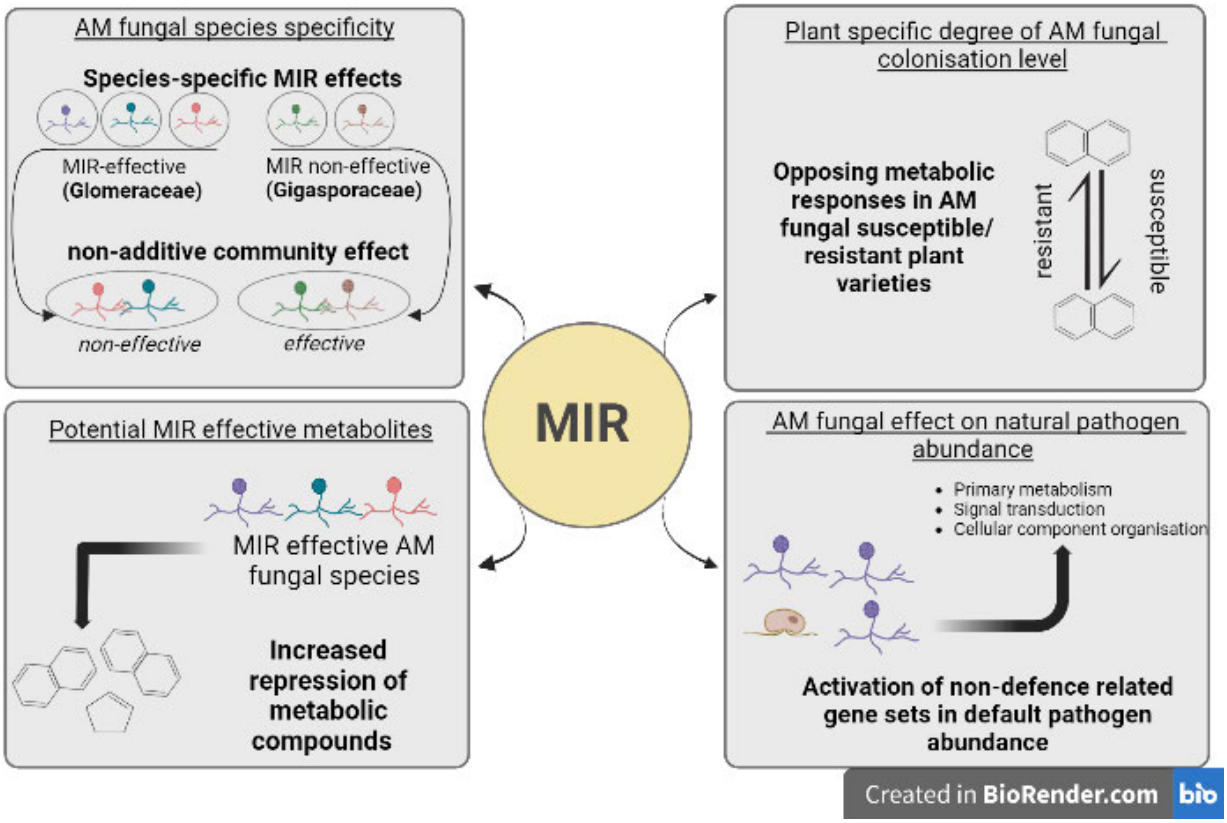


Figure 6. 1: *Illustration of MIR-related findings in this thesis*

6.2 AM FUNGAL COMMUNITIES CHANGE MIR POTENTIAL

The findings in this thesis showed non-additive MIR community responses when compared to single-species responses (Figure 6.1). AM fungi-conferred beneficial effects have been reported in studies utilising community treatments (Hafez et al. 2013; Al-Askar & Rashad 2010; Rahou et al., 2021). Enhanced resistance was observed in a comparison of single species to inoculations with mixed communities in a previous study (Jaiti et al., 2007). Therefore, I expected additive AM fungal community effects, however, these expectations were not met. The experiments in this thesis (Chapter 4) depicted non-additive effects where MIR-effective species were not always able to enhance MIR resistance responses in an additive or synergistic way. On the other hand, the combination of non-effective species led to an increased MIR response. These results showed that observation of MIR-related single-species abilities may not accurately predict community settings in natural environments. The composition of AM fungi in natural communities therefore could be an important driver for MIR and requires consideration in future studies. Competition between diverse AM fungal species could contribute to non-additive effects within communities (Engelmoer et al., 2014b). These natural communities harbour varying proportions of AM fungal species (Alimi et al., 2021), likely including both MIR-effective and non-effective species. Moreover, different abilities to colonise plant roots might influence the impact on MIR responses in the plant. While further studies are needed to better understand the complexity of these interactions, my findings provided important insights into MIR when a common plant is colonised by a diverse AM fungal community. Through a direct comparison between a variety of individual AM fungal species treatments and mixed species treatments, my thesis gave an important insight into the changing capacities of AM fungi and pointed out the importance of investigations within community settings. Future studies should include in-depth analyses of communities including biochemical alterations in plants colonised by multiple AM fungal species. While the investigation of biochemical changes upon colonisation by single species offers important insights into MIR, the non-additive phenotyping results in Chapter 4 showed the importance of comparing findings within community settings. Biochemical modifications could differ in community settings depending on the AM fungal composition. The presence of both MIR-effective and non-effective species might further shape the extent and direction of biochemical changes at the community level.

Chapter 2 depicted the involvement of non-defence-related biological processes on early MIR transcriptomic responses under conditions with reflected natural compositions of microbial communities. In Chapter 3 I also observed contrasting responses of metabolite profiles among plant varieties that mirrored differences in their AM fungal colonisation potential. In natural

environments, the plant rhizosphere usually harbours complex microbial communities, including different types of pathogens and AM fungi (Mendes et al., 2013). The unpredictable change in how single AM fungi versus AM mixtures affect MIR, together with the findings of the influence of plant type and natural abundance of microbes, demonstrates the need to investigate a variety of experimental settings such as AM fungal composition and plant varieties that could also influence the colonisation abilities of AM fungi. This thesis started to investigate some of these issues, however, certain limitations such as the integration of more diverse and complex AM fungal communities could be investigated in future studies and other stress conditions could be tested. Conducting investigations including larger AM fungal communities, especially those collected from field sites would provide important data to understand MIR. In particular, isolating single species from these same communities and directly contrasting them with the complexity of natural communities could be useful in understanding AM fungal-mediated resistance. A direct comparison of fungal pathogens including bacteria, nematodes and viruses and even including abiotic stress conditions could enhance our understanding of the varying capacities of AM fungi to mediate resistance against a range of stressors. Research regarding MIR could be taken to the next step by enhancing the knowledge regarding AM fungal community-mediated MIR.

6.3 MIR IN NATURAL PATHOGEN ABUNDANCE SITUATIONS DOES NOT REFLECT CONVENTIONAL DEFENCE RESPONSES

One goal of this thesis was to expand the understanding of MIR-related molecular pathways including transcriptional levels. Relating gene expression in *B. distachyon* to Oomycete abundance and AM fungal colonisation in roots (Chapter 2), I found that AM fungi presence influenced gene expression of biological processes that are associated with growth and maintenance more than those connected to plant defence responses. These results indicated distinct MIR plant responses to reduced pathogen levels, compared to previously tested high pathogen abundance conditions. Interestingly, previous research has shown that AM fungal-conferred MIR is most beneficial under high pathogen abundance (Sikes et al., 2009). Studies performed with high pathogen pressure showed transcriptomic changes in *Medicago trunculata* including elevated defence gene transcripts in shoots of mycorrhized plants and therewith connected enhanced protection against *Xantomonas campestris* (Liu et al., 2007). The low pathogen conditions used in my thesis, modelled after natural environments without imminent pathogen pressure, imply a pathogen-load-dependent alteration in MIR-related transcriptional responses. This furthermore could pinpoint the importance of resource allocation linked to priming and underscore the role of trade-off plays in MIR. My work found that transcriptional responses of biological processes previously associated with AM fungal colonisation

were more responsive to an inoculum of pathogenic and beneficial microbes taken directly from a soil environment, compared to responses of processes frequently linked to defence responses (Figure 6.1).

Natural and managed environments usually harbour a broad diversity of AM fungal communities and are not restricted to a single or few species (Alimi et al., 2021) and, along with the phenotypic responses discussed above, my findings reiterate the importance of implementing additional natural context into MIR research. The enhancement of defence-related molecular plant responses in mycorrhized plants has been reported frequently (Hill et al., 2018; Piasecka et al., 2015; Cosme et al., 2014). The involvement of down-regulated metabolic compounds in MIR responses in two of my chapters (Chapters 3 & 5) could however indicate that MIR is a much more complex process with the involvement of non-conventional defence-related responses. Previous observations of MIR involve improved plant performance upon pathogen attack, local and systemic defence responses and a frequent mention of the phenomenon of priming (Pozo et al. 2002; Trotta et al. 1996; Fritz et al. 2006). Although priming could be one possible explanation leading to these results, I found here that the low abundance of pathogenic microbes in AM presence affects gene sets not primarily connected to defence responses. Metabolomic profiling of the priming phase suggests the central involvement of compounds from the primary metabolism in priming for defences (Pastor et al., 2014). These findings reflect the importance of primary biological processes in gene expression related to AM fungal presence. To detangle the complex network of MIR and tackle involved pathways inclusion of simultaneous phenotypic observations and physiological responses as well as downstream processes such as gene expression and the impact on biochemical changes, under different environmental conditions and in different symbiotic stages. Future studies should include primary pathways, such as targeted analysis of genes, proteins and metabolites related to primary metabolism, and investigate their link to MIR.

6.4 The role of Down-regulated biochemical pathways in MIR

So far, most previous studies reported an increase in metabolite abundance upon AM fungal colonisation (Kaur & Suseela, 2020). It is accepted that the enhancement of defence-related compounds is involved in the MIR network, however, not much attention was paid to the involvement of down-regulated metabolic compounds in AM fungal-mediated defence responses. Therefore, their role in the regulation of defence responses might have been overlooked and it cannot be ruled out that the metabolomic regulation of the MIR network might be also strongly influenced by downregulated compounds. The findings in this study showed the repression of

metabolic compounds in species that also depicted phenotypical evidence for MIR, which reflects the potentially important role of down-regulated compounds in MIR. This discovery is an important contribution to MIR research, highlighting an aspect of molecular mechanisms in MIR that has not received enough attention so far.

The experiments from two chapters in this thesis (Chapter 3, Chapter 5) showed the possible involvement of metabolic repression during MIR (Figure 6.1). The results in the chickpea experiment (Chapter 3) showed opposing metabolomic responses in two varieties with differing AM fungal colonisation level potential. In addition, as shown in Chapter 5 the fact that MIR-effective AM fungal species were associated with stronger repression of tomato root metabolic pathways suggests that repression of biochemical pathways might play an important role in MIR. Although only a few previous studies have reported the downregulation of metabolic compounds upon AM fungal colonisation, these results are in line with this observation (Geneva et al., 2010; Maier et al., 2000). I would speculate that the mechanism by which this may occur is via the influence of AM effector proteins. Genomic studies predict a variety of potential AM fungal secreted effector-like proteins that might play an important role in this symbiosis (Zeng et al., 2018), and a vast number of common as well as specific putative secreted proteins between *R. irregularis* and *Gigaspora rosea* (Kamel et al., 2017). Previous studies have already identified several AM fungal effectors (Kloppholz et al., 2011; Tsuzuki et al., 2016; Voß et al., 2018; Wang et al., 2021) two of which translocate into the host nucleus and subsequently suppress defence responses (Wang et al., 2021; Kloppholz et al., 2011). This scheme could apply to the manipulation of metabolic pathways, including compounds that are more favourable in downregulated conditions under defence.

6.5 No significant evidence for trade-offs connected to MIR

Nutrient exchange as the main effect of the AM fungal symbiosis and changes in chemical compounds from the primary metabolic pathway have been reported linked to induced plant resistance (Pastor, Balmer, et al., 2014). Although a recent study showed that MIR can function uncoupled from nutritional effects (Pozo de la Hoz et al., 2021), MIR metabolic profiles were shown to be strongly altered but still functional upon nitrogen depletion (Sanchez-Bel et al., 2016). The similarity between AM fungal colonisation and pathogen infection, and the fact that AM fungi do not always have positive effects but can also result in zero or negative effects in growth and defence, depict the potential for trade-off effects regarding growth or defence-related benefits (Grace et al., 2009, Koide, 1985; Pieterse et al., 2014; Güimil et al.,2005; Pieterse et al., 2012; Wang

et al., 2012). The low-cost benefits of MIR reflect the huge potential of AM fungi as biocontrol agents, however, there is a need to understand the mechanisms to allow efficient and appropriate usage (Jacott et al., 2017; Filho, 2022). Therefore, the consideration and investigation of potential trade-off effects of MIR is an important part of future MIR research. By measuring alteration in nutrient composition within the plant to complement phenotypic observations, enhanced understanding of MIR-related trade-offs in MIR could enable to establish a link to phenotypes.

I investigated the variation in phenotypically enhanced plant defence responses mediated by different symbiotic AM fungal species and communities and their growth-related trade-offs (Chapter 4). This thesis did not observe phenotypically related evidence of trade-offs in the presence of MIR. MIR responses varied in the presence of different AM fungal species without significant observable trade-off effects and the presence of AM fungal communities depicted non-additive effects. Although I was not able to detect signs of growth-related trade-offs in plants mediated by AM fungi, other studies have previously reported disadvantageous impacts of AM fungi on plant growth and also on disease development (Gange & Ayres, 1999; Johnson et al., 1997; Liu et al., 2018; Miozzi et al., 2019). As I did not determine nutrient concentrations, I cannot rule out eventual impacts from different AM fungal species. Therefore, further research should be undertaken including nutrient allocation in varying plant tissues colonised with varying AM fungal species, to confirm trade-off theories in different environmental settings including a variety of plant species and AM fungal species.

6.6 Conclusion and future directions

In conclusion, the results of this thesis provide new insight into several MIR aspects (Figure 6.1). Phenotypical MIR species-specific effects confirmed typical AM fungal family responses previously observed and strengthened the theory of strong Glomeraceae members having stronger MIR potential (Sikes et al., 2009; Sikes, 2010; Sikes et al. 2010; Maherali & Klironomos, 2007). Systemic insight into the involved biochemical mechanisms and whether the metabolomic landscape is reflecting these observations confirmed these patterns and additionally revealed the potential importance of down-regulated metabolic compounds involved in MIR. The family of Glomeraceae also showed an increase in non-defence-related gene sets, while known defence-related genes were hardly influenced. These findings show the importance of considering the variation in plant-fungal interactions that exists within natural and managed environments to fully understand the molecular machinery of MIR and its impact on plant fitness. Natural environments always contain a plethora of different AM fungal species, depending on the environment (Mendes et al., 2013). The fact that AM

fungal communities showed non-additive MIR responses emphasizes the complexity of MIR, but also the importance of investigating not just single AM fungal species effects but also AM fungal community effects that reflect natural environments. Furthermore, the opposing responses in the metabolite profile of plant varieties and therewith connected alterations of AM fungal colonisation level to pathogen presence confirm MIR molecular modifications and suggest that it might be useful to consider plant varieties with higher beneficial microbe compatibility in crop production.

It is important to keep in mind that although it is essential to understand the underlying molecular mechanisms, MIR is a highly complex phenomenon that also includes physiological changes. Different physical defences like waxy structures (cuticula) on the outer cell surface, trichomes on the epidermis, and the accumulation of minerals like silicon in epidermal cells can be promoted by AM fungi (Serrano et al., 2014; Reynolds et al., 2009; Frew et al., 2016; Barton, 2016; Vannette & Hunter, 2013; Calo et al., 2006). Beneficial microbes also compete with pathogens for space and nutrients as they colonise the same habitat (Ghorbanpour et al., 2018). AM fungal colonisation can lead to a change in plant root exudates (Jung et al., 2012; López-Ráez et al., 2010). Decreased mycotoxin production of a soil-borne pathogen was observed in the tissue of potato plants upon mycorrhiza colonisation (Ismail et al., 2013). Other microbes such as endobacteria in AM fungi and mycorrhiza helper bacteria have therefore supported effects on bioprotection (Cruz & Ishii, 2012; Cameron et al., 2013). Studies found that mycorrhizal networks can communicate with neighbouring plants via an underground messaging system about an existing attack and therefore transfer resistance and induce defence signals to the infected plant (Giovannetti et al., 2001; Song et al., 2010; Babikova et al., 2013). This exchange of information within AM fungal species might enhance the ability to improve plant growth during a pathogen attack and the mechanism that leads to disease suppression might differ drastically in communities compared to single species. Therefore, the extent of mechanisms beneficial microbes can improve plant health such as morphological and nutritional changes, interaction with other microbes in the rhizosphere and competition shows that MIR is not just a complex phenomenon on a molecular basis but an ensemble of physiological and molecular events (Weng et al., 2022). It will be important to implement more factors into future studies that add complexity, as well as assessments of combinations of molecular and physiological responses in these studies, in order to understand these dimensions of MIR.

As sustainability awareness rises, the importance of plant-microbe interactions for crop production is becoming increasingly important. The identification of mechanisms in plant-microbe interactions is essential to improve the understanding and use of AM fungi in agricultural systems and to maintain and improve healthy soils (Vishwakarma et al., 2020; Basu et al., 2018; Jacott et al., 2017;

Averill et al., 2022). While the findings in my thesis contributed important knowledge to the complex topic of MIR, further comprehensive research focusing on the molecular landscape as well as in-depth investigations of potentially involved molecular players is as important as the incorporation of natural settings and AM community effects on MIR. The combination of omics and targeted analysis in controlled as well as field conditions will provide acquaintances that subsequently allow the efficient and sustainable use of AM fungi.

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CHAPTER 2: SUPPLEMENTARY INFORMATION

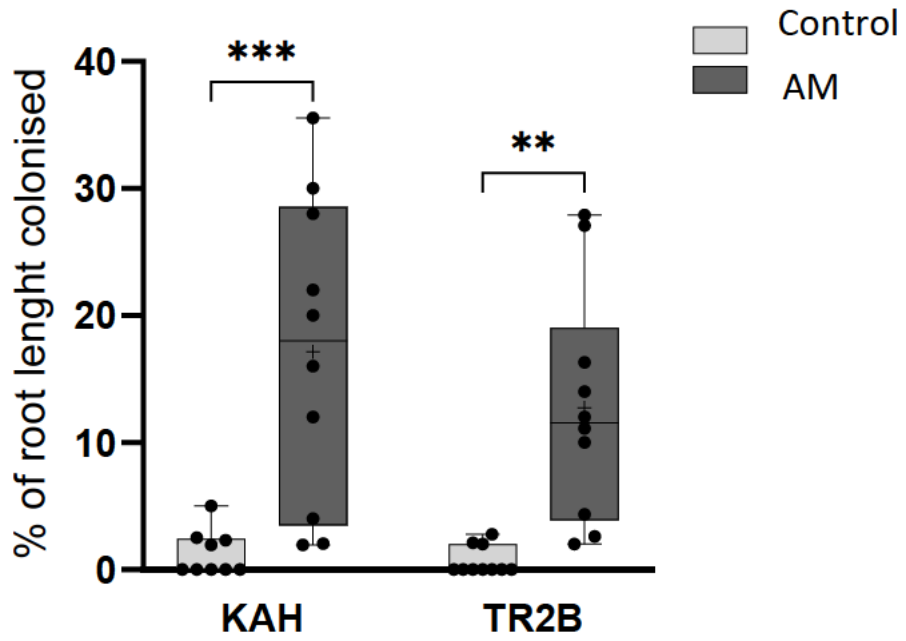


***Figure S2.1**: AM fungal root colonisation in* B. distachyon *lines KAH and TR2B. Two-way ANOVA revealed 46% of variation explained by treatment (p=0.0001). KAH adjusted p-value = 0.0002, TR2B adjusted p-value = 0.0027. Dots represent single measurements, lines represent minimum, maximum values and median, + represents the mean value.*

Table S2.1: *Proportion explained by CCA1, CCA2 and CCA3 in each of the GO-Term classified biological processes*

Biological process	CCA1	CCA2	CCA3
Primary metabolic compounds	0.501358	0.286843	0.211799
Signal transduction	0.436711	0.359156	0.204132
Cellular component	0.55939	0.281925	0.15868
Response to biotic stimulus	0.48215	0.29905	0.2188
Secondary metabolic compounds	0.565243	0.361023	0.073735
Hormone metabolic process	0.566834	0.257589	0.175577

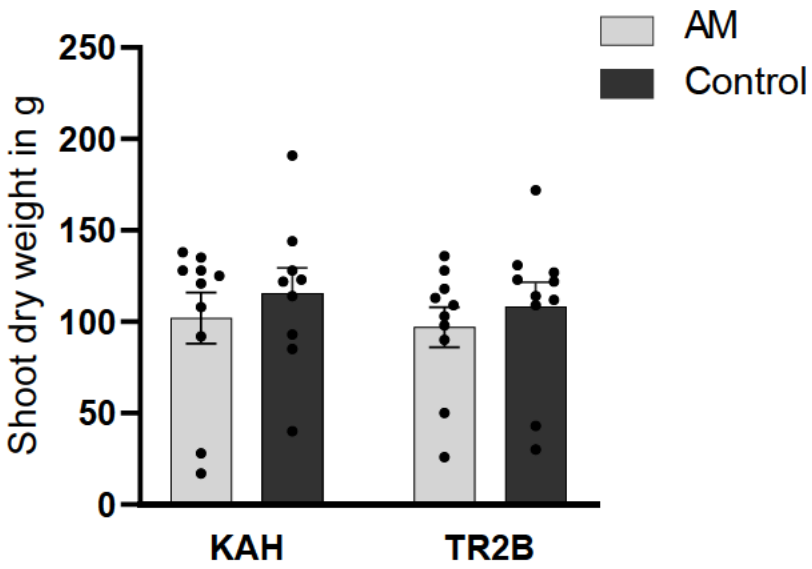


Figure S2.2: *Shoot biomass of both* B. distachyon *lines (KAH, TR2B) under AM fungal inoculated conditions and control conditions. Dots represent a single measurement.*

Table S2.2: *Information of MIR-influential genes in the biological process "Primary metabolic compounds".*

Gene ID	Gene name	GO Molecular Function
Bradi_4g40620v3	Uncharacterized protein	cysteine-type endopeptidase activity(GO:0004197);peptidase activity(GO:0008233); cysteine-type peptidase activity(GO:0008234);hydrolase activity(GO:0016787)
Bradi_2g46400v3	Uncharacterized protein	polygalacturonase activity(GO:0004650);hydrolase activity(GO:0016787);hydrolase activity, acting on glycosyl bonds(GO:0016798)
Bradi_1g35317v3	Uncharacterized protein	mRNA binding(GO:0003729);protein tag(GO 0031386);ubiquitin protein ligase binding(GO:0031625)
Bradi_3g16790v3	Protein kinase domain-containing protein	protein kinase activity(GO 0004672);ATP binding(GO:0005524)
Bradi_3g17403v3	F-box domain-containing protein	
Bradi_4g42700v3	RING-type domain-containing protein	ubiquitin protein ligase activity(GO 0061630)
Bradi_1g66510v3	Uncharacterized protein	structural constituent of ribosome(GO:0003735)
Bradi_1g18920v3	Uncharacterized protein	nucleotide binding(GO:0000166);nucleic acid binding(GO:0003676);RNA binding(GO 0003723); RNA helicase activity(GO:0003724);helicase activity(GO:0004386);ATP binding(GO:0005524);hydrolase activity(GO:0016787)
Bradi_3g45000v3	Uncharacterized protein	structural constituent of ribosome(GO:0003735);mRNA 5'-UTR binding(GO:0048027);small ribosomal subunit rRNA binding(GO:0070181)
Bradi_1g67020v3	D-fructose-1,6-bisphosphate 1-phosphohydrolase	hydrolase activity(GO:0016787);phosphatase activity(GO:0016791);fructose 1,6-bisphosphate 1-phosphatase activity(GO:0042132) ;phosphoric ester hydrolase activity(GO 0042578);metal ion binding(GO:0046872)
Bradi_1g36740v3	Uncharacterized protein	transferase activity(GO:0016740);glycosyltransferase activity(GO:0016757); cellulose synthase activity(GO 0016759);cellulose synthase (UDP-forming) activity(GO 0016760)
Bradi_1g28520v3	Protein kinase domain-containing protein	nucleotide binding(GO:0000166);protein kinase activity(GO:0004672); protein serine/threonine kinase activity(GO 0004674);cyclin-dependent protein serine/threonine kinase activity(GO 0004693); ATP binding(GO:0005524);RNA polymerase II CTD heptapeptide repeat kinase activity(GO 0008353);kinase activity(GO:0016301);transferase activity(GO:0016740)
Bradi_1g64090v3	Uncharacterized protein	phosphatidylinositol-3-phosphate binding(GO:0032266);phosphatidylinositol-3,5-bisphosphate binding(GO:0080025)
Bradi_5g04010v3	Protein kinase domain-containing protein	protein kinase activity(GO 0004672);cyclin-dependent protein serine/threonine kinase activity(GO 0004693); ATP binding(GO:0005524);RNA polymerase II CTD heptapeptide repeat kinase activity(GO 0008353)
Bradi_2g05260v3	IPPc domain-containing protein	catalytic activity(GO 0003824);phosphatidylinositol-4,5-bisphosphate 5-phosphatase activity(GO:0004439); inositol-polyphosphate 5-phosphatase activity(GO 0004445);hydrolase activity(GO:0016787); phosphatase activity(GO:0016791);phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase activity(GO:0034485)
Bradi_1g21230v3	Cyclin N-terminal domain-containing protein	cyclin-dependent protein serine/threonine kinase regulator activity(GO:0016538)
Bradi_2g55150v3	Uncharacterized protein	hydrolase activity(GO:0016787);phosphatase activity(GO:0016791); fructose 1,6-bisphosphate 1-phosphatase activity(GO:0042132);phosphoric ester hydrolase activity(GO:0042578);metal ion binding(GO 0046872)
Bradi_3g06350v3	Uncharacterized protein	arylformamidase activity(GO:0004061)
Bradi_3g19216v3	RING-type domain-containing protein	zinc ion binding(GO:0008270);ubiquitin protein ligase activity(GO 0061630)
Bradi_2g56180v3	Uncharacterized protein	serine-type endopeptidase activity(GO:0004252);peptidase activity(GO 0008233);serine-type peptidase activity(GO:0008236);hydrolase activity(GO:0016787)
Bradi_2g51310v3	LOB domain-containing protein	
Bradi_2g13410v3	Uncharacterized protein	tRNA dimethylallyltransferase activity(GO:0052381)
Bradi_2g50815v3	AA_kinase domain-containing protein	kinase activity(GO:0016301)
Bradi_2g57940v3	GRAS domain-containing protein	DNA-binding transcription factor activity(GO 0003700);sequence-specific DNA binding(GO:0043565)
Bradi_1g69760v3	Uncharacterized protein	polygalacturonase activity(GO:0004650);hydrolase activity(GO:0016787);hydrolase activity, acting on glycosyl bonds(GO:0016798)
Bradi_5g23060v3	Receptor-like serine/threonine-protein kinase	nucleotide binding(GO:0000166);protein kinase activity(GO:0004672); protein serine/threonine kinase activity(GO 0004674);ATP binding(GO:0005524);kinase activity(GO:0016301);transferase activity(GO:0016740)
Bradi_2g49196v3	RING-type domain-containing protein	ubiquitin protein ligase activity(GO 0061630)
Bradi_2g42280v3	Uncharacterized protein	

Table S2.3: *Information of MIR-influential genes in the biological process "Signal transduction".*

Gene ID	Gene name	GO Molecular Function
Bradi_1g58491v3	Tify domain-containing protein	
Bradi_1g35390v3	Uncharacterized protein	
Bradi_3g42770v3	HPt domain-containing protein	
Bradi_2g06960v3	Uncharacterized protein	
Bradi_1g06270v3	Uncharacterized protein	calcium ion binding(GO:0005509);calmodulin binding(GO 0005516);ATP binding(GO:0005524); calcium-dependent protein serine/threonine kinase activity(GO:0009931);kinase activity(GO:0016301);transferase activity(GO:0016740);metal ion binding(GO:0046872);;
Bradi_3g22510v3	LOB domain-containing protein	
Bradi_4g10998v3	Protein kinase domain-containing protein	ATP binding(GO:0005524);kinase activity(GO:0016301);transferase activity(GO:0016740)
Bradi_2g52870v3	Uncharacterized protein	calcium ion binding(GO:0005509);calmodulin binding(GO 0005516);ATP binding(GO:0005524); calcium-dependent protein serine/threonine kinase activity(GO:0009931);kinase activity(GO:0016301);transferase activity(GO:0016740);metal ion binding(GO:0046872);;
Bradi_2g31200v3	Uncharacterized protein	
Bradi_3g28710v3	Uncharacterized protein	ATP binding(GO:0005524);kinase activity(GO:0016301);transferase activity(GO:0016740)
Bradi_1g62710v3	Uncharacterized protein	
Bradi_2g21390v3	Uncharacterized protein	calcium ion binding(GO:0005509);calmodulin binding(GO 0005516);ATP binding(GO:0005524); calcium-dependent protein serine/threonine kinase activity(GO:0009931);kinase activity(GO:0016301);transferase activity(GO:0016740);metal ion binding(GO:0046872);;
Bradi_3g23180v3	Tify domain-containing protein	
Bradi_4g39870v3	Uncharacterized protein	calcium ion binding(GO:0005509);calmodulin binding(GO 0005516);ATP binding(GO:0005524); calcium-dependent protein serine/threonine kinase activity(GO:0009931);kinase activity(GO:0016301);transferase activity(GO:0016740);metal ion binding(GO:0046872);;
Bradi_2g15610v3	Auxin efflux carrier component	
Bradi_2g44987v3	Auxin efflux carrier component	
Bradi_2g37621v3	Protein kinase domain-containing protein	kinase activity(GO:0016301);transferase activity(GO:0016740)
Bradi_2g58917v3	Auxin efflux carrier component	
Bradi_1g37304v3	Protein kinase domain-containing protein	kinase activity(GO:0016301);transferase activity(GO:0016740)
Bradi_1g36977v3	Protein kinase domain-containing protein	
Bradi_2g51310v3	LOB domain-containing protein	
Bradi_1g56950v3	VQ domain-containing protein	
Bradi_3g23190v3	Tify domain-containing protein	
Bradi_3g41080v3	Uncharacterized protein	
Bradi_3g41770v3	Uncharacterized protein	calcium ion binding(GO:0005509);calmodulin binding(GO 0005516);ATP binding(GO:0005524); calcium-dependent protein serine/threonine kinase activity(GO:0009931);kinase activity(GO:0016301);transferase activity(GO:0016740);metal ion binding(GO:0046872);;
Bradi_2g52530v3	Uncharacterized protein	
Bradi_2g42920v3	Uncharacterized protein	

***Table S2.4:** Information of MIR-influential genes in the biological process "Cellular component organisation.*

Gene ID	Gene name	GO Molecular Function
Bradi_1g28513v3	Clathrin light chain	structural molecule activity(GO:0005198);clathrin heavy chain binding(GO:0032050)
Bradi_1g74170v3	Exocyst complex component	
Bradi_1g57240v3	Uncharacterized protein	
Bradi_4g25572v3	Protein kinase domain-containing protein	nucleotide binding(GO:0000166);protein kinase activity(GO 0004672);structural molecule activity(GO:0005198); ATP binding(GO:0005524);kinase activity(GO:0016301);transferase activity(GO 0016740);clathrin light chain binding(GO 0032051)
Bradi_3g14050v3	Uncharacterized protein	phosphatidylinositol-3-phosphate binding(GO:0032266);phosphatidylinositol-3,5-bisphosphate binding(GO:0080025)
Bradi_2g31611v3	Uncharacterized protein	structural constituent of ribosome(GO:0003735)
Bradi_3g40510v3	Exocyst subunit Exo70 family protein	phosphatidylinositol-4,5-bisphosphate binding(GO:0005546)
Bradi_4g41980v3	Exocyst subunit Exo70 family protein	phosphatidylinositol-4,5-bisphosphate binding(GO:0005546)
Bradi_3g18412v3	30S ribosomal protein S19, chloroplastic	RNA binding(GO:0003723);structural constituent of ribosome(GO 0003735)
Bradi_3g51430v3	Uncharacterized protein	protein transmembrane transporter activity(GO:0008320);transmembrane transporter activity(GO 0022857)
Bradi_2g07721v3	Uncharacterized protein	GTPase activity(GO:0003924);GTP binding(GO 0005525);protein kinase binding(GO:0019901)
Bradi_3g45000v3	Uncharacterized protein	structural constituent of ribosome(GO:0003735);mRNA 5'-UTR binding(GO:0048027);small ribosomal subunit rRNA binding(GO:0070181)
Bradi_1g61810v3	Uncharacterized protein	double-stranded DNA binding(GO 0003690)
Bradi_1g36740v3	Uncharacterized protein	transferase activity(GO 0016740);glycosyltransferase activity(GO:0016757);cellulose synthase activity(GO 0016759); cellulose synthase (UDP-forming) activity(GO:0016760)
Bradi_1g64090v3	Uncharacterized protein	phosphatidylinositol-3-phosphate binding(GO:0032266);phosphatidylinositol-3,5-bisphosphate binding(GO:0080025)
Bradi_3g10870v3	Uncharacterized protein	
Bradi_5g14930v3	G-patch domain-containing protein	nucleic acid binding(GO:0003676)
Bradi_3g27289v3	Ribosomal protein S7	RNA binding(GO:0003723);mRNA binding(GO:0003729);structural constituent of ribosome(GO:0003735);rRNA binding(GO:0019843)
Bradi_1g21230v3	Cyclin N-terminal domain-containing protein	cyclin-dependent protein serine/threonine kinase regulator activity(GO:0016538)
Bradi_4g44730v3	eRF1_1 domain-containing protein	translation release factor activity(GO:0003747);translation release factor activity, codon specific(GO:0016149); sequence-specific mRNA binding(GO:1990825)
Bradi_1g74109v3	DUF223 domain-containing protein	damaged DNA binding(GO:0003684);single-stranded telomeric DNA binding(GO:0043047)
Bradi_3g35771v3	Uncharacterized protein	ATP binding(GO:0005524);hydrolase activity(GO:0016787);ATP hydrolysis activity(GO:0016887);polyubiquitin modification-dependent protein binding(GO:0031593)
Bradi_2g60940v3	DUF223 domain-containing protein	damaged DNA binding(GO:0003684);single-stranded telomeric DNA binding(GO:0043047)
Bradi_4g09970v3	Uncharacterized protein	nucleotide binding(GO:0000166);DNA binding(GO 0003677);ATP binding(GO:0005524); ATP-dependent activity, acting on DNA(GO:0008094);nucleosome binding(GO:0031491);ATP-dependent chromatin remodeler activity(GO 0140658)
Bradi_1g25150v3	Uncharacterized protein	transferase activity(GO 0016740);glycosyltransferase activity(GO:0016757);cellulose synthase (UDP-forming) activity(GO 0016760); mannan synthase activity(GO:0051753)

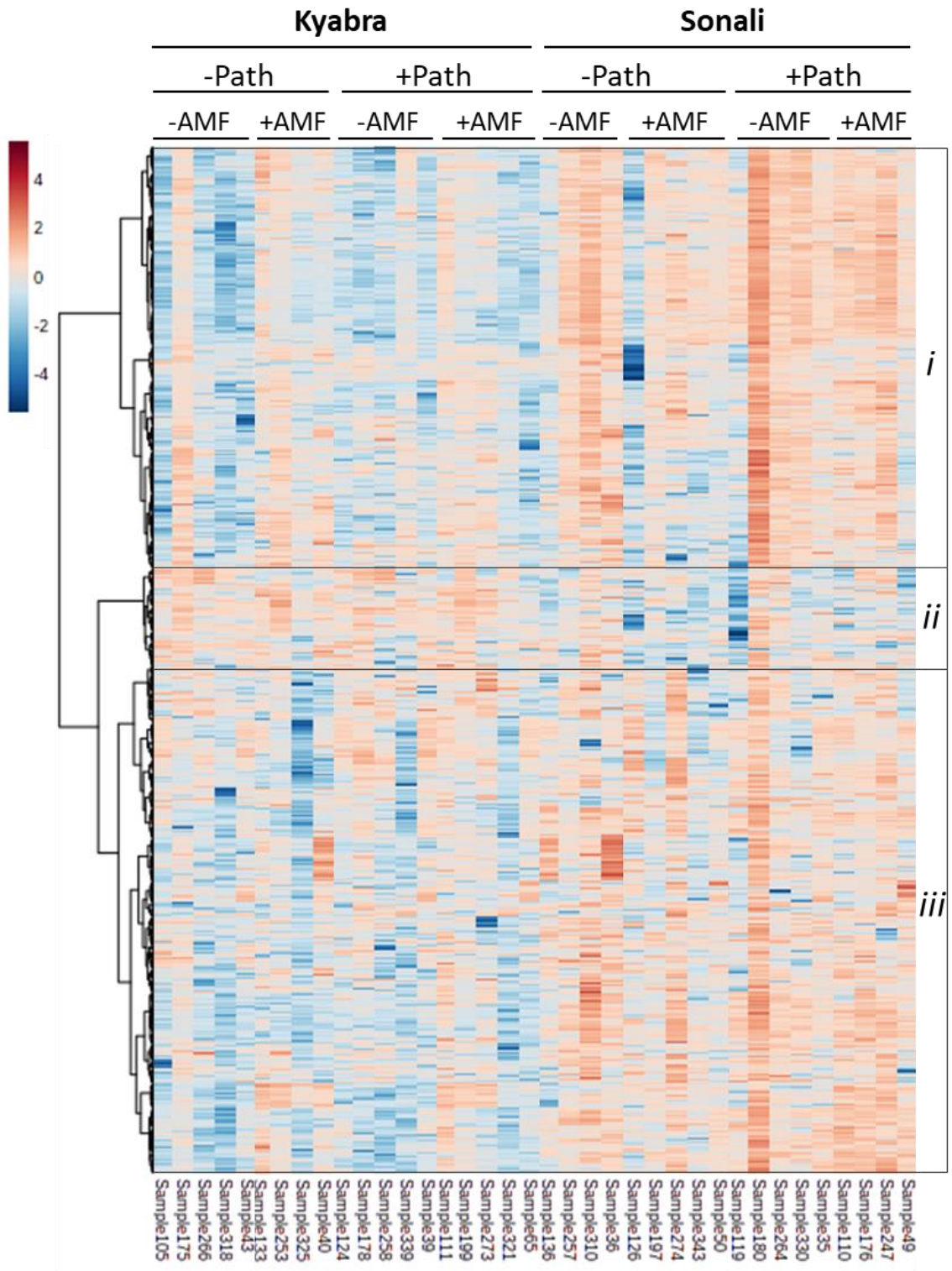


Figure S3.1: *Hierarchical clustering of the normalized data points of all 300 significant featuress in negative ion mode, detected in the two varieties Kyabra and Sonali with (+AMF) or without (-AMF) AM fungal inoculation and/or pathogen treatment with Phytophthora medicaginis (+/-Path) ranked by ANOVA (P<0.05) using log10 transformed and weight-specific normalized data. Euclidean distance measure and Ward method were used for clustering. Red values indicate high metabolite occurrence while blue indicates low metabolite occurrence.*

Table S3.1: *Information of the ANOVA and post-hoc results for each of the 143 analysed significant feature*

Compound number	Molecular weight	f.value	p.value	LOG10(p)	FDR
1	4.66_311.0539m.z	12.652	2.55E-07	6.5942	0.000144
2	4.93_681.0824m.z	11.72	5.58E-07	6.2537	0.000144
3	4.84_657.0706m.z	11.427	7.19E-07	6.143	0.000144
4	2.80_248.0666n	10.918	1.13E-06	5.9458	0.00017
5	5.16_202.1326n	9.7286	3.46E-06	5.4605	0.000409
6	4.75_305.0983m.z	9.5602	4.09E-06	5.3888	0.000409
7	4.40_162.0134m.z	8.7094	9.69E-06	5.0137	0.000681
8	4.93_694.0605n	8.6808	9.98E-06	5.0007	0.000681
9	4.41_607.1787m.z	8.6587	1.02E-05	4.9907	0.000681
10	3.85_198.5102m.z	8.3366	1.44E-05	4.8424	0.000804
11	3.83_299.2067m.z	8.3136	1.47E-05	4.8317	0.000804
12	4.93_221.0188n	8.1198	1.82E-05	4.7407	0.000908
13	4.75_573.1683m.z	7.7409	2.76E-05	4.5591	0.001219
14	4.62_440.0052m.z	7.6191	3.17E-05	4.4996	0.001219
15	4.40_190.5129m.z	7.6188	3.17E-05	4.4995	0.001219
16	5.12_314.0410m.z	7.5445	3.44E-05	4.463	0.001219
17	0.65_618.1008n	7.5212	3.54E-05	4.4515	0.001219
18	4.65_281.2234m.z	7.4916	3.66E-05	4.4368	0.001219
19	4.18_198.5103m.z	7.393	4.09E-05	4.3879	0.001293
20	3.57_312.1579m.z	7.197	5.14E-05	4.2894	0.001541
21	4.45_155.4995m.z	7.0893	5.82E-05	4.2347	0.001664
22	5.14_874.1734n	6.9775	6.65E-05	4.1775	0.001812
23	4.93_133.4809n	6.8194	8.02E-05	4.0957	0.002093
24	5.97_553.1157m.z	6.7176	9.07E-05	4.0424	0.002254
25	3.83_215.0321m.z	6.6888	9.39E-05	4.0273	0.002254
26	3.29_434.9997m.z	6.2294	0.000165	3.7814	0.003705
27	5.10_437.0851m.z	6.2233	0.000167	3.7781	0.003705
28	4.67_630.1709n	6.1751	0.000177	3.7517	0.003796
29	4.59_204.9956m.z	6.0856	0.000198	3.7026	0.004104
30	4.90_870.1802n	6.0492	0.000208	3.6824	0.004155
31	5.18_451.0458m.z	5.9538	0.000235	3.6295	0.004542
32	4.93_512.9713n	5.7661	0.000299	3.5242	0.005608
33	5.57_539.1240m.z	5.7375	0.00031	3.508	0.005645
34	4.93_132.9877n	5.6869	0.000332	3.4792	0.005684
35	4.17_597.1020m.z	5.6753	0.000337	3.4726	0.005684
36	2.77_313.0138m.z	5.6659	0.000341	3.4672	0.005684
37	4.30_328.2237n	5.6078	0.000368	3.4341	0.005969
38	4.10_402.2606n	5.4737	0.00044	3.3568	0.006943
39	5.07_350.0987n	5.4375	0.000462	3.3358	0.006972
40	3.37_742.1444n	5.4245	0.00047	3.3282	0.006972
41	3.33_673.2013n	5.4138	0.000476	3.322	0.006972
42	5.87_234.1238n	5.3521	0.000518	3.286	0.007394
43	4.93_330.6592m.z	5.3317	0.000532	3.2741	0.007423

44	3.33_132.0408n	5.3143	0.000545	3.2639	0.007427
45	3.07_508.1798m.z	5.2688	0.000579	3.2371	0.007514
46	3.53_533.3838m.z	5.2587	0.000587	3.2312	0.007514
47	3.31_961.2096m.z	5.2452	0.000598	3.2232	0.007514
48	4.18_301.2267m.z	5.2278	0.000612	3.2129	0.007514
49	5.15_562.1264m.z	5.2263	0.000614	3.2121	0.007514
50	5.82_574.1801n	5.1439	0.000687	3.1632	0.008242
51	4.02_317.2210m.z	5.1052	0.000724	3.1401	0.008459
52	5.30_255.0920m.z	5.0963	0.000733	3.1348	0.008459
53	4.92_223.0378m.z	5.0704	0.00076	3.1193	0.008583
54	4.35_373.0889m.z	5.0558	0.000775	3.1106	0.008583
55	5.18_584.0861m.z	5.0451	0.000787	3.1041	0.008583
56	4.66_374.9995m.z	4.9712	0.000872	3.0597	0.009231
57	3.59_338.1343m.z	4.9668	0.000877	3.057	0.009231
58	4.82_665.0747m.z	4.943	0.000906	3.0426	0.009378
59	4.07_315.2080m.z	4.9213	0.000934	3.0295	0.009389
60	4.97_151.0735m.z	4.9178	0.000939	3.0274	0.009389
61	4.26_618.1362n	4.8234	0.001072	2.9699	0.010541
62	3.29_401.9823n	4.7835	0.001134	2.9455	0.010971
63	4.88_326.0153n	4.7696	0.001156	2.937	0.01101
64	3.61_563.1051m.z	4.7226	0.001236	2.9081	0.011584
65	3.63_175.5075m.z	4.6713	0.001329	2.8764	0.012269
66	3.67_803.0933m.z	4.64	0.00139	2.8571	0.012634
67	3.31_537.0242m.z	4.6226	0.001425	2.8463	0.012759
68	5.97_539.1302m.z	4.58	0.001515	2.8197	0.013363
69	2.59_552.1161m.z	4.5475	0.001587	2.7995	0.013799
70	3.22_370.9832m.z	4.5202	0.001651	2.7824	0.013865
71	2.27_368.9900m.z	4.5169	0.001659	2.7803	0.013865
72	5.75_368.2532n	4.5147	0.001664	2.7789	0.013865
73	2.96_494.0756n	4.5028	0.001693	2.7715	0.013912
74	5.22_299.2796n	4.3884	0.001998	2.6994	0.016144
75	5.58_599.1532m.z	4.3816	0.002018	2.6951	0.016144
76	4.40_659.0433m.z	4.3661	0.002064	2.6853	0.016296
77	3.83_565.3342m.z	4.3276	0.002184	2.6608	0.017017
78	5.45_188.0485n	4.3153	0.002224	2.6529	0.017104
79	6.15_563.0970m.z	4.2883	0.002313	2.6358	0.01757
80	4.92_283.2089m.z	4.2668	0.002388	2.622	0.01791
81	4.07_408.9853n	4.2455	0.002464	2.6084	0.018084
82	3.25_316.2079n	4.2388	0.002488	2.6041	0.018084
83	5.36_372.0831n	4.2353	0.002502	2.6018	0.018084
84	0.58_355.1884n	4.2017	0.002629	2.5803	0.018602
85	5.09_381.0830n	4.2001	0.002635	2.5792	0.018602
86	3.39_336.0459n	4.1915	0.002669	2.5737	0.01862
87	4.81_405.9975n	4.1636	0.002782	2.5557	0.019183
88	3.52_462.3317n	4.1486	0.002844	2.546	0.019298
89	6.03_219.1356m.z	4.1443	0.002863	2.5432	0.019298

90	3.37_359.0728m.z	4.1063	0.003029	2.5186	0.020121
91	3.96_192.5744n	4.1014	0.003052	2.5155	0.020121
92	2.54_302.9928m.z	4.0927	0.003091	2.5098	0.020139
93	3.62_250.9873n	4.0811	0.003146	2.5023	0.020139
94	4.64_327.0450m.z	4.079	0.003155	2.501	0.020139
95	3.80_842.1918n	4.0291	0.003401	2.4684	0.021477
96	3.57_312.1334n	4.0029	0.003537	2.4514	0.022027
97	5.65_582.1164n	3.9979	0.003564	2.4481	0.022027
98	4.87_349.1487m.z	3.9916	0.003598	2.444	0.022027
99	3.63_154.9943m.z	3.9262	0.003971	2.4011	0.024066
100	5.20_316.2433m.z	3.8457	0.004488	2.348	0.026925
101	5.56_496.2089n	3.8359	0.004555	2.3415	0.02706
102	4.87_312.2274n	3.8268	0.004619	2.3355	0.027168
103	1.98_242.6463m.z	3.767	0.005061	2.2958	0.029482
104	2.00_225.0832m.z	3.7123	0.005505	2.2593	0.0317
105	2.28_404.7250m.z	3.7022	0.005591	2.2525	0.0317
106	4.83_320.9481n	3.7011	0.0056	2.2518	0.0317
107	5.81_552.1337m.z	3.6709	0.005867	2.2316	0.032901
108	3.93_356.1114n	3.6566	0.005999	2.222	0.033265
109	0.60_315.1553m.z	3.6518	0.006043	2.2187	0.033265
110	3.80_620.1496m.z	3.6124	0.006424	2.1922	0.035038
111	4.80_378.0923n	3.5742	0.006817	2.1664	0.036843
112	0.55_242.6463m.z	3.5685	0.006877	2.1626	0.036843
113	4.02_400.0590m.z	3.5602	0.006967	2.1569	0.036994
114	3.67_592.1583m.z	3.5488	0.007092	2.1492	0.037326
115	3.95_204.0401n	3.5159	0.007466	2.1269	0.038955
116	2.30_374.2267m.z	3.4861	0.007823	2.1067	0.040058
117	2.42_387.2357n	3.4821	0.007871	2.104	0.040058
118	3.85_398.9859m.z	3.4767	0.007938	2.1003	0.040058
119	2.05_566.2827n	3.4762	0.007945	2.0999	0.040058
120	6.04_605.3862m.z	3.4397	0.008413	2.075	0.041481
121	3.37_406.2657n	3.4373	0.008445	2.0734	0.041481
122	4.16_409.0214n	3.4371	0.008447	2.0733	0.041481
123	4.93_173.5143n	3.4329	0.008504	2.0704	0.041481
124	4.48_397.9718m.z	3.4167	0.008724	2.0593	0.041506
125	2.28_426.2358n	3.4156	0.008738	2.0586	0.041506
126	2.25_237.1707n	3.4146	0.008752	2.0579	0.041506
127	2.33_378.0742n	3.4122	0.008786	2.0562	0.041506
128	2.20_484.2481n	3.4035	0.008907	2.0503	0.041633
129	0.58_261.6362m.z	3.4003	0.008951	2.0481	0.041633
130	6.04_517.3337m.z	3.3807	0.009233	2.0347	0.042381
131	2.87_372.9995m.z	3.3746	0.009322	2.0305	0.042381
132	5.17_587.1777m.z	3.3745	0.009324	2.0304	0.042381
133	5.27_869.1804m.z	3.3605	0.009533	2.0208	0.042838
134	2.23_374.1610n	3.3582	0.009567	2.0192	0.042838
135	0.56_342.1764m.z	3.3504	0.009686	2.0138	0.04305

136	5.45_523.1360m.z	3.3426	0.009806	2.0085	0.043263
137	5.68_494.2443n	3.3087	0.010348	1.9851	0.045321
138	4.87_232.0950n	3.297	0.010542	1.9771	0.045774
139	2.33_441.2209n	3.2933	0.010604	1.9745	0.045774
140	3.75_637.0537m.z	3.2628	0.011131	1.9535	0.047365
141	0.96_254.0629n	3.2624	0.011138	1.9532	0.047365
142	4.53_449.9690n	3.2584	0.01121	1.9504	0.047365
143	3.54_600.0181n	3.2298	0.011733	1.9306	0.049231

Table S3.2: *Number of up/down regulated features in all three treatment comparisons in Kyabra and Sonali, in positive ion mode. Downregulated features are shaded in blue.*

	Treatment contrast	Number of MFs	
		KYABRA	SONALI
Positive mode	Pathogen vs Control	15	36
		15	25
	AMF vs Control	10	79
		82	2
	AMF vs Pathogen	10	46
		63	5

Table S3.3: *Number of up/down regulated features in all three treatment comparisons in Kyabra and Sonali, in negative ion mode. Downregulated features are shaded in blue.*

	Treatment contrast	Number of MFs	
		KYABRA	SONALI
Negative mode	Pathogen vs Control	19	24
		9	84
	AMF vs Control	29	148
		156	18
	AMF vs Pathogen	35	217
		164	8

Table S3.4: *Fresh weight of root pieces in gramms used for each sample to normalise the data for feature analysis with univariate analysis (ANOVA) followed by a post-hoc analysis (Fisher's LSD, p-value 0.05)value 0.05)*

Sample Name	Weight in gramm
2_K_A_1	0.0629
2_K_A_2	0.0286
2_K_A_4	0.0345
2_K_A_5	0.0984
2_K_AP_1	0.0398
2_K_AP_2	0.0253
2_K_AP_3	0.0311
2_K_AP_4	0.0181
2_K_AP_5	0.0498
2_K_P_1	0.0237
2_K_P_2	0.0359
2_K_P_3	0.0183
2_K_P_4	0.0253
2_K_P_5	0.0489
2_K_X_1	0.0304
2_K_X_2	0.0461
2_K_X_3	0.0389
2_K_X_4	0.0342
2_K_X_5	0.0399
2_S_A_1	0.0516
2_S_A_2	0.0106
2_S_A_3	0.0554
2_S_A_4	0.0101
2_S_A_5	0.0352
2_S_AP_1	0.0322
2_S_AP_2	0.025
2_S_AP_3	0.0269
2_S_AP_4	0.0148
2_S_P_1	0.0246
2_S_P_2	0.0293
2_S_P_3	0.0092
2_S_P_4	0.0259
2_S_P_5	0.0302
2_S_X_1	0.0354
2_S_X_2	0.0533
2_S_X_4	0.035
2_S_X_5	0.0087

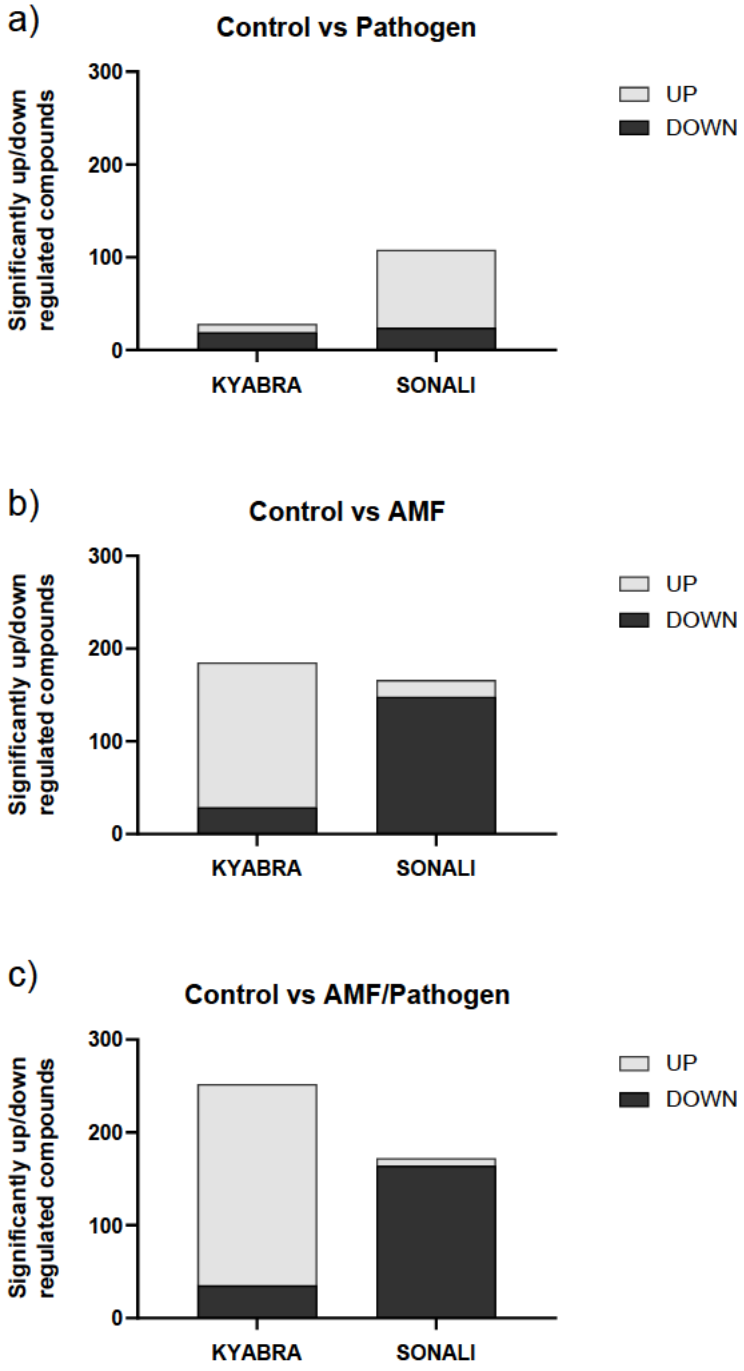


Figure S3.2: *Analysis of the number of features in each of the three treatment contrasts in negative ion mode.* ***a)*** *Pathogen contrast (Contr vs Path treatment)* ***b)*** *AMF contrast (Contr vs AMF treatment)* ***c)*** *tripartite contrast of AMF/Pathogen (Control vs AMF/Path treatment)*

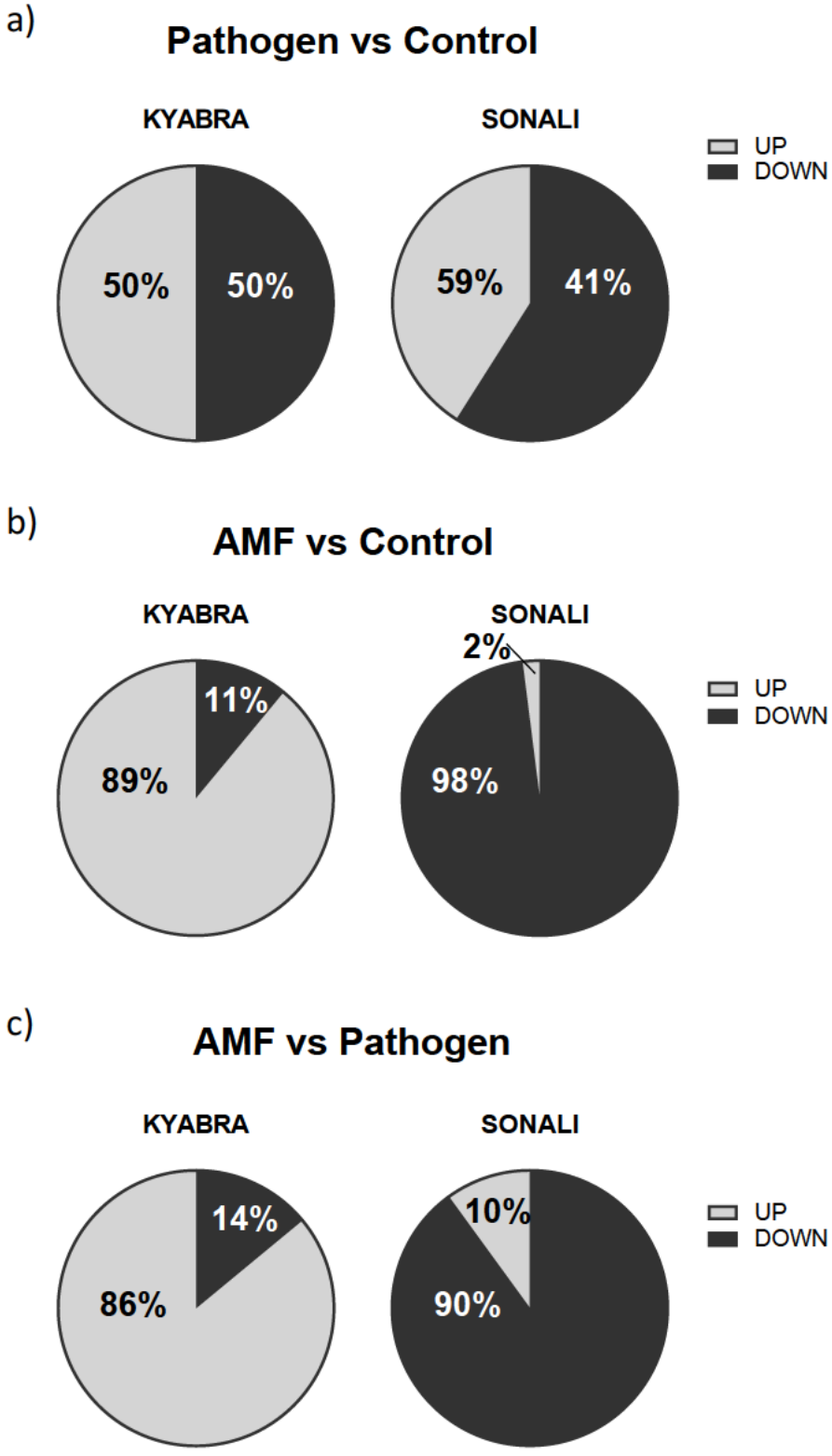


Figure S3.3: *Analysis of the percentage of features in each of the three treatment contrasts.* ***a)*** *Pathogen contrast (Contr vs Path treatment)* ***b)*** *AMF contrast (Contr vs AMF treatment)* ***c)*** *tripartite contrast of AMF/Pathogen (Control vs AMF/Path treatment)*

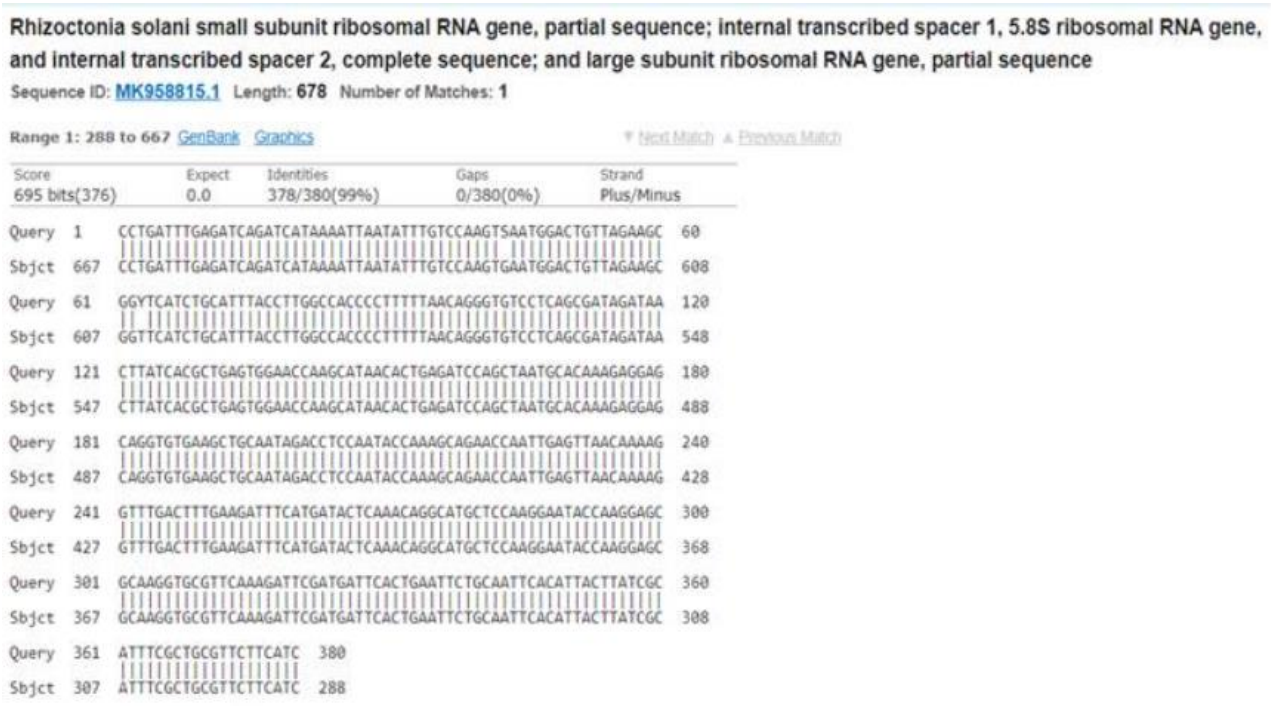
Table S3.5: *Information of the ANOVA and post-hoc results for each of the 28 analysed significant putatively identified molecular features*

Compound number	Molecular features	Molecular weight	f.value	p.value	-log10(p)	FDR
1	Aromatic hydrocarbon	2.28_426.2358n	3.4156	0.0087382	2.0586	0.0099431
2	Unknown	0.55_242.6463m.z	3.5685	0.0068773	2.1626	0.0099431
3	Aromatic derivate	1.98_242.6463m.z	3.7672	0.0050597	2.2959	0.0099431
4	Steroid derivate	2.20_484.2481n	3.4035	0.0089071	2.0503	0.0099431
5	Aromatic derivate	0.58_261.6362m.z	3.4003	0.0089511	2.0481	0.0099431
6	Aromativ hydrocarbon	2.33_441.2209n	3.2933	0.010604	1.9745	0.010604
7	Indol derivate	0.58_355.1884n	4.2017	0.0026287	2.5803	0.0077681
8	Aromativ derivate	0.56_342.1764m.z	3.3504	0.0096862	2.0138	0.010045
9	Organophosphate	2.05_566.2827n	3.4762	0.0079448	2.0999	0.0099431
10	Polyol	2.28_404.7250m.z	3.7022	0.0055908	2.2525	0.0099431
11	Aromatid pyrimidine derivate	0.60_315.1553m.z	3.6518	0.0060432	2.2187	0.0099431
12	Carboxylic acid	3.37_406.2657n	3.4373	0.0084447	2.0734	0.0099431
13	Unknown	6.04_517.3337m.z	3.3807	0.0092329	2.0347	0.0099431
14	Pyrethroid	6.04_605.3862m.z	3.4397	0.0084134	2.075	0.0099431
15	Polyol	4.93_330.6592m.z	5.3318	0.000532	3.2741	0.0025417
16	Peptide	5.16_202.1326n	9.7286	3.46E-06	5.4605	4.85E-05
17	Unknown	4.83_320.9481n	3.7011	0.0056003	2.2518	0.0099431
18	Carboxyl acid	3.33_132.0408n	5.3143	0.0005447	3.2639	0.0025417
19	Polyol	4.84_657.0706m.z	11.427	7.19E-07	6.143	2.01E-05
20	Unknown	3.96_192.5744n	4.1014	0.0030517	2.5155	0.0077681
21	Unknown	4.45_155.4995m.z	7.0893	5.82E-05	4.2347	0.0005437
22	Carboxylic acid	6.03_219.1356m.z	4.1443	0.0028625	2.5433	0.0077681
23	Polyol	5.75_368.2532n	4.5147	0.0016638	2.7789	0.0058232
24	1-O-feruloyl-beta-D-glucose	3.93_356.1114n	3.6566	0.0059985	2.222	0.0099431
25	Unknown	3.29_401.9823n	4.7835	0.0011337	2.9455	0.0045348
26	Aromatic derivate	3.67_592.1583m.z	3.5488	0.0070918	2.1492	0.0099431
27	Polyol	5.10_437.0851m.z	6.2233	0.0001667	3.7781	0.0011669
28	Carboxylic acid	3.95_204.0401n	3.5159	0.0074663	2.1269	0.0099431

CHAPTER 4: SUPPLEMENTARY INFORMATION

Table S4. 1: *Pathogen (*Rhizoctonia solani*) culture information*

Accession NO	Qualified Name	Qualified Name: (Host Details)	Organ	Common Name	Host Family	Precise Location	Town	State	Country	Collection Date	Collectors	Determiner	Living Culture	DNA
DAR 61830	Rhizoctonia solani	Lycopersicon esculentum "UC84"	Fruit rot	Tomato	Solanaceae	property, Field 1	Cowra	NSW	Australia	16/03/1988	Letham, Dr David	Letham, Dr David	Freeze dried	NO



Rhizoctonia solani small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

Sequence ID: MK958815.1 Length: 678 Number of Matches: 1

Range 1: 288 to 667 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
695 bits(376)	0.0	378/380(99%)	0/380(0%)	Plus/Minus

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Query  1    CCTGATTTGAGATCAGATCATAAAATTAATATTTGTCCAAGTSAATGGACTGTTAGAAGC  60
            ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  667  CCTGATTTGAGATCAGATCATAAAATTAATATTTGTCCAAGTGAATGGACTGTTAGAAGC  608

Query  61   GGYTCATCTGCATTTACCTTGGCCACCCCTTTTTAACAGGGTGTCCTCAGCGATAGATAA  120
            || |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  607  GGTTCATCTGCATTTACCTTGGCCACCCCTTTTTAACAGGGTGTCCTCAGCGATAGATAA  548

Query  121  CTTATCACGCTGAGTGGAACCAAGCATAACACTGAGATCCAGCTAATGCACAAAGAGGAG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547  CTTATCACGCTGAGTGGAACCAAGCATAACACTGAGATCCAGCTAATGCACAAAGAGGAG  488

Query  181  CAGGTGTGAAGCTGCAATAGACCTCCAATACCAAAGCAGAACCAATTGAGTTAACAAAAG  240
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Sbjct  487  CAGGTGTGAAGCTGCAATAGACCTCCAATACCAAAGCAGAACCAATTGAGTTAACAAAAG  428

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Query  301  GCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGC  360
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Figure S4. 1: *Blast search alignment obtained from sanger sequencing of the* R. solani *cultures used in this study*

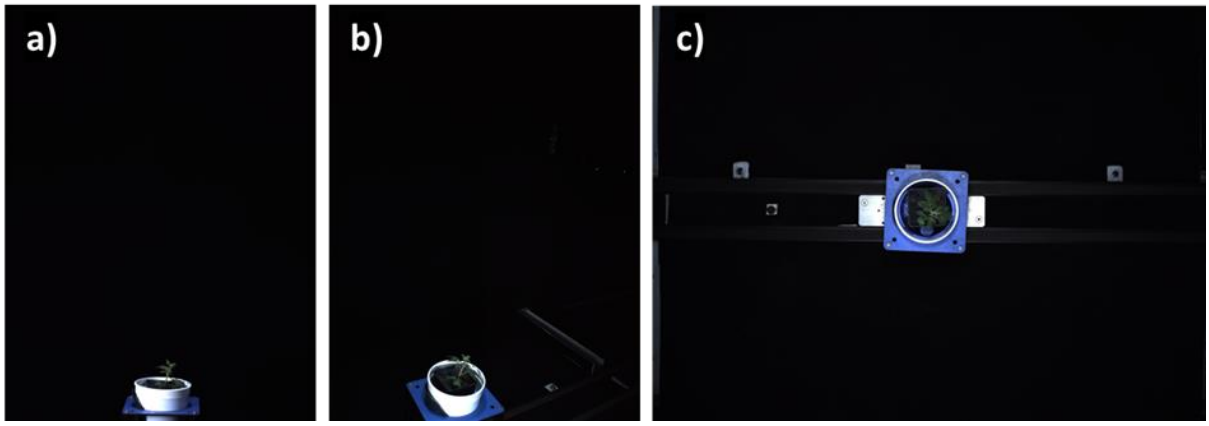


Figure S4. 2*: Red Blue Green images used to calculate the projected shoot area from* ***a)*** *side view* ***b)*** *side view 80° angle and* ***c)*** *top view****.***

Table S4. 2: *Pathogen re-isolation from tomato root and stem tissue on PDA + streptomycin agar plates with or without pathogen addition after 9 days of growth at room temperature.*

Sample treatments	Agar plate	Pathogen addition	Pathogen growth after 9 days
R. irregularis 23	PDA + Streptomycin	YES	YES
R. irregularis 10 + 23	PDA + Streptomycin	YES	YES
	PDA + Streptomycin	YES	NO
S. calospora	*PDA*	YES	YES
All	PDA + Streptomycin	YES	NO
S. calospora	PDA + Streptomycin	NO	NO
No AMF	PDA + Streptomycin	NO	NO

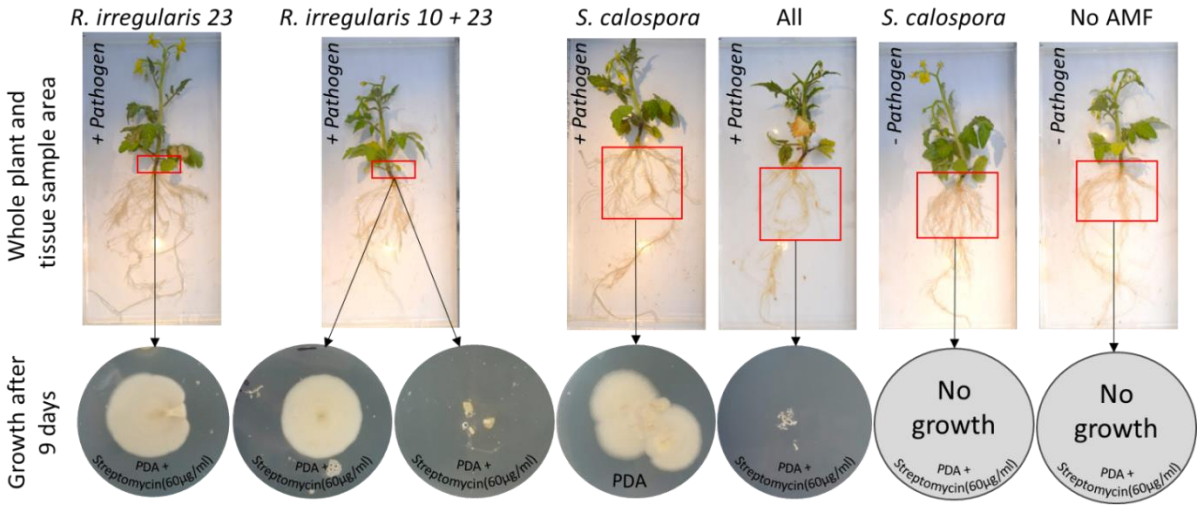


Figure S4. 3: *Illustration of pathogen reisolating from the different samples (top row) with and without pathogen addition. Red squares indicate the sample area of shoot/root tissue or both. The bottom row shows the growth of* Rhizoctonia solani *on Potato dextrose agar (PDA) plates, with and without added Streptomycin from the harvested.*

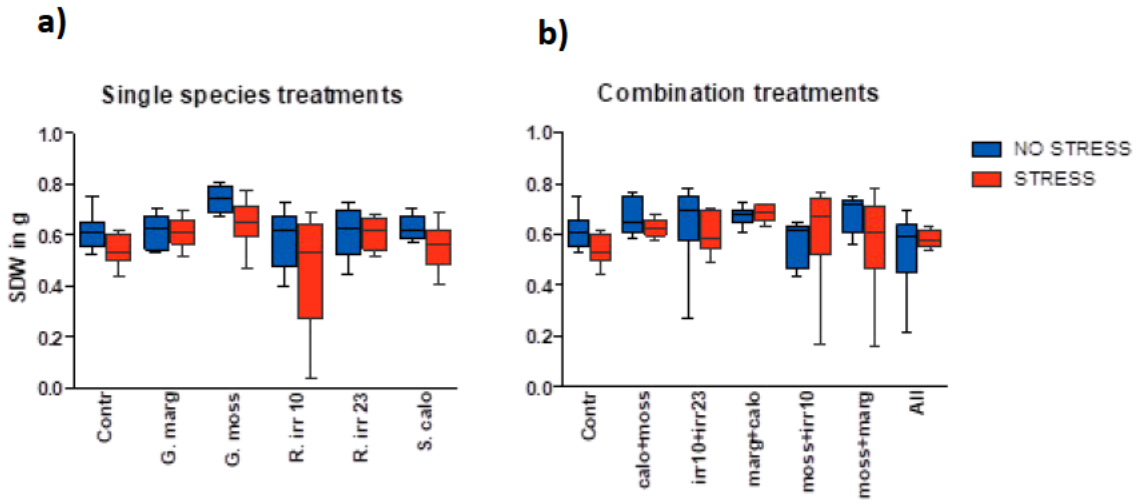


Figure S4. 4: *Shoot dry weight data of all treatments of* ***a)*** *single species treatments and* ***b)*** *combination treatments. Whiskers show 10-90% percentile, and the black line shows the median.*

Table S4. 3 : *ANOVA (type II), R_2= 0.7321992, tested responses of PSA with different variables, Significant value in bold, Signif. codes: 0 *** 0.001 ** 0.01 * 0.05 . 0.1 ‘ ’ 1*

Tested Variables	*Sum*	*Df*	*F value*	*Pr(>F)*	*Significance*
SDW	**4972.7**	**1**	**168.4874**	**<2e-16**	***
Mycorrhiza	352.2	12	0.9945	0.4596	
Stress	25.7	1	0.8698	0.3532	
SDW:Mycorrhiza	515	12	1.4543	0.1544	
SDW:Stress	3	1	0.1021	0.75	
Mycorrhiza:Stress	266.7	12	0.753	0.6965	
SDW:Mycorrhiza:Stress	353.1	12	0.9971	0.4573	
Residuals	2980.9	101			

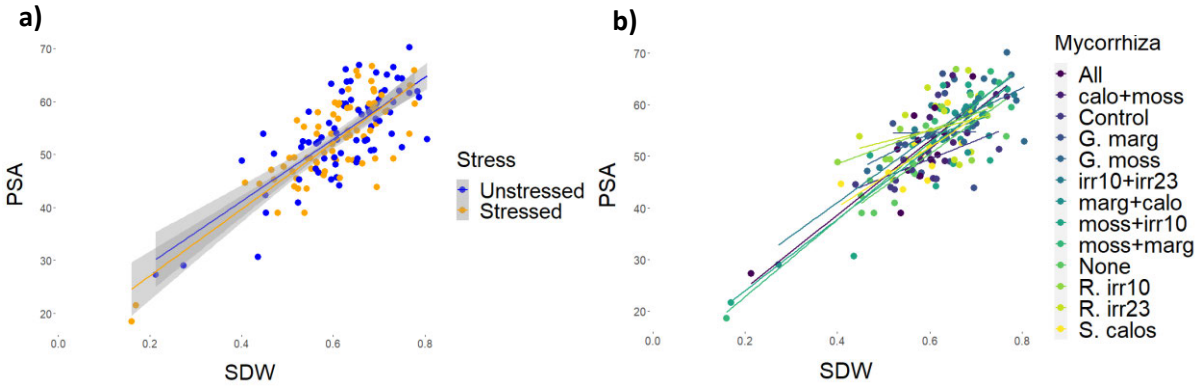
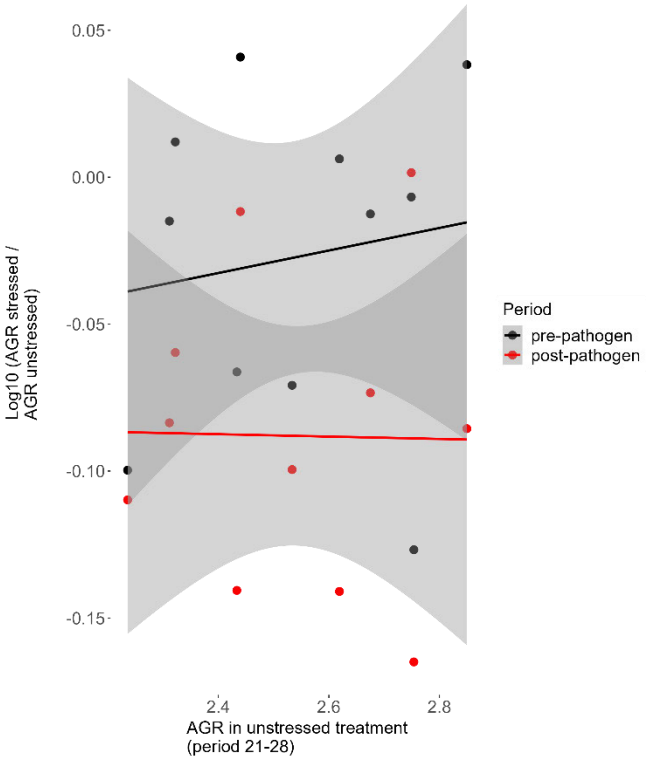


Figure S4.5: *Linear regression model of projected shoot area (PSA) and shoot dry weight (SDW)* ***a)*** *under stressed and unstressed conditions* ***b)*** *with all AM fungal treatments.*



Supplementary Figure S4.6: *Log10 of the absolute growth response in stressed versus unstressed conditions. The pre-pathogen period reflects days 28-34, post-pathogen addition reflects days 34-39.*

***Table S5.1**: Total number of up-and downregulated features in the control and each of the five AM fungal single species treatments, divided into effective and non-effective species.*

	control	effective			non-effective	
Compounds	**no AMF**	**G. moss**	**R. irr23**	**R. irr.10**	**G. marg**	**S. calo**
DOWN	25	324	113	202	205	118
UP	130	159	114	141	45	99
Total	155	483	227	343	250	217

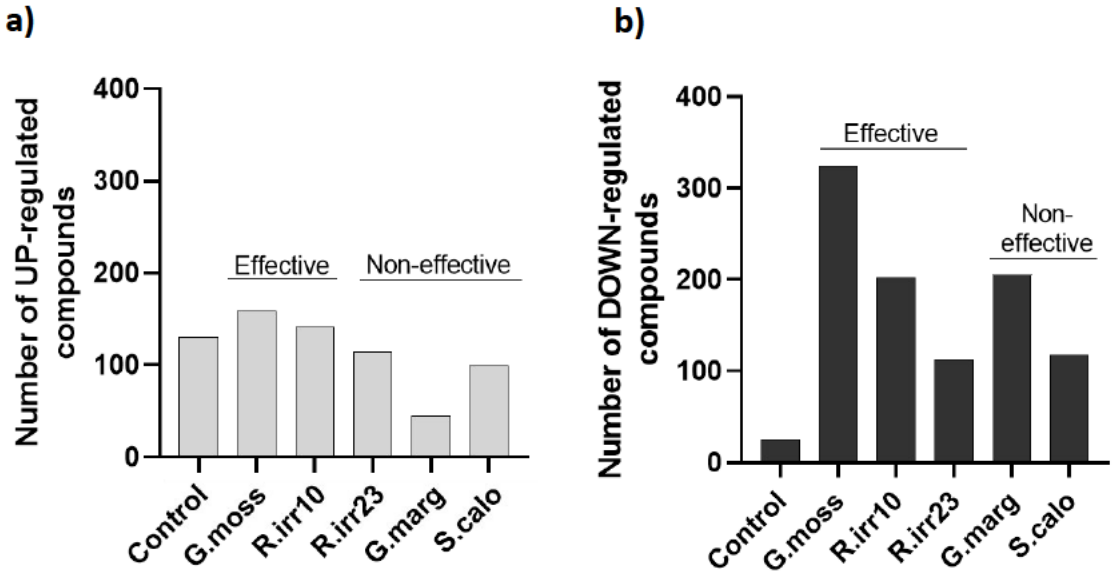


Figure S5.1: *Number of* ***a)*** *Up-regulated and* ***b)*** *down-regulated metabolomic compounds in response to the pathogen treatment in control and all four selected single species AM fungal inoculation treatments. Effective and non-effective species are highlighted above the bars.*