Symbiotic interactions between the parasitic weed *Striga hermonthica* and the arbuscular mycorrhizal fungus *Rhizophagus irregularis* in rice

A thesis submitted by

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Declaration

No portion of the work referred to in this thesis has been submitted in support of another application for another degree or qualification at this or any other university or institute of learning.

Abstract

The parasitic weed *Striga hermonthica* and the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* are both obligate root symbionts that appear to have opposing functions *in planta*. *S. hermonthica* is a parasite that presents a serious threat to subsistence agriculture in sub-Saharan Africa (SSA), whereas *R. irregularis* can enhance host fitness by providing nutrients and inducing plant defence against a range of pathogens and parasites. Previous research into the interaction between AM fungi and *Striga* has found a suppressive effect of the fungus on parasite infection. This has been attributed to a reduction in parasite-seed germinating strigolactones (SLs) released from the host roots as a result of AM-facilitated nutrient enhancement. Given their requirement for association with host roots, *Striga* and AM fungi enter an inherent competition to acquire resources, and alter host physiology in order to achieve their optimum fitness. It is therefore likely that the interaction will involve more than just the pre-attachment SL signalling mechanism. The aim of this thesis was to develop our understanding of this interaction by exploring symbiont success and the effect of the interaction on host growth and physiology.

In chapter 2, the host plant, rice, was grown in pots with both *S. hermonthica* and a commercially available *R. irregularis* inoculum. *R. irregularis* acted as a highly mutualistic symbiont. However, in combination with *S. hermonthica*, there was an increase in *S. hermonthica* infection. Root metabolome analysis indicated opposing effects of each symbiont on host defence chemistry at the time of harvest. I hypothesised that the down-regulation of host defences known to occur during the early stages of AM colonisation were responsible for the increase in *S. hermonthica* infection, and that high nutrient supply coupled with nutrient provision by the fungus allowed the host plant to support the increase in parasite demand for host nutrients.

In chapter 3, I manipulated the nutrient supply to the host to see how this alters the ability of the host to support *S. hermonthica*. Hairy root cultures of *R. irregularis* isolate 09 were used instead of the commercial inoculum used in chapter 2. Large differences in root growth between host plants supplied with high and low nutrient treatments made comparisons of symbiont success problematic. Interestingly though, the isolate of *R. irregularis* used in this chapter had a commensal relationship with the host in contrast to the mutualistic isolate used in chapter 2. Furthermore, there was little alteration in *S. hermonthica* infection in the presence of the fungus. These observations showed the importance of AM fungal identity for host plants and on subsequent competition with *S. hermonthica*.

In chapter 4, I used rhizotrons to investigate how the order of colonisation/infection alters symbiont success. *R. irregularis* isolate 09 was used to colonise plants in rhizotrons before, after and at the same time as *S. hermonthica*. Early arrival afforded a priority effect, which suppressed the invading symbiont, and each symbiont appeared to exclude the other from specific roots.

Overall, this thesis has expanded our knowledge of the AM-*Striga*-host interaction by exploring mechanisms beyond SL signalling. I have shown that the impact of an AM fungus on *Striga* is context-dependent and may not always be beneficial by reducing parasite infection. Furthermore, the order of symbiont arrival and its effects on host physiology may determine symbiont success.

Abbreviations

2,6-DMBQ	2,6-dimethoxy-benzoquinone
ABA	Abscisic acid
AM	Arbuscular mycorrhizal
ANOVA	Analysis of variance
C	Carbon
DAC	Days after colonisation
DAI	Days after infection
DAS	Days after sowing
ESI	Electrospray ionisation
HIF	Haustorial initiation factor
HR	Hypersensitive response
IRRI	International Rice Research Institute
JA	Jasmonic acid
N	Nitrogen
OPLS-DA	Orthogonal partial least squares- Discriminant analysis
P	Phosphorus
PCA	Principle component analysis
PGPR	Plant growth-promoting rhizobacteria
PLS-DA	Partial least squares- Discriminant analysis
SA	Salicylic acid
SL	Strigolactone
SSA	SSA
WAS	Weeks after sowing

Contents

Chapter 1

General introduction

1.1 Plant symbiosis

Symbiosis describes the intimate interaction between different organisms (de Bary 1879), where genes that exist in another organism are utilized to allow individuals to overcome their own genetic limitations and carve out a new niche for themselves as symbionts (Douglas, 1994). Symbioses can be described along a complete spectrum of associations from mutualism to competition according to the relative benefit for the organisms involved (Johnson et al., 1997). Competition describes interactions where both parties are inhibited, whereas mutualism describes symbioses where both symbionts benefit. Other possible outcomes are; commensalism, where one partner benefits but the other is not affected; parasitism, where one symbiont benefits at the expense of the other; neutralism, where neither is affected; and amensalism, where one partner is not affected but the other is inhibited (Johnson et al., 1997). The cost-benefit currency is fitness (reproductive success), for which multiple measurement proxies such growth and disease resistance as well as seed production are used in scientific studies.

Plants host a diverse array of symbionts, from mutualistic arbuscular mycorrhizal (AM) fungi (Smith and Read, 2008) and plant growth-promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova, 2009) through to pathogenic bacteria (Mansfield et al., 2012), fungi (Rodriguez et al., 2009), nematodes (Holbein et al., 2016), and parasitic plants (Westwood et al., 2010, Tesitel, 2016). Based on the impacts of each symbiont, the classification of many symbioses can be obvious. For example, parasitic plants like *Striga hermonthica* extract nutrients and water from their host, severely reducing host growth in the process (Frost et al., 1997, Gurney et al., 1999, Oswald and Ransom, 2004, Cissoko et al., 2011). However, the outcome of many symbiotic interactions can be less predictable, particularly in symbioses that have the potential to be mutualistic (Jones and Smith, 2004, Jones et al., 2015). For example, the AM symbiosis, in which plants receive nutrients, mainly phosphorus (P), from an associated fungus in return for plant-fixed carbon (C) (Smith and Read, 2008), is generally considered to be mutualistic (Jones and Smith, 2004). In reality, because AM fungi impose a C demand on the host plant, requiring as much as 20 % of host photosynthate (Jakobsen and Rosendahl, 1990), the AM symbiosis can lead to host

exploitation (Klironomos, 2003). AM fungi are obligate symbionts that require a host to complete their life cycle, so they can be considered beneficiaries of the symbiosis by default, so the AM symbiosis is normally classified as being either mutualistic, commensal, or parasitic depending on the benefit received conferred to the host plant (Johnson et al., 1997).

Exploitation can occur in both directions in the in plant-fungus associations. Plant parasitism of an associated fungus, known as mycoheterotrophy, illustrates this (Leake, 1994). Symbioses not only differ in their nature depending on the partners involved, but their relative benefit to each symbiont can also fluctuate in a temporal manner. For example, mycoheterotrophs which develop through achlorophyllos to photosynthetic life stages can use a 'take now, pay later' strategy; taking nutrients from the fungus during the achlorophyllous stages of their lifecycle, but giving C back when they reach a photosynthetic stage (Cameron et al., 2008b, Field et al., 2015).

The story of plant symbiosis is one of the coevolution of individual genomes resulting from genome interactions. The phenotype of an interaction depends on the genotypes (G) of the individuals involved, and so are referred to as genotype by genotype (GxG) interactions (Hamilton, 1980). Symbioses rarely occur in simple pairs in isolation. Co-infections and the interaction with the environment (GxGxGxE), make plant symbioses complex and challenging to study (Bose and Schulte, 2014). However, their importance in nature and in agriculture makes our understanding of them vital.

Symbiosis is so widespread in nature that it is considered the norm (Smith et al., 2011). For example, over 80% of terrestrial plant species form symbioses with AM fungi (Parniske, 2008). Plants are constantly challenged by potential symbionts and, as sessile organisms, their ability to regulate them is critical for plant fitness. Due to their widespread nature and significant effects on plant fitness, plant symbioses are major influencers of natural ecosystems (Press and Phoenix, 2005, Cameron, 2010). In fact, symbioses between ancient plant and fungal ancestors are believed to have been vital for the colonisation of land by plants (Pirozynski and Malloch, 1975). Researchers consider symbionts such as AM fungi and parasitic plants to be highly

influential ecosystem engineers by altering the fitness of host plants relative to neighbouring competitors (Press and Phoenix, 2005, Cameron, 2010). Plant symbioses, both beneficial and detrimental, are also important in agroecosystems, particularly as their effects on plant fitness can be very different in anthropogenic environments compared to natural environments.

1.2 Plant symbiosis in agriculture

The domestication of plants leading to agriculture has itself been described as a symbiosis between man and plants (Rindos, 1980). As a 10,000 year old anthropogenic practise, agriculture represents a relatively recent and dramatic shift in selection pressures for plant symbioses (Doebley et al., 2006). Agricultural practices such as the use of fertilisers, biocides, tillage and monoculture are the major driving factors behind the generation of agroecosystems (Verbruggen and Kiers, 2010).

Arable soils, which are constantly re-used in a cycle of input and harvest, differ markedly from most soils in natural ecosystems and this can lead to alterations in symbiont-host relationships (Verbruggen and Kiers, 2010). High nutrient supply has been known to both increase and decrease plant disease severity (Dordas, 2008, Veresoglou et al., 2013). N addition has been shown to decrease disease symptoms caused by *Fusarium verticilliodes* in sugarcane compared to plants growth without N addition (Lin et al., 2016). On the other hand, the susceptibility of tomato to the powdery mildew-causing *Oidium lycopersicum* can increase significantly with increasing N supply and leaf N concentration (Hoffland et al., 2000). In line with this, a meta-analysis on 57 articles has shown that in the majority of cases, N fertilisation increases the severity of plant diseases (Veresoglou et al., 2013).

However, the opposite is true in the case of the parasitic plant *Striga*, for which high nutrient conditions are known to decrease emergence and biomass of the parasite and the negative impact on the host (Cechin and Press, 1993b, Cechin and Press, 1994). Increased nutrient supply (predominantly N but also P) can reduce parasite attachment in rice (Riches et al., 2005, Adagba et al., 2002, Jamil et al., 2011a), maize (Kamara et al., 2009, Ahonsi et al., 2002, Jamil et al., 2012), pearl millet (Jamil et al., 2014), and sorghum (Cechin and Press, 1993b, Showemimo et al., 2002,

Jamil et al., 2013). However, low input practices such as those used in many areas of sub-Saharan Africa (SSA) by subsistence farmers generate nutrient poor fields, and this is where *Striga* infestation is most severe (Ejeta, 2007). *Striga* parasitises most of the staple cereal crops grown in SSA and is provided with a constant supply of fresh host plants with each growing season (Scholes and Press, 2008).

As well as altering disease severity, high input practices are also known to influence the diversity of beneficial symbionts like AM fungi (Bender et al., 2016). In general, diversity in high intensity agroecosystems is lower than in natural ecosystems, and greater management intensity increases this trend (Hole et al., 2005). Consistent with this, AM fungal diversity has been shown to decrease in conventional high input agricultural systems compared to lower input organic systems or natural systems (Helgason et al., 1998, Kohl et al., 2014, Hijri et al., 2006, van der Gast et al., 2011, Dai et al., 2014). For example, using 454 pyrosequencing analysis of 18s rRNA gene fragments, Lin et al. (2012) found that AM diversity and richness significantly decreased in soils under long-term nutrient fertilisation conditions (Lin et al., 2012). As with *Striga,* nutrient addition has been shown to decrease the symbiotic success of AM fungi. For example, Mader et al. (2000) measured AM colonisation in wheat, vetch-rye and grass clover grown in a long term field trial comparing low and high input sites differing in the amount of fertiliser input. It was found that % root length colonisation was significantly higher (by 30-60%) in plants grown in low input soils than in those grown in conventional soils (Mader et al., 2000). Therefore, in a similar way to the parasitic plant *Striga,* low input practices such as those used in SSA may actually benefit mycorrhizal colonisation.

It is therefore likely that AM fungi and *Striga* will interact in agricultural systems in SSA, making it prudent to seek an in-depth understanding of their interaction. If both symbionts occur in the same environment and are enhanced by similar conditions, they will enter an inherent competition for host association, space and resources. As part of this competition, both AM fungi and *Striga* need to manipulate the physiology of the host plant in order to achieve their optimum fitness. Currently, very little is known about how the two organisms interact with each other at a physiological level and the consequences of this for the host plant. I will therefore

review what is known about the individual symbioses before considering the interaction.

1.3 The arbuscular mycorrhizal (AM) symbiosis

The AM symbiosis is over 450 million years old, and is a relationship which is formed between fungi of the phylum Glomeromycota with over 80% of terrestrial plant species (Redecker et al., 2000, Schussler et al., 2001, Parniske, 2008). Many AM fungi within the Glomeromycota have recently been reclassified (Table 1.1), aided by new molecular evidence resulting in the rejection or merger of a number of species whose description was previously restricted to the analysis of spore morphology (Schüßler and Walker 2010, Schüßler et al. 2011, Redecker et al. 2013). Examples of important newly named AM fungi are shown in Table 1.1.

Table 1.1 Example list of AM fungal species showing previous and new names. This table shows the new names currently being used after Schüßler and Walker (2010), Schüßler et al. 2011 and Redecker et al (2013).

Previous name	**New name**
Gigaspora decipiens	Unchanged
Gigaspora gigantea	Unchanged
Gigaspora margarita	Unchanged
Gigaspora rosea	Unchanged
Glomus claroideum	*Claroideoglomus claroideum*
Glomus clarum	*Rhizophagus clarus*
Glomus etunicatum	*Claroideoglomus etunicatum*
Glomus geosporum	*Funneliformis geosporum*
Glomus hoi	*Unchanged*
Glomus intraradices	*Rhizophagus irregularis*
Glomus irregulare	*Rhizophagus irregularis*
Glomus mosseae	*Funneliformis mosseae*
Glomus monosporum	Unchanged
Glomus versiforme	*Diversispora epigaea*
Scutellospora calospora	Unchanged
Scutellospora dipurpurescens	Unchanged
Scutellospora fulgida	Unchanged

In the AM symbiosis, the host plant receives nutrients, mainly phosphorus (P) but also nitrogen (N) in return for plant-fixed carbon (C) (Govindarajulu et al., 2005, Smith and Read, 2008). The problem with P in plant nutrition is that it is highly immobile in the soil. This means that the uptake of P results in a depletion zone around the roots which cannot be restored sufficiently by mass flow and diffusion (Lambers et al., 2006). AM fungal hyphal networks in the soil effectively extend the root system of the host plant outside of this depletion zone. In return, plants can allocate around 5-30% of photosynthate to mycorrhizal symbionts (Staddon and Fitter, 1998). This is an obligatory arrangement for the fungus that is required to complete its lifecycle. The bi-directional transfer of resources represents an opportunity for reciprocal reward for both partners, increasing the fitness of both as part of a mutualistic association. Nutrient transfer occurs inside the host root cortex via two major development patterns observed in AM fungi; the *Arum*-type and *Paris*-type. The *Arum*-type is defined by extensive intercellular growth and branching in the root cortex, whereas the *Paris*-type is defined by cell-to-cell growth of intracellular hyphal coils (Smith and Smith, 1997). The *Arum*-type is the most widely studied and is formed in the association between rice and *R. irregularis* (Gomez and Harrison, 2009), which is the AM interaction studied in this thesis.

In this thesis the fungal species used in all experiments is *Rhizophagus irregularis*. This species was selected because *R. irregularis* is a widely used AM fungus in mycorrhizal studies, and it is widely available in both commercial and axenic culture forms. The use of a limited number of species and isolates in scientific studies prevents us from making assumptions about the effects of AM fungi on host plants in general. Because of this, care must be taken to avoid making general assumptions about AM fungi in the field based on controlled experiments, and to recognise that a single isolate under experimental conditions gives us a limited idea of the effects of AM fungi in the field where environmental conditions, AM and host communities are highly variable. It is noted throughout this thesis that experimental contexts are likely to significantly alter the result of the AM interactions. For example, a particularly crucial factor in the field may be the diversity of AM fungi and the presence of other plant growth-promoting rhizobacteria in the soil compared to highly controlled experiments. This will be discussed in the following sections and

throughout this thesis. Despite these problems, previous research has resulted in a detailed mechanistic overview of the colonisation process by AM fungi, and this colonisation process will be discussed next.

Before nutrient exchange can occur, the AM symbiosis needs to be established between two compatible partners. To this end, the establishment of the AM symbiosis requires complex signalling between plant and fungus. The establishment of the symbiosis and the role of plant defences will therefore be considered first, followed by consideration of nutrient exchange between host and symbiont.

1.3.1 Detection and pre-symbiotic signalling during AM colonisation

Before AM-specific signalling takes place, it is likely that AM fungi appear to a plant as a potentially parasitic invader looking to exploit it as a nutrient resource. Plants use pattern recognition receptors (PRRs) to detect microbes via pathogen-associated molecular patterns (PAMPs) (Zipfel, 2008). Upon detection by plant PRRs, PAMPs induce PAMP-triggered immunity (PTI), the first layer of plant immunity which includes the production of reactive oxygen species (ROS), callose deposition at the cell wall, expression of defence-related genes, and the production of defence hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (Zhang and Zhou, 2010). Examples of PAMPs include the glucans, chitins and proteins which are major components of fungal cell walls (Eckardt, 2008). A more useful term for these molecular patterns is microbe-associated molecular patterns (MAMPs), since they can originate from commensal and mutualistic microbes as well as pathogens (Sanchez-Vallet et al., 2015). It is therefore possible that MAMPs like these in AM fungi can be recognised by PRRs causing MAMP-triggered immunity in potential hosts. In order to suppress host immune responses, a symbiosis-specific signalling dialogue is required between the plant and fungus. This is initiated by chemicals released from plant roots.

For the process of AM colonisation to begin (Fig. 1.1), the release of branching factors called strigolactones (SLs) from plant roots represents the initial signal to which the fungi respond by initiating hyphal branching (Akiyama et al., 2005). SLs degrade rapidly in the rhizosphere resulting in a steep concentration gradient which

provides a guide for the fungal hyphae towards the root (Akiyama et al., 2005, Ruyter-Spira et al., 2013). AM fungi can respond to SLs down to picogram and nanogram levels, suggesting that AM fungi use a highly sensitive mechanism for detecting SLs at such low levels (Akiyama and Hayashi, 2006).

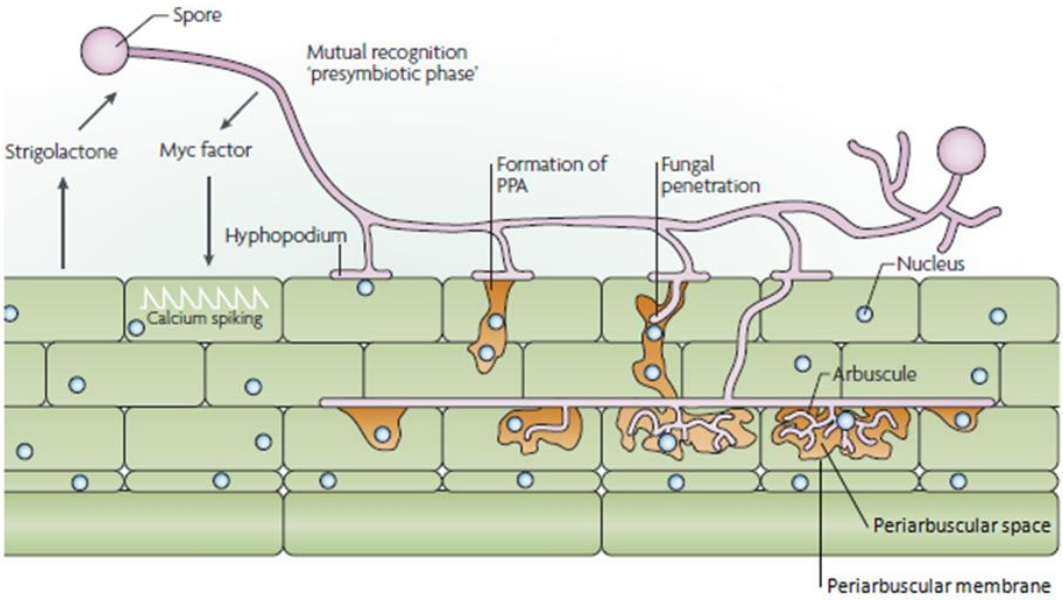


Fig. 1.1 The AM fungi infection process from SL perception to arbuscule formation (Genre et al., 2008, Parniske, 2008). PPA = prepenetration apparatus.

Ideally for the plant, specific blends of SLs will signal to beneficial AM fungi but not to parasites and pathogens (Akiyama et al., 2010). Over 20 different SLs have been reported so far (Kisugi et al., 2013, Xie et al., 2013), and they differ significantly in their ability to induce hyphal branching in AM fungi, but also in their ability to induce parasitic plant seed germination (Akiyama et al., 2010, Nomura et al., 2013, Cardoso et al., 2014). For example, the SL orobanchol is known as a particularly strong inducer of hyphal branching in AM fungi (Akiyama et al., 2010). Cardoso et al. (2014) analysed the SL content of root exudates from rice, and measured their stimulatory effects on hyphal branching in the AM fungus *Gigaspora margarita* and seed germination in the parasitic plant *Striga hermonthica*. It was found that particular SLs such as (-)-orobanchol and *ent*-2-*epi*-5-deoxystrigol strongly stimulated *G. margarita* hyphal branching, but had little germination stimulating activity on *S. hermonthica* seeds (Cardoso et al., 2014).

Structurally, SLs consist of four rings (A-D), with the C and D rings connected by an enol ether bond (Fig. 1.2). Most variation between SLs results from different side groups and modifications at the A and B rings, while the C and D rings appear highly conserved (Tsuchiya and McCourt, 2012). Structural requirements of SLs, such as the enol ether bridge between the C and D rings, are essential for germinating parasitic plant seeds, but bridge identity is thought to be less important for inducing hyphal branching in AM fungi (Akiyama et al., 2010). For example, Akiyama et al. (2010) showed that SLs with aloxy or imino C-D bridges both stimulate hyphal branching.

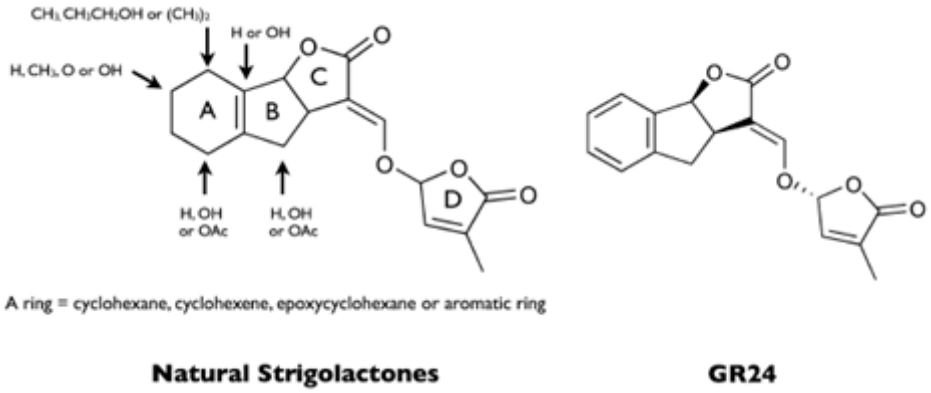


Fig. 1.2 The general structure of strigolactones (left), and the structure of the synthetic SL, GR24 (Tsuchiya and McCourt, 2012).

Plants produce and release more SLs under N and P stress (Lopez-Raez et al., 2008, Yoneyama et al., 2012). This allows plants to enhance recruitment of AM fungi so as to exploit their hyphal network, and is also used by leguminous plants to form nodulating symbioses with N-fixing *Rhizobium* spp. (Akiyama et al., 2005, Foo et al., 2013). Sufficient nutrient levels act to suppress SL production and release; mycorrhizal colonisation also reduces SL production and release by enhancing host nutrient status (Jamil et al., 2013, Lopez-Raez et al., 2011). Reduced SL production and release can in turn reduces AM recruitment, as seen by the reduced levels of AM colonisation seen in plant mutants defective in SL biosynthesis (Kohlen et al., 2012). For example, *Pisum sativum* (pea) mutants defective in SL biosynthesis (*rms1*) show reduced AM colonisation by *Rhizophagus irregularis*, and this phenotype can be

restored by the application of the synthetic SL analogue GR24 (Gomez-Roldan et al., 2008).

As well as SLs, flavonoids have also been shown to act as rhizosphere signals by inducing AM spore germination and hyphal branching in the rhizosphere, where they also have roles as allelochemicals that can inhibit the germination and growth of parasitic plant seeds (Tsanuo et al., 2003, Hooper et al., 2010). Endogenous host flavonoid accumulation varies throughout AM colonisation. For example, flavonoids are induced before *R. irregularis* colonisation in response to fungal signals (Larose et al., 2002). Akiyama et al. (2002) used high performance liquid chromatography (HPLC) to show that P-deficient melon roots accumulate C-glycosylflavonoid to promote colonisation by the AM fungus *Glomus caledonium*. In the same study, colonisation in P-deficient plants was significantly higher than in plants grown with high P, but application of C-glycosylflavonoid increased colonisation in plants supplied with high P to levels seen in P-deficient plants (Akiyama et al., 2002).

The perception of host-released signals sets the pre-symbiotic growth phase of the fungus in motion, increasing the number and activity of mitochondria in fungal cells leading to the characteristic increase in branching (Besserer et al., 2006). An increase in hyphal branching increases the chances of root contact by the fungus, and also initiates a complex signalling dialogue between the plant and fungus (Parniske, 2008). In response to host signals, AM fungi synthesise and release mycorrhizal (Myc) factors, chito-oligosaccharides (COs) and lipo-chitooligosaccharides (LCOs) (Kosuta et al., 2003, Genre et al., 2013, Maillet et al., 2011). Myc factors, COs and LCOs trigger a range of host responses including calcium spiking and the expression of symbiosis-related genes (Kosuta et al., 2008, Czaja et al., 2012, Genre et al., 2013). At this stage, plant-derived cutin monomers are released from the root surface which stimulate the formation of a specialised type of fungal appressoria called the hyphopodium which leads to the penetration stage of the symbiosis (Wang et al., 2012, Murray et al., 2013). The prepenetration apparatus (PPA) is then formed by the plant to determine the path of the infecting fungal hyphae through the epidermis and cortical cells (Genre et al., 2005).

Plant signals sent into the rhizosphere can stimulate a number of organisms including parasites and pathogens, so the point of hyphal contact is critical because the plant needs to determine if the prospective symbiont is beneficial or not, and therefore must regulate its immune system to encourage or dissuade its progression (Hayashi et al., 2014, Bulgarelli et al., 2013). AM fungi have been shown to transiently induce SA at the early stages of infection, potentially due to host recognition of AM fungal MAMPs. For example, transient SA accumulation has been observed during mycorrhizal colonisation by *Funneliformis mosseae* (syn. *Glomus mosseae*) of *Pisum sativum*, *Nicotiana tobacum* and *Oryza sativa* (Blilou et al., 1999, Blilou et al., 2000a, Blilou et al., 2000b). Accumulation of SA is even greater in symbiosis-resistant *P. sativum* mutants (P2), indicating the involvement of SA in an early defence response during mycorrhizal colonisation (Blilou et al., 1999, Garcia-Garrido and Ocampo, 2002). Induction of SA biosynthesis and SA-dependent defences are known to occur in response to the perception of many biotrophic fungi (Glazebrook, 2005). As biotrophic fungi which rely on symbiosis with a plant partner, AM fungi must overcome these plant defences. The transient nature of the SA response suggests that the initial defence response is suppressed at later stages of mycorrhizal colonisation.

1.3.2 Regulation of host defence for successful AM colonisation

The suppression of plant defences by pathogens is mainly achieved via secreted effector proteins which are delivered into the host cell (Kamoun, 2007). An effector protein (SP7) has been identified in AM fungi (Kloppholz et al., 2011). During colonisation of *Medicago truncatula* roots by the AM fungus *R. irregularis*, this protein is secreted by the fungus and delivered to the host cell nucleus, where it binds to and inhibits the pathogenesis-related transcription factor, ERF19. ERF19 is highly induced in response to fungal pathogen infection and fungal extracts, and is transiently induced during AM infection, so it is likely that *R. irregularis* secretes SP7 to suppress plant defences via the inhibition of ERF19 (Kloppholz et al., 2011).

Further evidence for defence suppression by AM fungi comes from the hormone fluxes observed in the host during colonisation. As discussed above, AM colonisation has been shown to cause transient accumulation of SA in plants, and SA is associated with defence responses to biotrophic pathogens. Herrera-Medina et al.

(2003) compared colonisation by *R. irregularis* and *F. mosseae* (syn. *G. mosseae*) of transgenic tobacco plants with reduced (NahG) and enhanced (CSA) levels of SA to wild type. NahG plants showed a higher level of colonisation compared to wild type, whereas colonisation in CSA plants was reduced (Herrera Medina et al., 2003). This effect was sustained temporally throughout infection by *R. irregularis*. However, given enough time, *F. mosseae* colonisation was able to reach wild type levels. These results suggest both that SA-dependent defence responses down-regulate mycorrhizal colonisation, and also that AM fungi may be able to overcome SA-dependent defences over time.

The suppression of host SA-dependent defence responses by AM fungi may occur via the induction of abscisic acid (ABA) synthesis in the host. ABA has been shown to be important for AM colonisation, and has also been shown to be involved in the suppression of SA-dependent defence. Herrera-Medina et al. (2007) compared colonisation by *R. irregularis* of wild type *Lycopersicon esculentum* to ABA *sitiens* mutants which contain lower ABA concentrations. *Sitiens* plants were found to be less susceptible to *R. irregularis* infection, with a significantly lower level of mycorrhizal colonisation than wild type (Herrera-Medina et al., 2007), suggesting that ABA is required for successful AM colonization. In tomato, SA accumulates in response to *Pseudomonas syringae* as part of a resistant response. However, exogenous application of ABA prevents accumulation of SA, suppressing resistance to *P. syringae* (Mohr and Cahill, 2007b), suggesting that ABA is involved in the suppression of SA-dependent defences. Overall, the early stages of AM colonization therefore induce transient systemic priming SA-dependent defences followed by transient localized suppression of host defences which allow colonisation to proceed.

In a compatible interaction, AM hyphae branch to form arbuscules in the inner cortical cells of the root. Successful colonization by AM fungi subsequently results in long term systemic priming of host JA- and ethylene-dependent defences (Jung et al., 2012, Van der Ent et al., 2009, Van Wees et al., 2008) as a result of induced systemic resistance (ISR) caused by rhizobacteria also present around the host roots (Berendsen et al., 2012, Cameron et al., 2013). The induction of JA as a result of AM colonisation has been demonstrated by enhanced colonisation and decreased pathogen susceptibility in plants treated with JA and plants with elevated levels of

endogenous JA caused by wounding (Landgraf et al., 2012). The overall effects of AM colonization on host defence priming throughout the colonization process is known as mycorrhiza-induced resistance (MIR). MIR is therefore contributed to by both SAR and ISR effects in a complex spatiotemporal manner (Cameron et al., 2013).

The downstream consequences of AM colonisation on host defences involves key secondary metabolites. For example, as well as their involvement in signalling, flavonoid accumulation also varies throughout the later stages of AM colonisation. Larose et al. (2002) analysed three stages of colonisation by *F. mosseae* (syn. *G. mosseae*) of the host legume *Medicago sativa* at 7, 18 and 32 days after inoculation. The authors measured percentage AM colonisation and flavonoid concentration of host roots by high performance liquid chromatography (HPLC). At 7 days, the study observed a colonisation level of 10.5% with no collapsed arbuscules, however by 18 days 50% of arbuscules were collapsed, and by 32 days this rose to 78%. The accumulation of specific flavonoids (e.g. ononin) decreased after 18 days when arbuscules started to collapse. The authors also showed that the extent of flavonoid accumulation depends on the species of fungus colonising, with varying levels of accumulation observed in the hosts associated with either *F. mosseae*, *R. irregularis* and *G. rosea* (Larose et al., 2002).

Schliemann et al. (2008) carried out metabolite profiling via gas chromatography-mass spectrometry (GC-MS), HPLC and liquid chromatography-mass spectrometry (LC-MS) of the roots of *Medicago truncatula* colonised by *R. irregularis* over 56 days. It was found that the late stages of colonisation induced the biosynthesis of isoflavonoids e.g. ononin, daidzein, and malonylononin. Furthermore, high levels of cell wall-bound antioxidant Tyrosol was specific to colonised roots (Schliemann et al., 2008). Harrison and Dixon (1994) used gene expression analysis to show the elevation of transcripts for phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) enzymes (which are involved flavonoid biosynthesis) in cortical cells of the host *M. truncatula* containing arbuscules after colonisation by *Diversispora epigaea* (syn. *Glomus versiforme*). In the same study, isoflavone reductase (IFR) transcripts were very low in colonised compared to uncolonised roots, and CHS transcripts were lower in cells containing senescing arbuscules

compared to younger arbuscules, suggesting the cell specific involvement of flavonoids in AM colonisation (Harrison and Dixon, 1994).

In the study by Harrison and Dixon (1994), no lignification was observed in arbusculated cells, suggesting that cell wall reinforcement, which is often associated with host plant response to pathogenic fungi, does not occur during successful AM colonisation (Harrison and Dixon, 1994). Cell wall reinforcement can deter herbivores (Johnson et al., 2010), reduce pathogen severity (Xu et al., 2011, Miedes et al., 2014), and may negatively affect AM colonisation (De Deyn et al., 2009, Bennett et al., 2013). Successful colonisation may therefore involve the suppression of this defence response, whereas incompatible AM fungi may induce it. Consistent with this idea, the induction of host defences and cell wall strengthening by *Rhizobium* during ineffective nodulation of *P. sativum* has been reported (Ivanova et al., 2015).

The various effects of AM fungi on host defences raise the question of how they might alter the success of other symbionts. Throughout this thesis, the effects of AM colonisation on *Striga* infection via alterations to host physiology including defences will be explored, as it is possible that either antagonistic or synergistic effects could occur. However, a likely area of competition between AM fungi and *Striga* is in their demand for host resources. Individually, nutrient stoichiometry in the AM symbiosis is complex, and will therefore be covered in the next section.

1.3.3 Nutrient exchange and benefit in the AM symbiosis

After the establishment of the AM symbiosis, nutrient exchange takes place via arbuscules formed in the host root cortical cells (Cox et al., 1980, Peterson and Howarth, 1991). However, even after successful colonisation, the fungus is excluded from the plant cytoplasm by the periarbuscular membrane and the periarbuscular space (Parniske, 2008). The plant periarbuscular membrane contains symbiosis-specific phosphate transporters to acquire P from the fungus (Harrison et al., 2002, Javot et al., 2007). Arbuscules are in some ways similar to the haustoria formed by biotrophic pathogens (Harrison, 1999, Gomez and Harrison, 2009). A major difference is that arbuscules are transient structures with a life of approximately 10-

12 days (Brundrett et al., 1985). As well as arbuscules, AM fungi also form lipid-filled vesicles within roots which act as storage compartments. Resources acquired from the host plant enable the growth of extraradical hyphae in the surrounding soil to further mediate nutrient uptake towards the plant in return for C (George et al., 1995, Bago et al., 1998).

Nutrient exchange represents an area of competition between host and fungus for maximum benefit. Many factors ultimately determine benefit in the AM symbiosis, including plant and fungal identity (Verbruggen and Kiers, 2010, Xing et al., 2012), resource abundance (Johnson et al., 2013, Wyatt et al., 2014), order of fungal arrival (priority effect) (Werner and Kiers, 2015), and direct competition between fungi for host association and resources (Verbruggen and Kiers, 2010). Furthermore, by receiving the benefits of symbiosis without paying a fair price for them, mutualisms can be exploited by one of the partners involved (Ghoul et al., 2014). The AM symbiosis is particularly complicated in the field because a single plant can associate with multiple fungal species and vice versa (Johnson et al., 2013, Weremijewicz and Janos, 2013).

Depending on the two partners involved in an association and how cooperative they are, the benefit to either can vary (Johnson et al., 2012). A mycorrhizal state is the norm for plants which can form these symbioses, but host plants can still exhibit selectivity and preference towards fungal partners (Bever et al., 2009). For example, plant roots have been shown to allocate more photosynthate to more beneficial fungi than to non-beneficial fungi (Bever et al., 2009). However, the fungus also allocates nutrients to the host, with the value of nutrients increasing under more nutrient-limited conditions, as in supply and demand. Furthermore, AM fungi have additional benefits over nutrient allocation, including pathogen and abiotic stress protection (Parniske, 2008). This makes their value to a host plant difficult to define (Werner and Kiers, 2015).

On the other hand, AM fungi can show preference to specific plant partners, as it is known that AM fungi can preferentially allocate nutrients to hosts that are more beneficial when the choice is available (Lekberg et al., 2010, Kiers et al., 2011). For example, Fellbaum et al. (2014) showed that AM fungi allocate less N and P to

plants that were grown under shaded conditions compared to those that were not shaded, showing a preference for higher quality hosts (Fellbaum et al., 2014). However, Walde et al. (2012) showed that AM fungi associated with two hosts gave more nutrients to the less beneficial (in terms of C return) host flax (*Linum usitatissimum*) than to the more beneficial host sorghum (S*orghum bicolor*) (Walder et al., 2012). This simultaneously suggested exploitation of the fungus by flax and exploitation by the fungus of sorghum.

The ability of both the plant and fungus to select their partners has led to the idea of biological markets taking place which ultimately drives decision making and partner selection (Wyatt et al., 2014). If a fungus is trading with a plant that has easy access to P via its own root uptake mechanisms, then this puts the fungus in a position of weakness. In order to increase their own value to the host plant, it has recently been suggested that suppression of direct uptake of P by their partner's roots by AM fungi (Li et al., 2008, Smith et al., 2011), increases the value of their own P allowing them to enforce trade for plant C (Wyatt et al., 2016). Nutrient exchange in the AM symbiosis is therefore highly complex and context dependent. In experimental systems that use single genotypes of plant and fungi (i.e. single cultivars of host plants and isolates of fungi), the outcome of the AM symbiosis may differ greatly compared to natural soils where partner choice is possible. Nutrient exchange and the importance of partner identity are discussed more in chapter 3 of this thesis.

1.3.4 Modification and hijack of the AM symbiosis

The process of AM colonisation has some similarities with the relatively more recent, 60 million year old *Rhizobium*-legume symbiosis (Bonfante and Requena, 2011). It has therefore been proposed that the AM signalling pathway has been adapted for nodulation (Parniske, 2000). In summary of the nodulation process, plant released flavonoids induce the production and release of nodulation (Nod) factors by the bacteria (Downie, 2010). This is similar to how SLs trigger branching and Myc factor production in AM fungi. Nod factors trigger symbiosis related activity in the host root including calcium spiking and the formation of an infection thread, a similar process to Ca spiking and PPA formation during AM colonisation. The bacteria grow through the infection thread before being released into the nodule and

encapsulated in a plant membrane. The bacteria differentiate into N-fixing bacteroids which, together with the plant membrane, form organelle-like structures called symbiosomes (Oldroyd and Downie, 2008, Oldroyd et al., 2011). Similar to the AM symbiosis, the bacteria are excluded from the plant cytoplasm by the peribacteroid membrane and peribacteroid space (Lodwig and Poole, 2003).

Nodulating plants and rhizobial bacteria have therefore manipulated the more ancient AM interaction to develop a unique symbiosis which improves N supply to the plant. Parasitic plants such as *Striga* have also hijacked the process of AM colonisation, which was first made clear by the involvement of SLs in both symbioses, which are critical in their initiation. Furthermore, as with AM colonisation, host defences are regulated in a complex spatiotemporal manner during *Striga* infection. This suggests that AM fungi and *Striga* are also highly interactive at a physiological level beyond SL signalling in the rhizosphere. Parasitic plants, the process of *Striga* infection, and its individual impact on host physiology are reviewed below.

1.4 Parasitic plants

Flowering plants have evolved a parasitic lifestyle on at least 12 occasions (Westwood et al., 2010) giving rise to 4,500 parasitic species (1% of angiosperm species) belonging to 275 genera in 28 families (Joel et al., 2013, Yoshida et al., 2016a). Ninety percent of parasitic plants attach to the host root rather than the stem, and 60% are hemiparasites that can photosynthesise, whereas the holoparasites have lost this ability (Hibberd et al., 1998). Table 1.2 shows an example list of parasitic plants along with their parasitism type. Parasitic plants use a specialised organ called a haustorium to attach to and penetrate host tissues, form vascular connections, and obtain water and nutrients from the host (Estabrook and Yoder, 1998). This requires the establishment of a xylem bridge, which connects the xylems of the host and parasite (Heidejorgensen and Kuijt, 1995). Some parasites also form phloem connections (Dorr et al., 1979, Zhou et al., 2004, Birschwilks et al., 2006), and symplastic connections via plasmodesmata can also occur (Ayre et al., 2003, Dorr and Kollmann, 1995).

Table 1.2 Example list of the main parasitic plants mentioned in this thesis.

Family	Genus and species	Parasitism type
Convolvulaceae		
	Cuscuta (Dodder)	
	Cuscuta campestris	Holoparasite
	Cuscuta reflexa	Hemiparasite
Orobanchaceae		
	Orobanche (Broomrape)	
	Orobanche crenata	Holoparasite
	Orobanche cumana	Holoparasite
	Orobanche minor	Holoparasite
	Orobanche ramose	Holoparasite
	Phelipanche	
	Phelipanche ramosa	Holoparasite
	Rhinanthus	
	Rhinanthus minor	Hemiparasite
	Striga (Witchweed)	
	Striga asiatica	Hemiparasite
	Striga gracillima	Hemiparasite
	Striga hermonthica	Hemiparasite
	Triphysaria	
	Triphysaria versicolor	Hemiparasite

Parasitic plants of the family Orobanchaceae are highly influential in natural ecosystems where they can reduce host productivity and increase community diversity (Press and Phoenix, 2005), and in agroecosystems where they can severely reduce the growth of host crop plants (Frost et al., 1997, Gurney et al., 1999, Oswald and Ransom, 2004, Cissoko et al., 2011). *Striga*, also referred to as the witchweeds, is a genus within the Orobanchaceae which are devastating to cereal crops grown in the infertile soils of SSA (Fig. 1.3 and Fig. 1.4) (Parker and Riches, 1993, Ejeta,

2007, Scholes and Press, 2008). In SSA, over 50 million hectares of farmland are infested, with yield losses costing around US$ 10 billion a year (Ejeta, 2007, Parker, 2009). *S. hermonthica* and *S. asiatica* are two particularly damaging species, and infest important food crops including rice (*Oryza sativa*), maize (*Zea mays*), sorghum (*Sorghum bicolor*), cowpea (*Vigna unguiculata*) and pearl millet (*Pennisetum glaucum*) causing severe stunting and yield losses of 30-90% (Scholes and Press, 2008, van Ast et al., 2005). As with AM fungi, the lifecycle of parasitic plants like *Striga* is critical, particularly for obligate symbionts like *Striga*. Infection requires a precise and compatible interaction with the host plant, including signalling and the regulation of host defences.

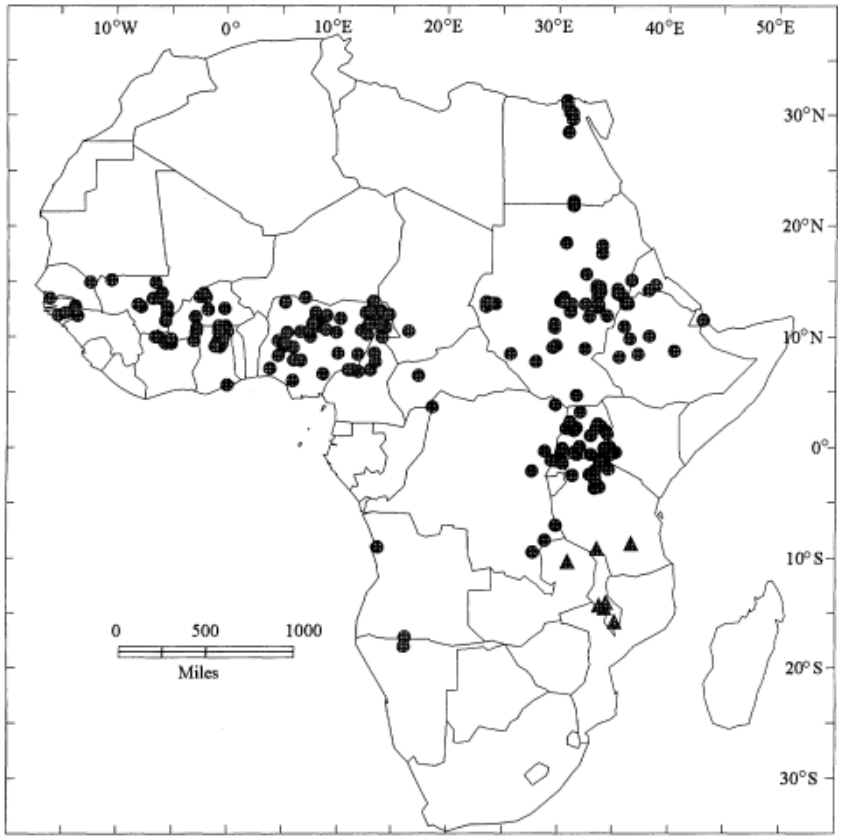


Fig. 1.3 Distribution map of *S. hermonthica* (circles) and *S. gracillima* (triangles, not studied in this thesis) (Mohamed et al., 2001).

Fig. 1.4 *S. hermonthica* attached to a rice root and parasitising rice in the field © AfricaRice (CC BY-NC-ND 4.0).

1.4.1 Pre-attachment stages of the *Striga* lifecycle and host defence

The *Striga* lifecycle involves several stages and it is intimately linked to the host plant lifecycle (Fig. 1.5a – g) (Scholes and Press, 2008). Individual *Striga* plants can produce 100,000 seeds which can persist in the soil for up to 20 years (Scholes and Press, 2008). Seed conditioning occurs under warm, moist conditions and over the course of about 14 days in which time seeds become increasingly sensitive to germination stimulants (Matusova et al., 2004, Dzomeku and Murdoch, 2007).

As with AM fungi, the timing of germination in close proximity to a host root is critical for *Striga* because its small seeds cannot support a long period of growth without attachment and exploitation of a host (Cardoso et al., 2011). Also like AM fungi, *Striga* detects a potential host via SLs in host root exudates (Cook et al., 1972, Matusova et al., 2005). In *Striga*, SLs are detected by a recently characterised diverged family of α/β hydrolase-fold proteins (Conn et al., 2015, Tsuchiya et al., 2015, Toh et al., 2015). SLs can be detected by *Striga* down to a concentration of just

10^{-12}M (Kim et al., 2010), where they stimulate germination by inducing ethylene biosynthesis in the seed (Sugimoto et al., 2003).

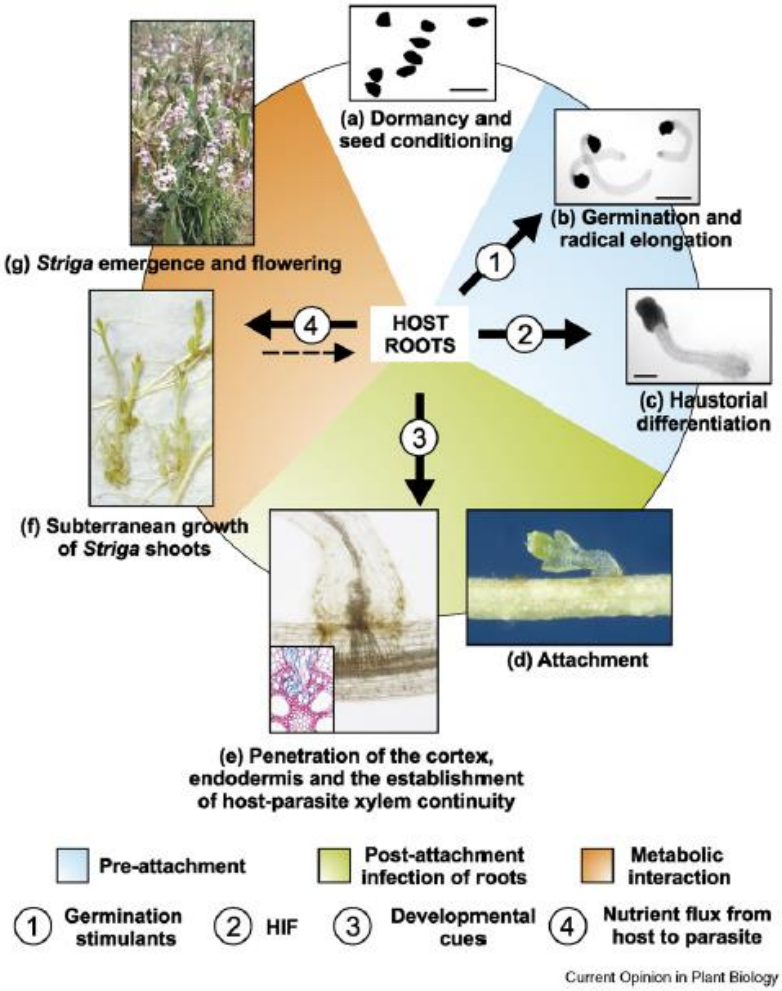


Fig. 1.5 The *Striga* lifecycle (Scholes and Press, 2008).

Germination is an early stage in the parasite lifecycle where host resistance can occur. Pre-attachment resistance to *Striga* is mostly associated with the differing SL profiles of host plants and a reduction in subsequent parasite seed germination (Jamil et al., 2011b). Qualitative and quantitative differences in SLs in different plant species cultivars have been shown, and some SLs are more potent germination stimulants than others (Yoneyama et al., 2008, Yoneyama et al., 2011, Yoneyama et al., 2012, Jamil et al., 2011a, Jamil et al., 2011b), and are also involved in host specificity (Xie et al., 2010, Fernandez-Aparicio et al., 2011). For example, Jamil et al. (2011)

analysed SLs in root exudates collected from New Rice for Africa (NERICA) cultivars of rice, and tested *Striga* germination, attachment and emergence rates in these cultivars. The quantity of SLs produced differed significantly between cultivars, and those cultivars which released lower amounts of SLs suffered lower levels of *Striga* infection. This showed that pre-attachment resistance varies between different cultivars, and that this is due to variations in SL exudation (Jamil et al., 2011b). As with AM fungi, differing structural properties of SLs have been shown to be a factor in their ability to initiate *Striga* seed germination (Nomura et al., 2013). Nomura et al. showed that 5-deoxystrigol and similarly configured SLs induced high germination in *S. hermonthica*, but inhibited germination in *S. gesnerioides*. Interestingly, the SLs with the structural requirements for *S. hermonthica* germination, like 5-deoxystrigol, also have high hyphal branching activity in the AM fungus *G. margarita* (Akiyama et al., 2010).

A reduction in parasite germination due to the nature of host exudates would represent an efficient avoidance strategy. Potential hosts may also alter parasite success before attachment via allelochemicals (Fernandez-Aparicio et al., 2013). Plants use allelochemicals to suppress the germination and growth of nearby competitors (Wu et al., 1999), and this role extends to the suppression of parasitic plants (Fernandez-Aparicio et al., 2013). For example, the flavonoid isoschaftoside isolated from *Desmodium uncinatum* root exudates has been identified as an allelochemical which inhibits growth of the *S. hermonthica* radicle (Hooper et al., 2010). Furthermore, germination and radicle development of *Orobanche crenata* is inhibited by a number of cereal-produced allelochemicals, with scopoletin producing necrotic darkening in radicles (Fernandez-Aparicio et al., 2013).

After the induction of germination, the *Striga* radicle extends from the seed and releases hydrogen peroxide (H_2O_2) and peroxidases which convert phenolic acids derived from host cell wall lignin to haustorium initiation factors (HIFs), which in turn initiates development of the parasite haustorium allowing attachment to the host root (Keyes et al., 2007b). For example, the HIF 2,6-dimethoxy*p*-benzoquinone (DMBQ) arises via the oxidation of the lignin derivative syringic acid (Chang and Lynn, 1986, Keyes et al., 2007a, Kim et al., 1998). The phenolic acid from which a HIF is derived can determine their ability to induce haustorial formation in different

parasitic plants, so the presence and abundance of different phenolic acids in host cell wall lignin may play a role in susceptibility (Albrecht et al., 1999). Flavonoids, which are also important in AM fungal signalling, can also act as HIFs (Albrecht et al., 1999). For example, the flavonoid peonidin initiates haustorium formation in the facultative parasitic plant *Triphysaria versicolor*, (Joel and Losnergoshen, 1994, Westwood et al., 2012).

The initiation of haustoria formation is critical for *Striga* attachment and penetration of the host, but it also represents another stage the infection cycle where the host plant can prevent infection. *Striga* produced H_2O_2 generates HIFs from host-derived compounds, so any process which disrupts or avoids HIF production can disrupt infection. For example, it has been shown that catalase enzyme activity can inhibit *Striga* haustorium induction by scavenging H_2O_2, thus preventing its oxidation of syringic acid to DMBQ (Kim et al., 1998). The pre-attachment processes of *Striga* infection leading up to root attachment can take place in a matter of hours (Scholes and Press, 2008).

1.4.2 Post-attachment stages of the *Striga* lifecycle and host defence

After attachment, the parasite has to penetrate between host root cells via an invasive structure known as the penetration peg. The attachment and penetration stage of the *Striga* lifecycle can involve various resistance responses by the host (Yoshida and Shirasu, 2009). For example, necrosis around the attachment site has been observed in more resistant hosts, which suggests active recognition and the onset of a hypersensitive response (HR). For example, *S. gesnerioides* infection of the resistant cowpea cultivar B301 results in HR, and is mediated by a defence gene which encodes a type of resistance (R) sensor protein (coiled-coil nuclear-binding site leucine-rich repeat (CC-NBS-LRR)) known to induce resistance against many plant pathogens in other plant species (Li and Timko, 2009). The presence of host R sensor proteins also suggests the presence of parasite effectors. Susceptible cultivars would either lack the R protein or it could be suppressed or avoided by parasite virulence factors (vir) in a manner more familiar in plant-bacterial/fungal/nematode gene-for-gene interactions (Asai and Shirasu, 2015). On the other hand, resistant cultivars

would recognise parasite avirulence factors resulting in effector triggered immunity (ETI) again as seen in other plant-pathogen interactions (Asai and Shirasu, 2015).

After attachment, *Striga* advances into the endodermis by fragmenting host cells and the Casparian strip, overcoming physical barriers such as lignin in the cortex and endodermis (Neumann et al., 1999). During this phase, *Striga* expresses genes encoding plant cell wall-degrading enzymes (PCWDEs) including pectate lyases (Yoshida et al., 2016a). Expansin proteins, which loosen cell walls (Cosgrove, 2000), are also produced in intrusive parasite cells (Honaas et al., 2013). Pectate lyases and expansins are both normal host-produced proteins involved in growth and development (Wing et al., 1990, Cosgrove, 2000), so their use by the parasite may allow them to avoid detection by the host. However, the fragmentation of host cells results in the presence of host apoplastic material and of the cell wall-degrading enzymes and their breakdown products in the host symplast, where they can act as elicitors which signal to the host that it is being damaged (Mitsumasu et al., 2015, Yoshida et al., 2016a). For example, plant-parasitic nematodes also use PCWDEs, and couple this with effector secretion to suppress subsequent host immune responses (Lozano-Torres et al., 2014, Mitsumasu et al., 2015). It is possible that *Striga* uses effectors in a similar way (Li and Timko, 2009, Huang et al., 2012, Mitsumasu et al., 2015).

Parasite development can be prevented at different areas of the root as the penetration peg approaches host vascular tissues. Prevention of development at the cortex is associated with the deposition of cell wall physical barriers such as lignin, and the accumulation of toxic phenolic compounds derived from phenylpropanoid metabolism (Perez-De-Luque et al., 2008). For example, reinforcement of cell walls by lignification has been shown to occur along with phytoalexin production at the host parasite interface between sunflower and *Orobanche cumana* (Echevarria-Zomeno et al., 2006).

Prevention of penetration at the endodermis has also been observed. For example, *S. hermonthica*, when infecting the resistant rice cultivar Nipponbare, is able to invade the cortex, but then cannot penetrate the endodermis and grows around the vascular cylinder (Gurney et al., 2006). When this was studied by Gurney et al. (2006), no

lignin deposition was observed, so it is thought that this response involves a lesion in the signalling pathways involved in penetrating the endodermis (Gurney et al., 2006). The endodermis can represent a significant physical barrier to parasite progress. For example, significant levels of lignification of cells can be seen at the endodermis at the host-parasite interface between resistant legume hosts and *O. crenata* (Perez-De-Luque et al., 2005).

Due to the importance of physical barriers such as lignin and the associated accumulation of phytoalexins during resistant interactions with parasitic plants, the biosynthetic pathways leading to their synthesis is a key maker of a defence response. Phenylpropanoid metabolism is essential in plant defence responses including the production of phytoalexins, and lignin, suberin and callose which are used for physical protection against penetration (Vogt, 2010).

Transcriptomic studies have detected genes involved in phenylpropanoid biosynthesis during host-*Striga* interactions. For example, a transcriptomic analysis of rice infected by *S. hermonthica* has shown that the resistance response in Nipponbare is associated with the up-regulation of the gene encoding phenylalanine ammonia lyase (PAL) which is involved in phenylpropanoid metabolism (Swarbrick et al., 2008). Transcriptomic evidence for the up-regulation of genes involved in lignin biosynthesis and cell wall modification in sorghum parasitised by *S. gesnerioides* has also been shown (Huang et al., 2012). Similarly, analysis of host plants during interactions with *Orobanche* has also shown the involvement of phenylpropanoid metabolism in resistance. For example, *O. cumana*-parasitised sunflower shows up-regulation of the *pal* gene during resistant interactions (Letousey et al., 2007).

Successful penetration of the root cortex and endodermis can allow *Striga* to form xylem continuity with hosts in the space of about three days (Gurney et al., 2006). After the parasite has established xylem continuity, there are still host resistance responses that can occur. For example, successful vascular connections made by *O. crenata* in resistant pea plants has been shown to be followed by the blocking of the nutrient and water supply through the vascular tissues by mucilage-like compounds leading to the death of the parasite (Perez-De-Luque et al., 2008). Another study

showed that successful xylem connections made by *S. hermonthica* in resistant *Tripsacum dactyloides* also resulted in death of the parasite, but this was attributed to the introduction of a toxin by the host to the parasite as no xylem blocking was observed (Gurney et al., 2003). Resistance after xylem connection establishment has also been shown in the rice cultivar Nipponbare infected by *S. hermonthica*, although it is unclear how this actually occurs (Yoshida and Shirasu, 2009).

While parasitic plants derive nutrients and water from their host, the bidirectional transfer of molecules including proteins, mRNA, metabolites, and viruses can also occur (Smith et al., 2013, Kim and Westwood, 2015). The bidirectional flow of molecules not only suggests manipulation of the host by the parasite, but it also represents another possible method of host defence. For example, small interfering RNA (siRNA), which cause targeted gene silencing, could be targeted to host defence-related genes providing a mechanism for host defence suppression by parasitic plants. On the other hand, they could be involved in host resistance when transferred to the parasite (Tomilov et al., 2008).

1.4.3 Host defence hormone responses to *Striga* infection

As with AM fungi, early defence hormone regulation is crucial for determining down-stream defence responses and compatibility during *Striga* infection. In particular, SA and JA regulation appears to be crucial in determining the success of infection. For example, a study by Hiraoka and Sugimoto (2008) analysed gene expression during the early stages of *S. hermonthica* parasitism (when attachment and tubercle formation was observed) and the effect of SA application on susceptible and less susceptible cultivars of sorghum. Susceptible interactions with *S. hermonthica* involved the induction of JA- and the suppression of SA-induced genes. In contrast, less susceptible interactions involved the induction of SA-induced genes. Application of SA (by inclusion of SA in nutrient solution) decreased the susceptibility of sorghum to *S. hermonthica*, as observed by induction of SA-induced genes and a reduction in *S. hermonthica* tubercle formation (Hiraoka and Sugimoto, 2008). In another example, the expression of the SA-responsive gene, *def* (defensin) in a resistant sunflower genotype (LR1) interaction with *O. Cumana* has also been reported (Letousey et al., 2007). SA-induced genes have also been shown to be

expressed in rice during resistant interactions with *S. hermonthica* (Swarbrick et al., 2008), although key SA markers such as the NPR1 (nonexpresser of pathogenesis related (PR) 1) gene which controls the onset of systemic acquired resistance (SAR) immunity in plants (Cao et al., 1997), were not up-regulated.

Recent RNA expression analysis of *S. hermonthica*-infected rice roots suggests the importance of a rapid JA-dependent response for resistance, which is followed by the induction of SA pathways. Mutuku et al., 2015 carried out RNA expression analysis of *S. hermonthica*-infected rice roots to investigate resistance (more resistant Nipponbare vs more susceptible Koshihikari). JA pathways were first induced, followed by SA pathways, indicating the importance of a rapid JA-dependent response for resistance. Foliar application of JA increased resistance, while the absence of JA biosynthesis gene *ALLENE OXIDE CYCLASE* increased susceptibility, and resistance was recovered by JA application. SA-deficient *NahG* plants showed JA pathway up-regulation and were more resistant, and knock-down of the SA/benzothiadiazole pathway regulator *WRKY45* resulted in JA pathway down-regulation and increased susceptibility which was rescued by foliar JA application (Mutuku et al., 2015).

Both SA and JA are therefore important in defence against parasitic plants, and susceptible interactions require the manipulation of these hormones and the down-stream defences responses. This draws an interesting parallel with the AM symbiosis, which requires the suppression of SA-dependent defences, and which can result in systemic priming of JA-dependent defences. As discussed in section 1.3.2, the regulation of host defence hormones during AM colonisation may be mediated by alterations to host ABA levels. Interestingly, ABA is also involved in plant parasitism.

ABA accumulates highly in both the host and parasite during infection. For example, Zhang et al. (2012) measured endogenous hormone levels in haustoria of *Santalum album* growing on host *Kuhnia rosmarnifolia*. Attached haustoria contained levels of ABA three times higher than in non-attached haustorial (Zhang et al., 2012). Taylor et al. (1996) measured ABA concentration in leaf tissue of both the host and parasite during maize interactions with *S. hermonthica*. Concentrations of ABA were

significantly higher in the leaves of infected maize compared to uninfected plants. In the *S. hermonthica* parasite, leaf tissue ABA concentration was an order of magnitude higher than in the host (Taylor et al., 1996). This is unusual, because ABA is known to have a role in the closing of stomata during drought stress, but parasite leaves transpire copiously in order to maintain the flow of water and nutrients from the host. The main assimilate in *Rhinanthus minor* and other parasitic plants like *S. hermonthica* is mannitol. High levels of mannitol increase the osmotic potential of the cell sap, so it was proposed that this may be the cause of the high levels of ABA found in parasitic plants (Taylor et al., 1996).

A number of studies have been carried out on ABA flows during the interaction between *R. minor* and its hosts (Jiang et al., 2010). Jiang et al. (2004) found ABA concentrations in shoots, roots and xylem sap of unattached *R. minor* to be many times higher, and leaf ABA up to an order of magnitude higher than in the potential host barley (Jiang et al., 2004). ABA has roles in plant response to drought, and increased biosynthesis is linked to the closing of stomata in leaves to reduce water loss via transpiration. Plant parasites like *R. minor* and *S. hermonthica* derive water and nutrients from the host via the xylem connection. To maintain a flow of nutrients from the host, host transpiration is reduced dramatically to establish a flow of xylem contents towards the transpiring parasite (Ackroyd and Graves, 1997, Taylor et al., 1996, Watling and Press, 2001). Increased ABA in parasite-infected plants could be caused by water stress via the loss of water to the parasite, by transfer of ABA from parasite to host, or by host ABA biosynthesis being manipulated by the parasite; it is not clear which is true.

The observations made on the involvement of plant defence hormones in *Striga* infection tie in well with their involvement in AM colonisation. It may be the case that suppression of SA-dependent plant defence by AM colonisation could make a plant more susceptible to parasitic plant infection. Both infections also appear to involve ABA, which could be responsible for regulating host changes in SA and JA.

1.4.4 The effect of *Striga* on host growth

Compatible interactions between host plants and *Striga* lead to the differentiation of the parasite haustorium and growth and development of the parasite towards emergence and flowering. To maintain a flow of nutrients from the host, host transpiration is reduced dramatically to establish a flow of xylem contents towards the transpiring parasite (Ackroyd and Graves, 1997). Exploitation of this nutrient source allows the parasite stem and leaves to grow underground before emerging and flowering to set seed after around 6 weeks of growth (Scholes and Press, 2008).

Obligate hemiparasites parasites such as *Striga* spp. receive about 30% of their carbon from their host plant (Irving and Cameron, 2009). This dependency means that *Striga* has a dramatic negative effect on host biomass. The loss of biomass is partly due to the loss of nutrients and water from the host to the parasite (Frost et al., 1997, Gurney et al., 1999). However, the effect of *Striga* infection on host plants cannot be fully accounted for by a source-sink relationship and occurs very soon after attachment (Frost et al., 1997). This has been attributed, at least in part, to a reduction in host photosynthesis as a result of elevated levels of ABA and stomatal closure in *Striga*-infected hosts (Frost et al., 1997, Cameron et al., 2008a). For example, reduced photosynthesis via reduced CO_2 assimilation and chlorophyll fluorescence (via electron transport through photosystem II and photochemical quenching) has been shown in *Striga*-infected *Sorghum bicolor*, but varies between tolerant and resistant genotypes (Rodenburg et al., 2008).

Morphologically, the overall negative effect of *Striga* on host biomass manifests itself through stunting, decreased stem diameter, decreased leaf area, leaf senescence, and decreased tiller number (Cechin and Press, 1994, Watling and Press, 2000, Cissoko et al., 2011, Echegoyen-Nava, 2012a). Root growth is also negatively affected by *S. hermonthica* infection but to a lesser extent than shoot growth, therefore reducing the root-to-shoot ratio in comparison to uninfected plants (Cechin and Press, 1994).

As discussed above, *Striga* infected plants show increased levels of ABA which may be involved in the regulation of defence hormones such as SA and JA, similar to theories about defence hormone regulation during AM colonisation. However, ABA may also interact with plant growth regulating (PGR) hormones. Alterations in PGRs are known to occur during *Striga* infection, resulting in the distinct effects of *Striga* on host morphology (Echegoyen-Nava, 2012a). At a mechanistic level, it has been suggested that the rapid and disproportionate *Striga*-effect on host growth could be due to the introduction of a toxin into the host (Scholes and Press, 2008). However, knowledge that parasitic plants transfer compounds to the host plant, such as siRNA, suggests that the parasite may use this mechanism to directly regulate PGRs as part of its effect on host growth and morphology.

SLs, which have already been mentioned for their signalling roles in the AM and *Striga* symbioses, are one of the PGRs likely altered by *Striga* infection (Echegoyen-Nava, 2012a). SLs act as endogenous plant hormones which are important in regulating many aspects of plant architecture, particularly in suppressing branching (tillering in rice). Indeed, this role for SLs was identified by the characterisation of low branching mutants in *Arabidopsis* and other models and low tillering mutants in rice (Gomez-Roldan et al., 2008, Umehara et al., 2008). Along with SLs, other PGRs including auxin, which is also involved in the suppression of shoot branching (Zhao, 2010), cytokinins (CKs), which act antagonistically to auxin by promoting shoot branching, and gibberellins (GAs), which promote stem elongation (Su et al., 2011) are likely altered by *Striga* infection. However, it is unclear if these hormones are directly altered by the parasite or if hormones can be introduced from the parasite to the host.

1.5 Previous work on the interaction between AM fungi and *Striga*

Earlier research into the interaction between AM fungi and *Striga* has indicated the involvement of SLs as summarised in the following studies. Lendzemo et al. (2005) used maize and sorghum grown in North Cameroon, Africa, to test the effect of AM inoculation on *Striga* infestation in the field. Plants grown in fields infested with *S. hermonthica* seeds were inoculated with AM fungi (*Rhizophagus clarus* syn. *Glomus clarum* and *Gigaspora margarita*), and it was found that inoculation significantly

reduced *Striga* emergence and dry weight on both crops (Lendzemo et al., 2005). This gave an early indication of the possible benefit of AM inoculation and colonisation in combating the *Striga* problem.

A later pot experiment by Lendzemo et al. (2007) used two cultivars; S-35, which is *Striga*-tolerant, and CK60-B, which is *Striga*-sensitive. Sorghum plants were grown with and without AM inoculum and harvested at 24 and 45 days after sowing (DAS). In agreement with Lendzemo et al., (2005), *S. hermonthica* attachment was reduced in AM-colonised sorghum compared to plants grown with the parasite alone. Additionally, root exudates were collected from controls and AM-colonised plants by immersing the roots in water for 36 hours, then using the subsequent solution in *S. hermonthica* seed germination assays. The germination of *S. hermonthica* seeds in root exudates from AM-colonised plants was found to be lower than in root exudates from un-colonised controls. It was proposed by Lendzemo et al. (2007) that the lower percentage germination of *S. hermonthica* seeds and lower attachments was due to decreased SLs in root exudates of AM-colonised plants (Lendzemo et al., 2007).

The involvement of SLs was confirmed in a study by Lopez-Raez et al. (2011), who reported reduced levels of SLs in the root exudates and root extracts of tomato plants infected with *Rhizophagus irregularis* syn. *Glomus intraradices* compared to uninfected plants. In this experiment, SLs were quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS), and the reduction in SLs was found to be dependent on the extent of AM colonisation. Root exudates and extracts from AM-colonised plants also reduced germination of *Phelipanche ramosa* parasitic plant seeds by about 50% compared to root exudates and extracts from un-colonised controls (Lopez-Raez et al., 2011). The evidence presented so far is consistent with the hypothesis that the reduction in SL exudation following colonisation and nutrient status enhancement of plant roots by AM fungi is an important factor in the interaction between AM colonisation and *Striga* infection. The SL story (Fig. 1.6) is therefore often used as the main mechanistic basis behind the effect of AM colonisation on *Striga* infection (Xie et al., 2010).

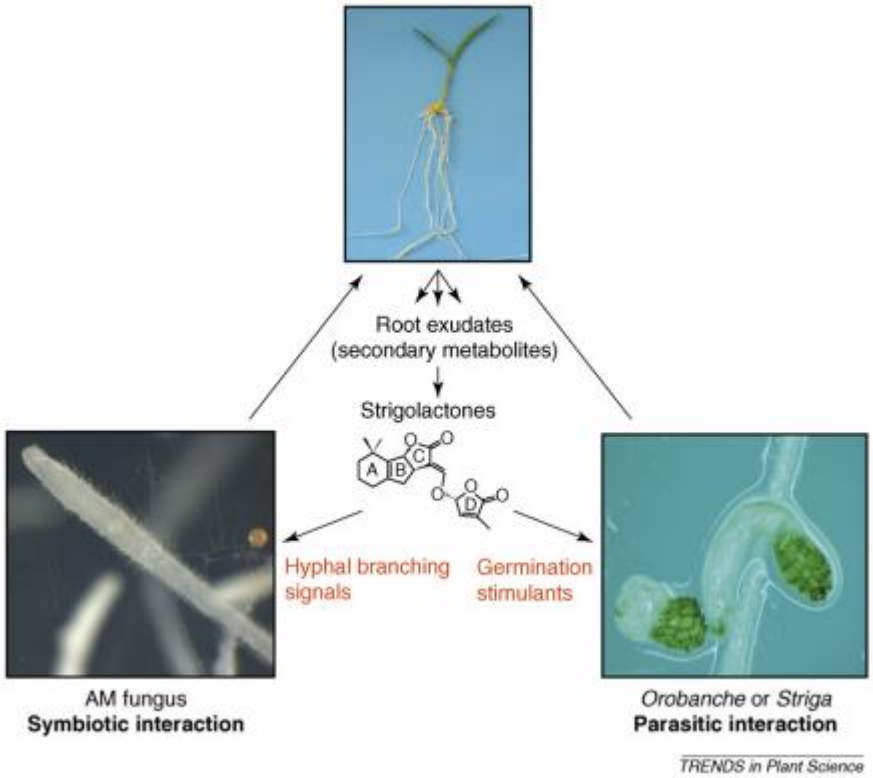


Fig. 1.6 The SL story in the individual AM-host and *Striga*-host symbioses (Bouwmeester et al., 2007).

In summary, there is currently good evidence in the literature that AM colonisation can reduce the germination-stimulating activity of host root exudates, and that this effect is due to reduced SLs. However, this is as far as our knowledge currently extends in this interaction, and it is likely that many more mechanisms alter symbiont and host success during the interaction. In this introductory chapter, many common features have been described for both AM fungal colonisation and *Striga* infection, including SL signalling, the involvement of plant hormones including SA, JA and ABA, and the involvement of secondary defence metabolism including flavonoids, phytoalexins and lignin. More research needs to be done to show how these common factors are involved in the interaction, how the success of *Striga* infection is altered by the presence of AM fungi and vice-versa, and what the consequences are for host growth. Critically, the SL mechanism described above makes a major assumption; that AM fungi are able to colonise the host plant first, supply the host with nutrients, and that SL biosynthesis is down-regulated in time to reduce *Striga* germination in the rhizosphere. It is likely that the interaction does not always follow this sequence

of events, and involves more than just SLs, so more needs to be done in order to understand the interaction in detail. In particular, it does not seem likely that AM fungi will always colonise the host plant well in advance of *Striga*, so the effect that the order and timing of colonisation / infection has on the interaction needs to be investigated.

1.6 Rice: A model host and staple crop

This introduction as so far introduced two obligate symbionts with the aim of examining their interaction. Of course, the individual symbioses and their interaction take place via a host plant. Throughout this thesis, the rice cultivar IAC165 (*Oryza sativa* L. subspecies *japonica*) is used in all experiments as the host plant.

Rice is arguably the most important food crop in the world, feeding over half of the world population (Matsumoto et al., 2005). As such, rice has a wide geographical distribution and a long history of cultivation which has led to the development of many genetically and phenotypically distinct cultivars (Matsumoto et al., 2005). Rice is a model cereal crop useful for its small genome size (389 Mb) (Matsumoto et al., 2005) which, while larger than the non-crop model *Arabidopsis thaliana* (125 Mb) (Kaul et al., 2000), is much smaller than that of other important cereal crops such as sorghum (730 Mb) (Paterson et al., 2009), maize (2300 Mb) (Schnable et al., 2009) and wheat (17000 Mb) (Brenchley et al., 2012), and there is an extensive mutant collection for rice (Hirochika et al., 2004). For example, the role of SLs in shoot branching (tillering in rice) inhibition has been identified by the characterisation of branching mutants, many of which exist for rice (Gomez-Roldan et al., 2008, Umehara et al., 2008). Its morphology gives it many easily measurable characteristics such as stem height, stem diameter, tiller number, internode length, and leaf number. This makes it useful in scientific studies and amenable to breeding by growth characteristics measured by eye in the field. In agriculture, rice has been selectively bred over many years for a range of traits such as reduced height and disease resistance.

Rice is grown in over 40 countries in Africa (Seck et al., 2010), and is an increasingly important crop in rain-fed fields (Rodenburg et al., 2015). In SSA, more

than 70% of the population make a living from farming (Balasubramanian et al., 2007). However, many constraints exist for subsistence farmers, including drought, poor soil fertility and weeds including *Striga* spp. (Waddington et al., 2010, Reynolds et al., 2015). The distribution patterns of rice (Fig. 1.7) and *S. hermonthica* (Fig. 1.3) in SSA are strikingly similar, which shows how much crop systems are under threat from this parasite (Balasubramanian et al., 2007, Mohamed et al., 2001).

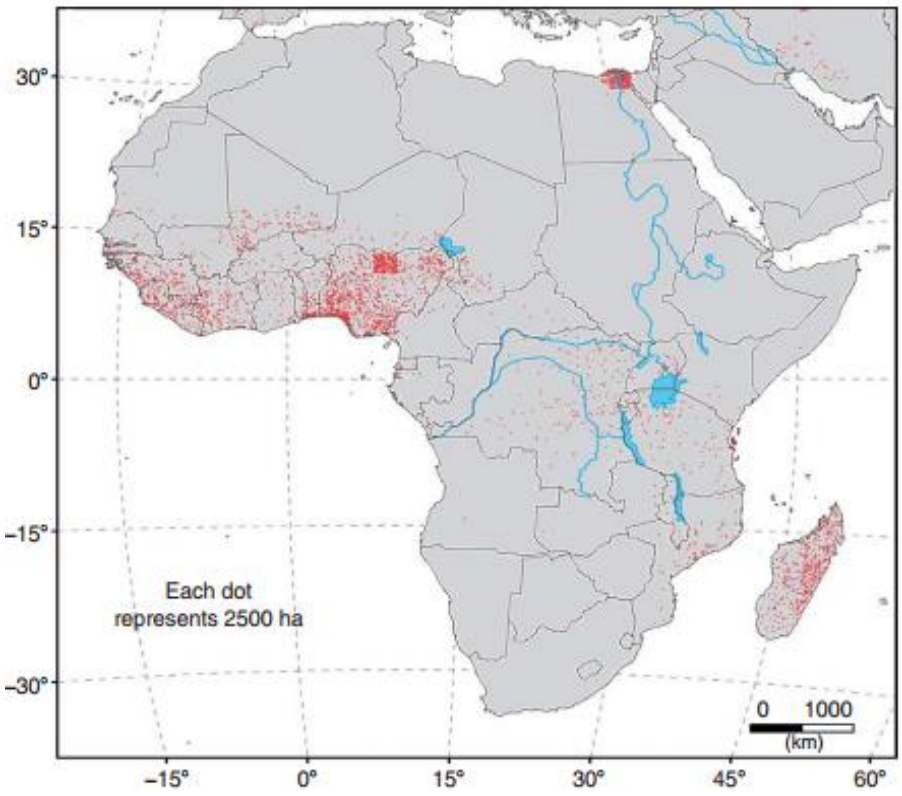


Fig. 1.7 The distribution of rice in SSA (Balasubramanian et al., 2007).

Rice, unlike the classic plant model *Arabidopsis thaliana*, is both mycorrhizal and subject to parasitism by *S. hermonthica* in laboratory and field conditions. Because of this, rice has been used to study gene expression during mycorrhizal colonisation (Guimil et al., 2005), and is often used in *Striga* research (e.g. Mutuku et al., 2015), and it is therefore ideal for studying the interaction between these two symbionts. Thus, rice is used as the host-symbiont system in this thesis.

1.7 Key considerations for this thesis

Throughout this thesis, the same three species of AM fungus, parasitic plant and host plant were used, although a different genotype of *S. hermonthica* and isolate of *R. irregularis* was used in the first experimental chapter. The ecotype of *S. hermonthica* used in the first experimental chapter was sourced from Korhogo Kuoto in 2008, whereas the ecotype used in subsequent experimental chapters was sourced from Kibos in 2013. The isolate of *R. irregularis* used in the first experimental chapter was an isolate from a commercially available source (Plant Works), whereas the isolate used in subsequent experimental chapters was *R. irregularis* isolate 09 maintained in axenic culture. It is widely known that both *R. irregularis* and *S. hermonthica* are highly variable, so it is important to define them from the outset.

With a complex three-way interaction such as the one studied in this thesis, it is also necessary for the purpose of clarity to define some commonly used terms. As the non-obligate player in this interaction, this thesis uses the term 'host' to describe the host plant rice. 'Parasite' always refers to *S. hermonthica*, where this characteristic is not disputed in this thesis. *R. irregularis* is simply described broadly as a fungus rather than as a mutualist, which avoids the assumption of mutualism which is often made of the AM symbiosis (Smith et al., 2011). Importantly, 'infection' refers to parasitism by *Striga*; whereas 'colonisation' refers to the presence of both extraradical and intraradical AM fungal structures associated with the host root.

1.8 Aims, hypotheses and objectives

The overall aim of this thesis is to further our knowledge and understanding of the interaction between the AM fungus *Rhizophagus irregularis* and the parasitic weed *Striga hermonthica*. This thesis will investigate the role of host defence, nutrient supply, and the order and timing of colonisation/infection in the success of these symbioses and the host plant. Understanding the interaction in more detail will allow us to begin assessing the possible impact of AM fungi on the *Striga* problem in SSA.

Chapter 2 aims, hypotheses and objectives

The aims of chapter two were to determine a) the interactive effects of *R. irregularis* and *S. hermonthica* on host defence metabolism; b) the consequences of this for the colonisation/infection success of each symbiont; and c) the downstream consequences on host growth and development.

Hypotheses:

1) *R. irregularis* decreases the amount of *S. hermonthica* infection in rice because they upregulate host defence physiology and/or because AM fungi increase host nutrient status.
2) *R. irregularis* alleviates the negative effects of *S. hermonthica* on the growth and development of rice because they enhance the nutrient status of the host plant.

Objectives:

1) Grow two cultivars of rice, IAC 165 and Shiokari in factorial combination with AM fungi and *S. hermonthica*.
2) Measure growth and development weekly over a 10-week period.
3) Harvest plants 10 weeks after sowing.
4) Measure nutrient status of the host.
5) Measure colonisation by *R. irregularis* and infection by *S. hermonthica*.
6) Analyse the defence metabolome of the host roots.

Chapter 3 aims, hypotheses and objectives

The aim of chapter 3 was to understand the effects of different amounts of substrate N/P levels on host colonisation/infection success of each symbiont, alone and in combination, and the subsequent effects on host growth and development.

Hypotheses

1) Decreasing N/P supply will enhance colonisation/infection of the host by both symbionts singly and in combination due to an increase in the host release of branching and germination stimulants.
2) Decreasing N/P supply will limit the ability of the host to support a high level of *S. hermonthica* infection alone and/or in combination with *R. irregularis*.

Objectives

1) Grow the rice cultivar IAC 165 in factorial combination with *R. irregularis* and *S. hermonthica* alone and in combination in pots.
2) Supply the pots with two levels of N/P concentration; one in which plants are likely to receive a sufficient supply ('high'), and the other where the plants are likely to be deficient ('low') in reference to the clearly sufficient supply used in chapter 2.
3) Measure growth and development weekly over a 10-week period.
4) Carry out harvests at 5 and 10 weeks after sowing.
5) Measure nutrient status of the host.
6) Measure colonisation by *R. irregularis* and infection by *S. hermonthica*.
7) Collect root exudates from IAC 165 grown alone and grown with *R. irregularis* and perform a bioassay on *S. hermonthica* seeds to analyse germination and haustorium formation. This will test the effect of AM colonisation on host root germination stimulating activity on parasite seeds.

Chapter 4 aims, hypotheses and objectives

The aim of chapter 4 was to determine whether the order of infection/colonisation of the symbionts affects the success and spatial distribution of either symbiont.

Hypothesis:

The first symbiont to colonise/infect the host plant will suppress infection by the other because early arrival will establish a priority effect via competition for space,

resources and effects on host defences which may act antagonistically on the invading symbiont.

Objectives:

1) Grow, colonise and infect the rice cultivar IAC 165 with *R. irregularis* isolate 09 and pre-germinated *S. hermonthica* in rhizotrons.
2) Co-colonise/infect with *R. irregularis* and *S. hermonthica* at the same time.
3) Pre-colonise with *R. irregularis* for five days before infecting with *S. hermonthica* for 14 days.
4) Pre-infect with *S. hermonthica* for five days before colonising with *R. irregularis* for 14 days.
5) Measure *S. hermonthica* infection on roots colonised by *R. irregularis* and roots not colonised by *R. irregularis* separately.
6) Measure colonisation on roots infected with *S. hermonthica* and roots not infected by *S. hermonthica* separately.

Chapter 2

Analysis of the interaction between *Rhizophagus irregularis* and *Striga hermonthica* in rice: consequences for the host defence metabolome and symbiont success.

2.1 Introduction

Arbuscular mycorrhizal (AM) fungi and the parasitic plant *Striga hermonthica* occupy a similar ecological niche. Both are obligate root symbionts which require association with a host plant to complete their life-cycle (Smith and Read, 2008, Joel et al., 2013). Colonisation/infection of the host by both symbioses is enhanced by plant signalling compounds exuded by the roots, particularly strigolactones (SLs) (Akiyama et al., 2005, Cook et al., 1972, Yoneyama et al., 2010). Strigolactones trigger germination of Striga seeds and promote branching of the mycorrhizal hyphae facilitating infection of the host by each symbiont. The exudation of strigolactones is increased under nutrient-limited conditions (Yoneyama et al., 2007a, Yoneyama et al., 2007b). Thus, the nutrient poor soils of sub-Saharan Africa (SSA) create a potential environment for both AM colonisation and *S. hermonthica* infection. Indeed, *Striga* infestation is most severe in nutrient poor conditions, to the point where infestation can be used as an indicator of low soil fertility (Oswald, 2005). Despite their similar niche, these symbionts have opposing strategies to obtain space within the host root and acquire nutrients from it. AM fungi trade N and P for C via specialized arbuscules in the inner cortical cells of colonized roots, which indicates a degree of cooperation or mutualism (Parniske, 2008, Smith and Read, 2008). On the other hand, *S. hermonthica* parasitises its host via a specialised organ, the haustorium, which connects it to the host vascular system allowing it to obtain nutrients and water (Joel et al., 2013, Yoshida et al., 2016a). In carrying out their respective symbioses, both AM fungi and *S. hermonthica* manipulate the growth, morphology, and nutrient status of their host in opposing ways. While AM fungi can be beneficial mutualists, increasing host nutrient status and growth despite imposing a carbon demand (Klironomos, 2003), *S. hermonthica* has negative effects on host growth and nutrient status (Gurney et al., 1999, Cissoko et al., 2011).

Once AM fungi have colonised the host, SL exudation from the roots is reduced, due to an increase in the N and P status of the host plant (Lopez-Raez et al., 2011). It has been suggested that this will cause a reduction in *Striga* seed germination and therefore infection of potential host plants (Lendzemo et al., 2007). This hypothesis has been supported by studies which have shown a reduction in parasitic plant infection when combined with AM fungi (Lendzemo et al., 2005, Lendzemo et al.,

2007, Othira et al., 2012), and that root exudates from mycorrhizal plants have lower SLs levels and a reduced capacity to induce parasitic plant seed germination (Lopez-Raez et al., 2011). However, a tripartite plant-fungal-plant interaction is likely to involve more than just one plant hormone, and is also likely to involve post attachment mechanisms. It is therefore necessary to consider the role that other plant processes have. A logical target is plant defence, because the recognition of foreign, non-self-material such as microbe-associated molecular patterns (MAMPs) initiates plant defence responses (Zhang and Zhou, 2010).

Phenylpropanoid metabolism is essential in plant defence as it is associated with defence responses such as the production of phytoalexins with antimicrobial activity, and deposition of lignin, suberin and callose which are used for physical protection against penetration (Vogt, 2010). Phytoalexins are low molecular mass secondary metabolites which have antimicrobial activity (Ahuja et al., 2012) but are also known to be involved in defence against the holoparasite *Orobanche cumana* (Serghini et al., 2001). Rice produces many phytoalexins (Cho and Lee, 2015). For example, the flavonoid phytoalexin sakuranetin has strong antimicrobial activity towards blast fungus as part of the resistance response in rice (Hasegawa et al., 2014). Vestitol is a legume-specific phytoalexin which is highly up-regulated in roots of the incompatible host *Lotus japonicus* after *S. hermonthica* attachment, and which has been shown to significantly inhibit radicle growth (Hiraoka et al., 2009).

Phenylpropanoid metabolism also provides the precursors necessary for lignin deposition. Lignin is a highly branched component of the cell wall formed by the polymerisation of the major monolignol monomers coumaryl alcohol, coniferyl alcohol, and syringyl alcohol. Polymerisation of these monomers occurs by oxidation by hydrogen peroxide (H_2O_2) catalysed by peroxidase enzymes to form p-hydroxyphenyl (H) lignin, guaiacyl (G) lignin and syringyl (S) lignin respectively (Rogers and Campbell, 2004, Vanholme et al., 2010). Plant lignin is composed mainly of the G- and S- units (Vanholme et al., 2010), and the ratio of these units in lignified cell walls appears to determine the effectiveness of lignin in the plant defence response (Hawkins and Boudet, 2003). For example, stem-wounding stress in *Eucalyptus gunii* has been shown to result in the vascular deposition of ‘defence lignin’ which contains less S-type lignin units compared to unwounded plants

(Hawkins and Boudet, 2003). However, *Triticum aestivum* (wheat) infected with the stem rust fungus *Puccinia graminis* has been shown to accumulate S-type rich lignin in the leaves during hypersensitive response (HR) resistance reactions (Menden et al., 2007). The regulation of monolignol monomers in lignin may therefore depend on the type of damage received or the compatibility between the two organisms.

Most studies on involvement of phenylpropanoid metabolism in AM colonisation use gene expression analysis. For example, AM fungi have been shown to increase PAL and CHS gene expression at the early stages of colonisation in *Lotus japonicas* roots (Deguchi et al., 2007). More recently, metabolite analysis has shown that many intermediates involved in phenylpropanoid metabolism are up-regulated in the roots of S*olanum lycopersicum* after 8 weeks of growth and colonisation by the AM fungus *R. irregularis* (Rivero et al., 2015). Isoflavonoids in legumes roots are also increased by AM colonisation as shown in early metabolomic studies (Schliemann et al., 2008, Laparre et al., 2014). Furthermore, flavonoids are involved in AM colonisation (Steinkellner et al., 2007) in pre-symbiotic signalling (Scervino et al., 2006), colonisation establishment stages (Larose et al., 2002) and in colonisation regulation (Catford et al., 2006).

During parasitic plant infection, lignification of host root tissues at the site of invading parasite structures is a key resistance mechanism in host plants which is used to prevent the establishment of vascular connections, as seen in histological studies. For example, Cameron et al. (2006) were able to show that resistance to *Rhinanthus minor* by the non-host forb *Leucanthemum vulgare* involves encapsulation of the invading parasite structures. The same study used Fourier-transform infrared micro-spectroscopy to identify lignin in the incompatible interaction, and show that lignin was not present in the parasite interface with the susceptible host plant *Cynosurus cristatus* (Cameron et al., 2006). Transcriptomic studies have shown that *S. hermonthica* alters phenylpropanoid metabolism, and that the nature of this regulation differs between susceptible and resistant rice cultivars (Swarbrick et al., 2008). Transcriptomic evidence for the up-regulation of genes involved in lignin biosynthesis and cell wall modifications in sorghum parasitised by *S. gesnerioides* has also been shown (Huang et al., 2012). Transcriptomic analysis of host plants during interactions with *Orobanche* has also shown the involvement of

phenylpropanoid metabolism in resistance. For example, *O. cumana*-parasitised sunflower shows up-regulation of the *pal* gene during resistant interactions (Letousey et al., 2007). Similar to their signalling role in AM fungi, a flavonoid (peonidin) also initiates haustorium formation in the facultative parasitic plant *Triphysaria versicolor*, although not in *Orobanche* spp. and *Phelipanche* spp. (Joel and Losnergoshen, 1994, Westwood et al., 2012). Furthermore, a flavonoid isolated from *Desmodium uncinatum* root exudates has been identified as an allelochemical which inhibits growth of the *S. hermonthica* radicle (Hooper et al., 2010).

Successful symbiosis requires regulation of host defences by both mutualistic AM fungi and parasitic plants like *S. hermonthica*. For example, successful AM colonisation involves systemic fluxes in plant defences (Blilou et al., 1999, Blilou et al., 2000a, Blilou et al., 2000b), but also localised defence suppression (Kloppholz et al., 2011), and results in the systemic priming of JA- and ethylene-dependent defences (Van Wees et al., 2008, Van der Ent et al., 2009, Jung et al., 2012, Gerlach et al., 2015). Infection by *S. hermonthica* also results in host defence responses throughout the infection process which ultimately determine the success of the parasite (Yoder and Scholes, 2010, Mutuku et al., 2015). While both JA and SA have been implicated in *S. hermonthica* infection, it is now thought that resistance to *S. hermonthica* requires a rapid JA-dependent response followed by the induction of SA-dependent responses (Mutuku et al., 2015).

While the individual effects of these symbioses on host defences are well studied, it is unclear if the effect that AM fungi have on plant defences has any influence on the ability of *S. hermonthica* to infect host plants, or vice-versa. However, because they interact so intimately with their hosts at the post-attachment level, AM fungi and *S. hermonthica* are likely to influence the relative fitness of each other. Still, the consequences of co-infection by *S. hermonthica* and AM fungi for host, AM and parasite performance remain poorly understood. In the first instance, studying such a complex interaction requires a global analysis of physiology.

Analysis of the host metabolome has the potential to provide a global view of the complex mechanisms underpinning host phenotype (Brunetti et al., 2013). The metabolome is the quantitative collection of molecular compounds (metabolites)

which are involved in metabolic reactions in a cell or organism, and metabolomics is the study of the metabolome (Oliver et al., 1998). Metabolites are the final downstream products of the genome, transcriptome, and proteome; and so, compared to the other omics fields, metabolomics provides a relatively complete representation of phenotype (Fiehn et al., 2000). Plant metabolomes can contain around 200,000 metabolites (Fiehn, 2001), compared to around 600 in the yeast *Sacchromyces cerevisiae* (Forster et al., 2003). The large number of metabolites, variations in chemical and physical properties, and the fact they can be found in picomolar (pM) to millimolar (mM) concentrations, makes analysis of the plant metabolome highly challenging (Dunn et al., 2005). Different strategies are required to study the complete range of metabolites in a biological sample. For example, metabolite fingerprinting describes the analysis of characteristic metabolites within a sample for screening or classification purposes, while metabolite profiling is used to analyse many more (often thousands) of metabolites in order to identify those in similar metabolic pathways (Kopka et al., 2004, Dunn et al., 2005). To this end, mass spectrometry is used in the detection of many metabolites in a complex mixture. In this chapter, matrix-assisted laser desorption/ionisation (MALDI)-MS was used to analyse the rice defence metabolome, focussing on the phenylpropanoid biosynthesis and flavonoid biosynthesis pathways, which are known to be involved in plant defence during the individual symbioses with AM fungi and parasitic plants.

With the above information in mind, the aims of chapter two were to determine a) the interactive effects of *R. irregularis* and *S. hermonthica* on host defence metabolism; b) the consequences of this for the colonisation/infection success of each symbiont; and c) the downstream consequences on host growth and development.

Hypotheses

1) *R. irregularis* decreases the amount of *S. hermonthica* infection in rice because AM fungi are known to upregulate host defence physiology and/or because AM fungi are known to increase host nutrient status.

2) *R. irregularis* alleviates the negative effects of *S. hermonthica* on the growth and development of rice because AM fungi are known to enhance the nutrient status of host plants.

Objectives

1) Grow two cultivars of rice, IAC 165 and Shiokari in factorial combination with AM fungi and *S. hermonthica*.
2) Measure growth and development weekly over a 10-week period.
3) Harvest plants 10 weeks after sowing.
4) Measure nutrient status of the host.
5) Measure colonisation by *R. irregularis* and infection by *S. hermonthica*.
6) Analyse the defence metabolome of the host roots.

2.2 Materials and Methods

2.2.1 Plant and fungal materials

Seeds of *Oryza sativa* ssp. *Japonica*, IAC 165 were obtained from the International Rice Research Institute (IRRI) Phillipines, whilst those of variety Shiokari were obtained from Prof Harro Bouwmeester, Wageningen University. *Striga hermonthica* seeds were collected from rice cultivar Iguape Cateto growing in Korhogo Kouto, Ivory Coast in 2010. The arbuscular mycorrhizal inoculum which contained *Rhizophagus irregularis* spores (1500-3000/L), hyphae, and infected maize and clover roots was a commercial product obtained from PlantWorks Ltd, UK.

2.2.2 Experimental set up

Rice seeds of IAC 165 and Shiokari were surface sterilised with 10% (v/v) bleach for 15 min followed by extensive washing with water. Seeds were then incubated on moist filter paper in Petri dishes in the dark at 25°C for 2 days to initiate germination. Once germinated, two seeds were transferred to individual cylindrical pots (length 25 cm; diameter 7.5 cm; volume 1.105 L), and thinned to one plant per pot after emergence.

As shown in Fig. 2.1, four different treatments (per cultivar) were established (1) rice plants alone (designated control (-AM-*Striga*)), (2) rice plants plus AM inoculum (designated +AM), (3) rice plants plus *S. hermonthica* seeds (designated +*Striga*), and (4) rice plants plus AM inoculum and *S. hermonthica* seeds (designated +AM+*Striga*). Pots for the control treatment (Fig. 2.1a) were filled with autoclaved Chelford sand only. Pots containing the +AM treatment (Fig. 2.1b) were made by filling the bottom 8 cm of the pot with autoclaved Chelford sand. The remaining 15 cm of substrate was made up of autoclaved Chelford sand mixed with 0.150 g L^{-1} of the *R. irregularis* mycorrhizal inoculum containing spores (1500-3000 L^{-1}), hyphae, and infected maize and clover roots as infective propagules. Pots for the +*Striga* treatment (Fig. 2.1c) were made up by filling the bottom 17 cm of the pot with

autoclaved Chelford sand. Three ml autoclaved Chelford sand mixed with 30 mg *S. hermonthica* seeds were sprinkled on top of the sand in a single layer. Autoclaved Chelford sand was then used for the remaining 6 cm of substrate. Pots containing the +AM+*Striga* treatment (Fig. 2.1d) were made by filling the bottom 8 cm of the pot with autoclaved Chelford sand. The next 9 cm of the pot was then filled with autoclaved Chelford sand mixed with 0.150 g L^{-1} of the *R. irregularis* mycorrhizal inoculum. Three ml autoclaved Chelford sand mixed with 30 mg of the *S. hermonthica* seeds were sprinkled on top of the sand, and the remaining 6 cm of substrate was again made up of autoclaved Chelford sand mixed with 0.150 g L^{-1} of the *R. irregularis* mycorrhizal inoculum. Eight biological replicates were established per treatment. In total, 64 pots were used for each of the two cultivars (128 in total), with the cultivars being planted a week apart to provide two separate repeat experiments, and also the time necessary to harvest.

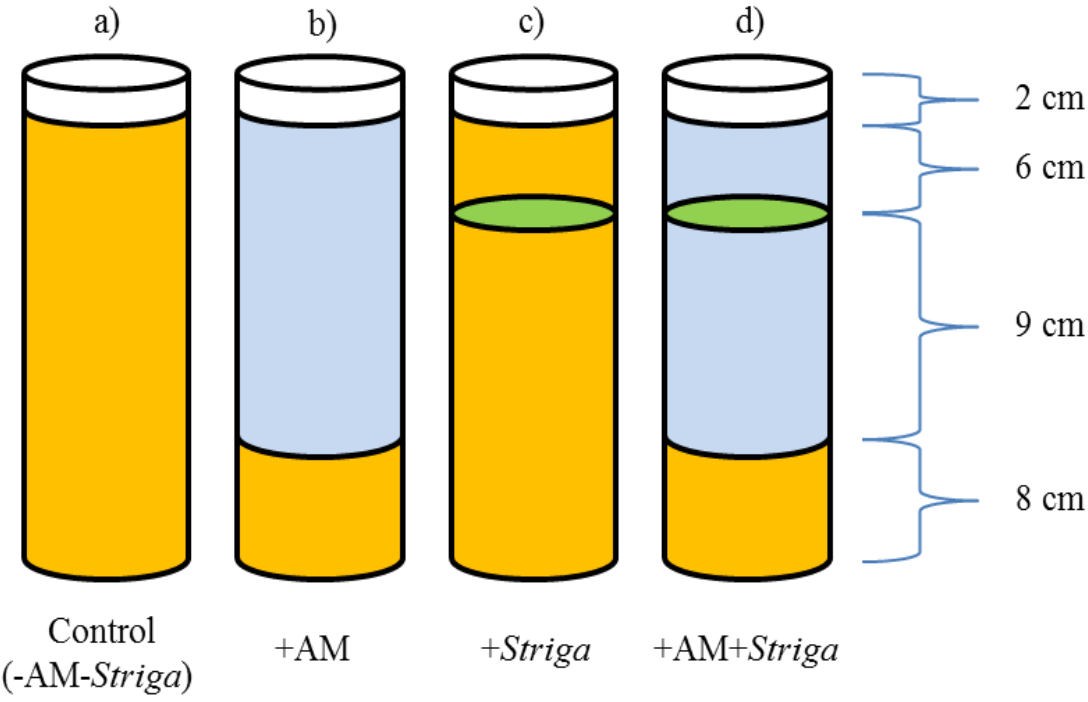


Fig. 2.1: Diagram of the treatments used in the experiment. a) The control treatment containing only autoclaved Chelford sand (yellow). b) The +AM treatment containing autoclaved Chelford sand (yellow), and autoclaved Chelford sand mixed with 0.150 g L^{-1} of the *R. irregularis* mycorrhizal inoculum (blue). c) The +*Striga* treatment containing autoclaved Chelford sand (yellow), and 3 ml autoclaved Chelford sand mixed with 30 mg of the *S. hermonthica* seeds (green). d) The +AM+*Striga* treatment containing both autoclaved Chelford sand mixed with 0.150 g L^{-1} of the *R. irregularis* mycorrhizal inoculum (blue), and 3 ml autoclaved Chelford sand mixed with 30 mg of the *S. hermonthica* seeds (green).

Plants were grown in a controlled environment greenhouse cubicle (Fig. 2.2) which was maintained at 28°C during the day and 24°C at night with 60 % relative humidity for 10 weeks during the months of July and August 2013. Plants were grown when the photoperiod was approximately 16h light and 8h dark. If the irradiance fell below 200 µmol m^{-2} s^{-1} supplementary lighting came on automatically. Plants were watered two times a day using an automatic dripper system delivering a total of 60 ml per day of 40 % Long Ashton solution containing 2mM N and 20 µM P. This equates to a total of 236.32 mg N and 1.26 mg P by the end of the experiment (Fig. 2.3). This volume of nutrient solution was chosen because it prevented the sand from drying out. The concentrations of N and P were in line with other mycorrhizal studies (e.g. Yoneyama et al., 2012, Foo et al., 2013), being reduced to encourage colonisation and infection. Nutrient solution was stored in a 300 L tank and pH adjusted to pH 5.5.

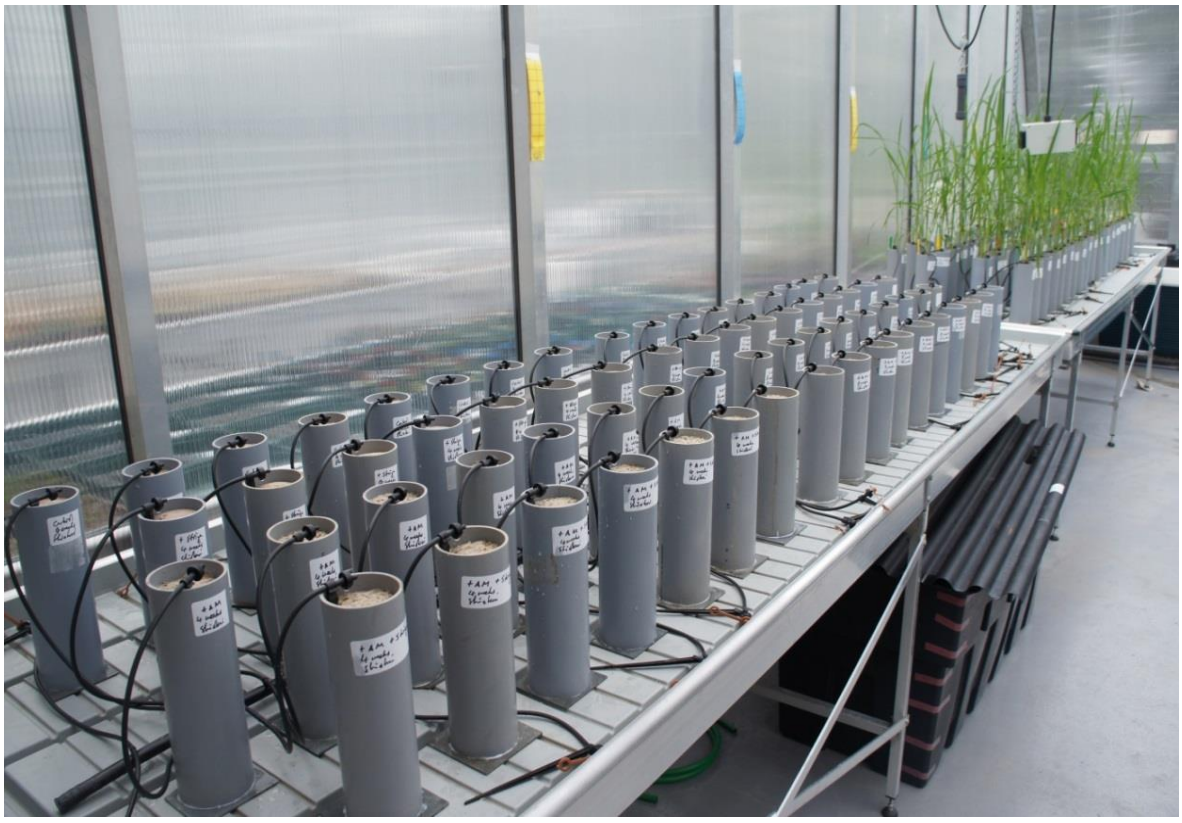


Fig. 2.2 Rice cultivars, IAC 165 (right) and Shiokari (left) growing alone, or with *R. irregularis* or *Striga hermonthica* singly and in combination. Plants were grown in a controlled environment greenhouse chamber.

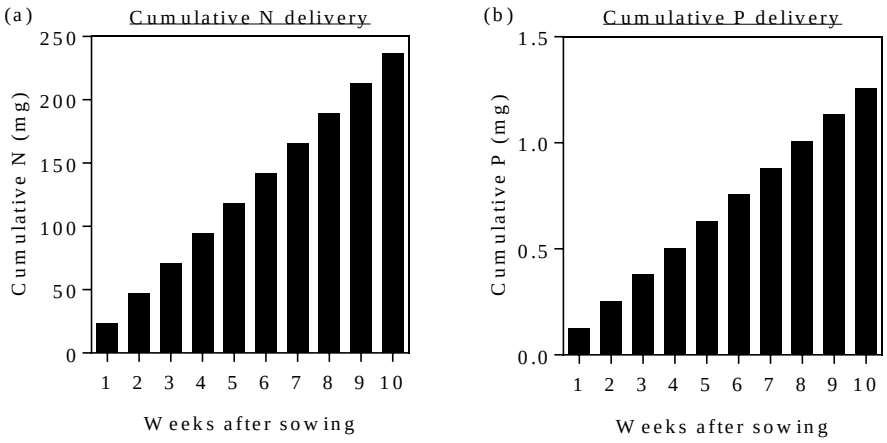


Fig. 2.3 The cumulative amount of N/P supplied to IAC 165 and Shiokari via nutrient solution during the 10 weeks of the experiment.

2.2.3 Measurements of plant morphology

During the experiment, a number of non-destructive measurements were taken once a week, beginning at the end of week 1 and ending 10 weeks after sowing (WAS). The measurements consisted of the height and diameter of the main stem, the latter measurement taken 3 cm above substrate surface using digital calipers (Mitutoyo Absolute Digimatic CD-6''C, England, UK) and the total number of leaves and tillers per plant.

2.2.4 Harvesting of plant material

Plants were harvested 10 weeks after sowing. At the time of harvest each individual plant was divided into roots, stems and leaves. Roots were cleaned by immersing in water above a 250 µm sieve to avoid losing plant material. After cleaning, roots from the +*Striga* and +AM+*Striga* treatments were laid flat in 2 cm water to separate the roots, and *S. hermonthica* parasites were harvested. The number of parasites was recorded for each replicate.

Roots were divided by length into top, middle and bottom thirds to create three sections. All subsamples used for root analysis in this chapter came from the middle section of the root system. The fresh weight of all samples and subsamples was recorded. After subsampling roots for mycorrhizal quantification and metabolite analysis, the remaining roots, and all of the leaves, stems, and harvested *S. hermonthica* were transferred to individually labelled paper envelopes and stored in a drying oven for two weeks at 80°C to obtain their dry weight. Root dry weight was corrected using the fresh weight data collected for the root subsamples. To do this, the proportion of weight lost after drying the fresh roots which remained after subsampling was calculated and applied to the fresh weight of the subsamples taken for mycorrhizal quantification and metabolite analysis. In this way the expected dry weight of the subsamples was calculated. The expected dry wright of the subsamples was then added on to the dry weight which had already been obtained.

2.2.5 Staining of roots and quantification of mycorrhizal colonisation

Approximately 1 g fresh weight of the harvested roots were preserved in 50 % ethanol and stained for quantification of mycorrhizal colonisation via microscopy. Many stages are involved in mycorrhizal staining and quantification (Fig. 2.4a). Before staining, root samples were cleared in 10 % KOH (Fig. 2.4b) for one autoclave cycle (35 minutes, 126°C, 1.4bar) to clear them of pigments and tannins which would obscure the view of fungal structures. After autoclaving, the roots were rinsed in water and immersed in 10 % HCL for 10 minutes. Roots were then rinsed again in water, then stained by immersion in trypan blue for 5 minutes (Phillips and Hayman, 1970). After staining, roots were rinsed with water and stored in 50 % glycerol (Fig. 2.4b) until they were needed for analysis. Total colonisation was measured using the grid line intersect method (Giovannetti and Mosse, 1980). To do this, approximately 0.1-0.2 g of stained roots were spread evenly on a plastic petri dish (9 cm diameter) with a 1.27 cm grid square pattern drawn on (Fig. 2.4c). This size of grid allows the number of centimetres of root analysed to be estimated via the number of intersects counted, where 1 intersect = 1 cm root (Giovanetti and Mosse, 1980). Roots were viewed under a dissecting microscope (Leica stereo, MZFLIII, Diagnostic Instruments Inc, USA), and the lines of the grid were traced vertically, then horizontally, until 150 intersections between the roots and grid lines were

counted. Intersect points were counted as mycorrhizal if intraradical mycorrhizal structures (intraradical hyphae, vesicles, or arbuscules) were observed (Fig. 2.4d). Colonisation was expressed as a percentage of intersects which were observed to be mycorrhizal.

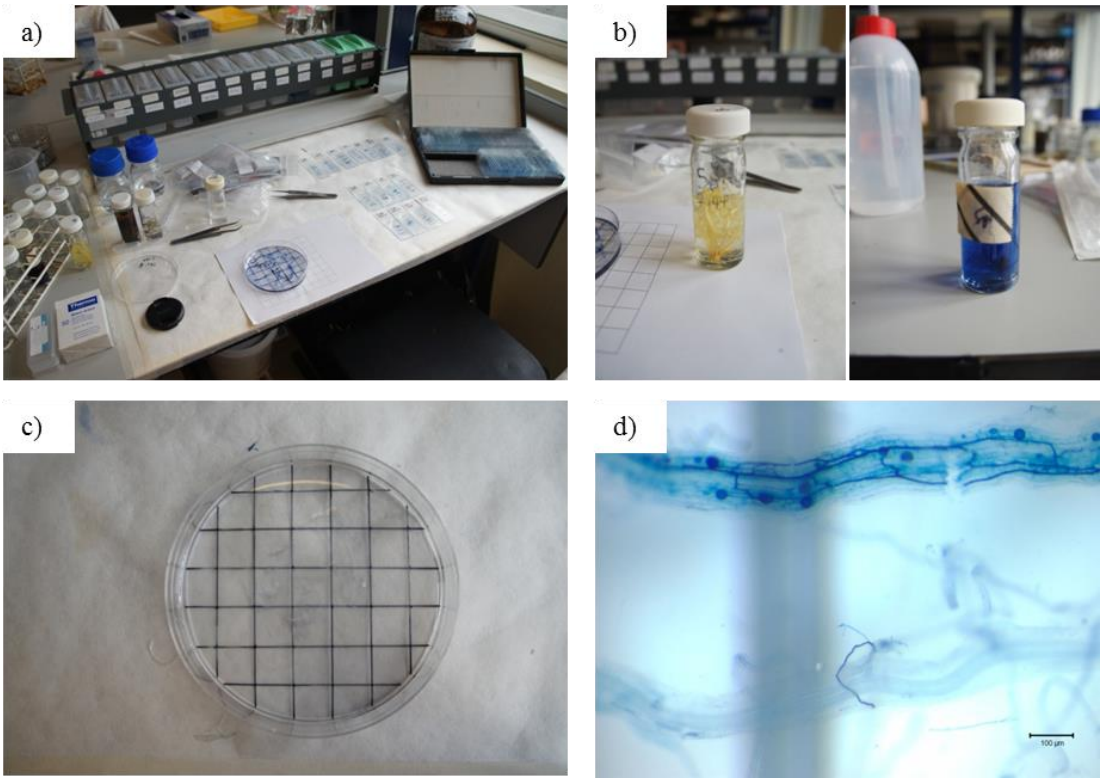


Fig. 2.4 Equipment used for staining and quantifying mycorrhizal colonisation. a) Picture of all equipment used. b) Roots in 10 % KOH before staining and in 50 % glycerol after staining. c) Grid lines drawn onto 9 cm petri dish to make 1.27 cm diameter squares. d) View of roots under dissecting microscope x80 magnification. The top root is colonised with visible intraradical hyphae and vesicles, while bottom root is not colonised. Scale bar = 100 µm.

2.2.6 Nutrient determination

Oven dried leaf and root material was homogenized using a Yellowline A10 Analytical Grinder (IKA, Staufen, Germany). A 50 mg subsample was digested using the Kjeldahl method (Allen, 1989). The Kjeldahl method uses a sulphuric acid and salicylic acid mix (33 g salicylic acid to 1 L sulphuric acid) with a lithium sulphate and copper sulphate catalyst (10:1 ratio respectively) to convert all nitrogen in samples to ammonium. Each 50 mg subsample was weighed into a digestion tube, to which 0.05 g catalyst and 1 ml acid was added, and then heated to 370°C in a digestion block (Techne DG-1) for approximately 6 hours until the solution cleared.

Blanks were prepared in the same way but without the addition of a sample. The resulting solution was diluted to 50 ml with ultra-high purity (UHP) water.

Digested samples were analysed for P concentration using the ascorbic acid colorimetric method (Murphy and Riley, 1986, Eaton et al., 2005). This method uses the reaction of ammonium molybdate and antimony potassium tartrate in an acid medium with dilute solutions of orthophosphate, and reduction of the resulting antimony-phospho-molybdate complex with ascorbic acid to form a blue coloured solution. To do this, 3.8 ml solutions containing 0.5 ml digest sample, 2.6 ml UHP water, 0.2 ml of 0.1 M L-ascorbic acid, and 0.5 ml developer solution (ammonium molybdate and antimony potassium tartrate) were prepared in cuvettes. A standard curve ($p < 0.98^{-1}$) was generated by using the same mixture but replacing the sample with a 10 ppm P standard (sodium dihydrogen orthophosphate) to final concentrations ranging from 0 to 5 ppm in 0.5 ppm increments, and making the volume up to 3.8 ml with UHP water. Solutions were left to develop for 45 minutes, and optical density was measured at 882 nm using a Cecil Ce 1 020 spectrophotometer zeroed against blanks.

Digested samples were analysed for N concentration using a phenol free modification of the Berthelot reaction (Krom, 1980) adapted for bench top use from the automated flow injection analysis (FIA) method (FIAflow2; Burkard Scientific, Uxbridge, UK). The unmodified Berthelot reaction uses the reaction of ammonia with phenol and hypochlorite to form indophenol blue. In the modified version, phenol is substituted for salicylate. Dichloroisocyanurate (DIC) is used as the source of hypochlorite ions. Sodium hydroxide in the DIC converts the ammonium in the digest to ammonia. To do this, 3.8 ml solutions containing 0.05 ml digest sample, 2.5 ml UHP water, 1 ml sodium salicylate, and 0.25 ml DIC were prepared in cuvettes. A standard curve ($p < 0.98^{-1}$) was generated by using the same mixture but replacing the sample with a 10 ppm N standard (ammonium chloride) to final concentrations ranging from 0 to 20 ppm in 2 ppm increments, and making the volume up to 3.8 ml with UHP water. Solutions were left to develop for 30 minutes, and optical density was measured at 650 nm using a Cecil Ce 1 020 spectrophotometer zeroed against blanks.

2.2.7 Metabolite extraction

Metabolomic analysis was carried out on the rice cultivar IAC 165. During the harvest, approximately 100 mg fresh weight of harvested root material was transferred to a foil packet and placed in liquid nitrogen before storage at -80°C. Extraction was performed from biologically replicated plant material (n = 4). Samples were transferred from a -80°C freezer storage to a SuperModulyo freeze dryer (Thermo Scientific, Waltham, USA) and freeze-dried for 3 days at -40°C and 20-40 mbar (2-4 atm). Freeze-dried samples were placed in a 2 ml grinding tube containing two 3 mm diameter ball bearings to aid in grinding the material. The tissue was ground to a powder in a tissue lyser (QIAGEN TissueLyser) for 2 minutes at a frequency of 30/s to produce a fine powder.

Fifty mg of sample powder was weighed into a 1.5 ml Eppendorf tube and used for metabolite extraction. Metabolites were extracted with a polar solvent consisting of MeOH:$CHCl_3$:UHPH_2O (2.5:1:1), an apolar solvent consisting of MeOH:$CHCl_3$ (1:1), UHP H_2O, and $CHCl_3$. Solvents were kept at -20°C and samples were kept on ice throughout the extraction procedure. Samples were first extracted with 500 µl of polar solvent, vortexed for 10 seconds and left on ice for 5 minutes. Samples were vortexed for 10 seconds and centrifuged at 14,000 rpm for 2 minutes at 4°C. The supernatant was removed and added to a new, pre-chilled Eppendorf tube. The pellet was re-extracted with 250 µl of apolar solvent, vortexed for 10 seconds and left on ice for 10 minutes. Samples were centrifuged at 14,000 rpm for 2 minutes at 4°C. The supernatant was then added to the supernatant from the polar extraction. Separation of polar and apolar phases was achieved by adding 175 µl of water and 100 µl of $CHCl_3$. This mixture was centrifuged at 14,000 rpm for 2 minutes at 4°C to form two clear phases separated by a cloudy layer. The bottom polar and top apolar phases were pipetted into new tubes and stored at -80°C.

2.2.8 Analysis of the root defence metabolome by MALDI-MS

Analysis was carried out on aqueous extracts in positive mode. Aqueous phase samples were diluted 100-fold with MeOH:H2O:HCOOH (50:49.9:0.1). Matrix solution (α-cyano-4-hydroxycinnamic acid; CHCA; Sigma-Aldrich; C2020) was

prepared at 5 mg ml^{-1} in MeOH:trifluoroacetic acid (TFA) in a 99.9:0.1 ratio. One µl of each diluted sample was mixed with an equal volume of matrix solution, and 0.5 µl of this mixture was spotted in triplicate onto 396-well stainless steel MALDI target plates (Waters) to provide three technical replicates of each biological replicate. Sample droplets on the target plate were allowed to air dry.

Mass spectra were obtained by matrix-assisted laser desorption/ionisation (MALDI) time of flight (TOF) MS analysis, using a Waters Synapt GS2 TOF mass spectrometer fitted with a MALDI orthogonal head (Waters; Manchester; UK). MassLynx data system (version 4.1; Waters) provided instrument control, data acquisition, and data processing. The mass spectrometer was operated in positive ionization mode with Repetition rate:1000 Hz, Laser energy for α-CHCA – 20% (2.3 µJ). Samples were ionised using a UV laser targeted at the plate and within each discreet spot a spiral pattern was drawn for 60 s. The spiral pattern is appropriate for accounting for heterogeneity within the spot. Mass ranges of scans were defined from 50–1,000 Da.

2.2.9 Metabolomics data processing

Peak lists in the form of mass to four decimal places versus ion counts were extracted using Masslynx software and transferred to Microsoft Excel (Microsoft Corp, USA) as text files. To avoid the loss of small peaks of potential interest, no peak threshold was imposed. The spectra for each of the three technical replicate runs for each sample were combined, and only peaks which were present in all three replicates were selected. Peaks were selected if the mass variance between the three technical replicates of a recorded mass fell within an accepted mass variance defined as a linear function of the mass-to-charge ratio (m/z). The mean of the three peaks in a selected triplicate was used as the mass for all three runs, giving a single m/z value for the biological replicate. The resultant m/z values with their ion intensity formed the metabolite profile for that sample. Data were rounded to 0.2 m/z ‘bins’ and the ion count as percentage of the total ion count (%TIC) was summed for each one and used as a measure of abundance. Thus, within the mass range of 50-1,000 Da, there were 4,750 m/z bins (m/z bin 50, 50.2 etc.), each with a %TIC.

M/z bin number and %TIC were imported into SIMCA-P+ 14 (Umetrics™) and pareto scaled for statistical analysis. Scaling is used on data where the variables have considerably different numerical ranges. Without scaling, data for a variable with a particularly large range dominates the modelling so that variance in data with a smaller range may not be revealed. Unit variance (UV) scaling can be used to scale variables so that even variables with a very small variance may be expressed in the model. However, metabolomics data sets are large and complex and can contain a lot of noise, particularly at lower masses and intensities. Pareto scaling is intermediate between no scaling and UV scaling (Eriksson et al., 2006a). This makes it more appropriate for metabolomics data because while it allows smaller peaks to influence the analysis, the influence of small and noisy peaks is reduced.

Principal component analysis (PCA) was used as an initial check for treatment separation and grouping. Principle component analysis (PCA) is a multivariate projection method designed to extract and display the systematic variation in an observation, doing so in a unguided manner. This is necessary because in the next analysis, Orthogonal Partial Least-Squares Discriminant Analysis (OPLS-DA), treatment identity is used to guide the analysis, resulting in separation between treatments even if there is no meaningful difference. OPLS-DA was used to compare the control treatment to each one of the other three treatments, +AM, +*Striga* and +AM+*Striga*. OPLS-DA is a regression extension of PCA with defined classes where structured noise has been separated from the Y-predictive X variation (Eriksson et al., 2006b). It therefore makes variation between treatments in a large, complex, noisy data set easier to visualise and interpret.

M/z bins of interest were selected using loading plots based on the OPLS-DA analysis carried out for each pairwise treatment comparison. For example, Fig. 2.5 shows the OPLS-DA model that was used to compare control to +AM in IAC165. Fig. 2.6 shows the resulting loading plots, which indicate m/z bins which were associated with either control (Fig. 2.6a) or +AM (Fig. 2.6b) treatments. M/z bins which were confidently associated with at treatment were defined by having a pq[1] value where the 95% confidence interval bar did not cross the x-axis. These m/z bins are therefore referred to here as confident m/z bins. Initially, to reduce the data to a

more manageable size, only the top 50 confident m/z bins (ranked by pq[1]) were investigated in more detail.

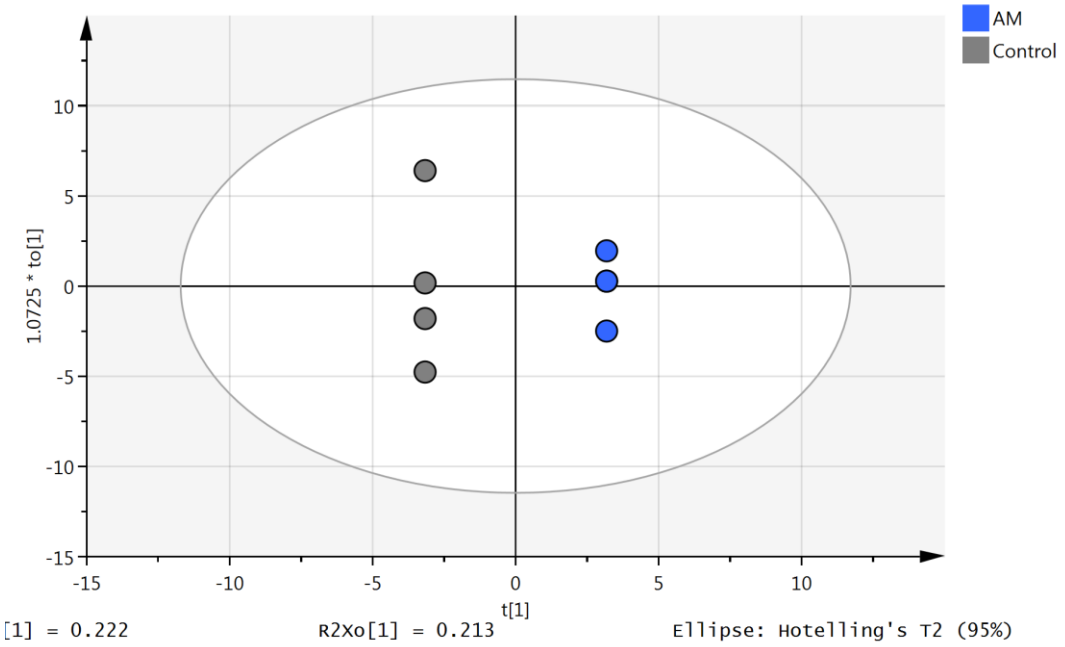
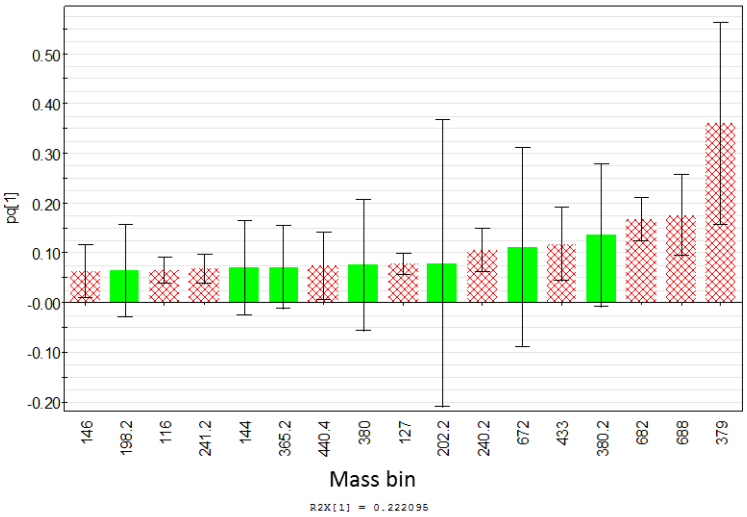


Fig. 2.5 Orthogonal partial least squares discriminant analysis (OPLS-DA). Example OPLS-DA of the rice root 0.2 Da and %TIC binned data for control (grey) and AM (blue) treatment comparison, n = 4. Note that two of the AM (blue) points are occupying the same space. The same analysis was carried out to compare *S. hermonthica* and co-infected samples to the un-infected control.

Putative identification of masses within these m/z bins was carried out using the online database METLIN. Masses were input with 4 decimal places, and the METLIN databased was searched in positive mode with [M+H], [M+Na], [M+K], adducts allowing a 10 ppm error and removing peptides from the search results. Matches in the Metlin database were searched in the KEGG database for verification and on KEGG Pathway to assign them to pathways. Pathways not relevant to plants (e.g. alcoholism) and exceedingly broad pathways (e.g. antibiotics) were not used in further analysis. Defence-related pathways were a target of this investigation, and many putatively identified metabolites mapped to pathways associated with defence such as flavonoid biosynthesis and phenylpropanoid biosynthesis. These pathways were analysed further by expanding beyond the initial 50 bin search to include any confident bin from the OPLS-DA analysis. Fold change for the putative metabolites within these pathways was calculated for each treatment compared to the control as the mean %TIC for the four biological replicates for a given treatment (for example +AM) divided by the mean %TIC for the four biological replicates of the control.

(a)



(b)

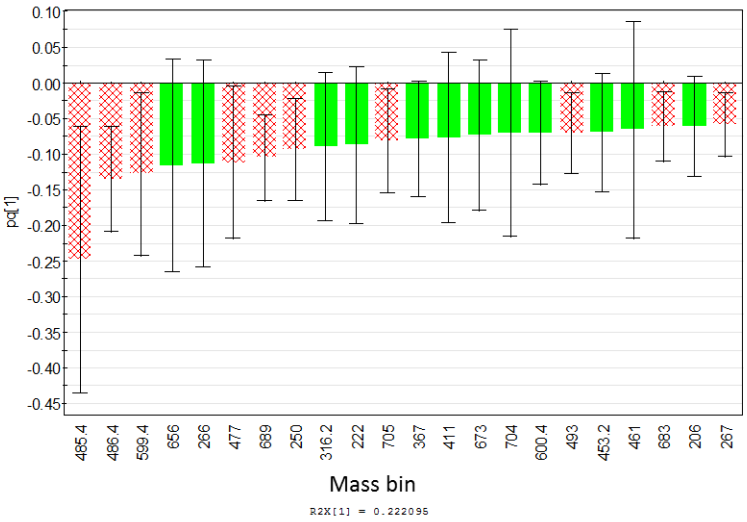


Fig. 2.6 Example column plots for the selection of bins of interest. Magnified view of plots created from the OPLS-DA model comparing un-infected control and *R. irregularis*-colonised polar root extracts of IAC165 roots analysed by MALDI-MS in positive mode. A magnified view of the plots corresponding to the bins positively (a) and negatively (b) associated with *R. irregularis*-colonised plants is shown. Columns for bins where the 95% confidence interval does not cross the origin were selected for the analysis. The same analysis was carried out to compare *S. hermonthica* and co-infected samples to the un-infected control.

2.2.10 Confirmation of select putative compounds by MS/MS

Tandem mass spectrometry (MS/MS) was used to confirm the identification of a selection of putative compounds. The list of for MS/MS analysis was chosen by only selecting putative compounds which were corrected with the [M+H] adduct, for which standards were available, and which had fragmentation information available on the METLIN database. Standards investigated were trans-Cinnamate, Luteolin, Sinapaldehyde, Sinapate, Vitexin and Chlorogenic acid. Fragmentation patterns of peaks for these putative metabolites were compared with those of standards (and those on the METLIN database). Standards were prepared to a 0.1 mg/ml^{-1} concentration using an appropriate solvent for each. Samples were diluted to 10-fold with MeOH:H2O:HCOOH (50:49.9:0.1).

Standards and samples were analysed using an electro-spray ionization (ESI) TOF mass spectrometer, API Sciex III Plus (AB Sciex UK Ltd, Warrington, UK), and Analyst software. Each sample was run by direct injection at 10 µl min−1 using a syringe pump (SP100iZ; WPI; UK). Spectra were collected at a rate of one spectrum s^{-1} 1 min (60 spectra total). Spectra were first obtained at the charge energy (CE) appropriate to show the presence of the parent ion, then at a CE to show the presence of the parent ion along with some fragmentation, and finally at a CE to only show the fragmentation pattern. One biological rep from each treatment (control, +AM, +*Striga* and +AM+*Striga*) was used for data collection and representation.

2.2.11 Statistical analyses

Measurements of growth taken at the end of the experiment at 10 weeks after sowing (WAS) were analysed using two-way ANOVA with Tukey's multiple comparisons (MC) post hoc analysis in Minitab 17 (version 17.2.1.0. Minitab Inc., Pennsylvania, USA). Statistics for the two cultivars, IAC 165 and Shiokari, which were planted sequentially as separate experiments, were carried out separately. Presence and absence of *R. irregularis* was included in the analysis as one factor, and presence and absence of *S. hermonthica* as another, with the interaction between the two factors then tested. For the weekly growth measurements, two-way ANOVA was used at the end of the experiment at 10 WAS, taking into account the fact that all seeds were

treated equally before sowing, so any effect on growth would be due to treatment (+AM, +*Striga*, or +AM+*Striga*). Assumptions of normality and homogeneity of variances were met through $\log_{10}$ or square root transformations of variables when necessary, and confirmed by analysis of residual vs fitted values variable histograms. ANOVA tables for each factor and interaction showing model fit, F-values, degrees of freedom and significance are shown in appendix A. Comparisons of AM colonisation and *S. hermonthica* infection levels were made using Student's t-test in Excel. Graphs were generated using GraphPad Prism 6. Binned metabolomics data were analysed using PCA and OPLS-DA in SIMCA-P+ 14 (Umetrics™).

2.3 Results

2.3.1 Morphology of rice cultivars IAC165 and Shiokari in the presence of *R. irregularis* and *S. hermonthica*

Fig. 2.7 shows the effect of *R. irregularis* and *S. hermonthica*, alone or in combination, on the morphology of the rice cultivars IAC 165 and Shiokari on a weekly basis from 1 week after sowing (WAS) to the end of the experiment at 10 WAS, and a representative image of the effect on Shiokari is shown in Fig. 2.8. ANOVA tables for each factor and interaction showing model fit, F-values, degrees of freedom and significance are shown in appendix A. The following results are analysed and presented using additional post-hoc Tukey's multiple comparison (MC) testing where necessary. There was no significant difference between the heights or diameters of the main stem of IAC 165 when grown in the presence or absence of *R. irregularis* (Fig. 2.7a and b). However, when IAC 165 was grown with *S. hermonthica* alone or in combination with *R. irregularis*, both stem height and diameter were significantly lower (two-way ANOVA, Tukey's multiple comparisons (MC), $p < 0.001$) compared to control plants and plants grown with *R. Irregularis* alone by 10 WAS (Fig. 2.7a and b). A similar pattern was observed for Shiokari, although the height of the main stem was significantly higher (two-way ANOVA, Tukey's multiple comparisons (MC), $p < 0.05$) in plants grown with *R. irregularis* alone compared to control plants by 10 WAS (Fig. 2.7e and f). Shiokari plants grown with *S. hermonthica* alone or in combination with *R. irregularis* again had significantly lower stem height and diameter (two-way ANOVA, Tukey's MC, $p < 0.05$) compared to control plants or plants grown with *R. irregularis* alone by 10 WAS (Fig. 2.7e and f).

In the presence of *R. irregularis* IAC 165 produced 4 tillers during the 10-week growth period compared to 2 tillers in all other treatments (Fig. 2.7c). After the number of leaves on IAC 165 when grown with *R. irregularis* alone peaked at 6 WAS, leaf number began to decrease due to leaf turnover, such that by week 10 plants grown with *R. irregularis* alone had similar but higher (two-way ANOVA, Tukey's multiple comparisons (MC), $p < 0.001$) numbers of leaves than control plants and plants grown with *S. hermonthica* alone (Fig. 2.7d). In Shiokari, there was

no significant effect of any treatment on tiller number compared to control plants (Fig. 2.7g). Similarly, there was little effect of treatment on total leaf number with the exception of plants grown with *R. irregularis* alone where leaf number was slightly but significantly higher compared to plants grown alone and with *S. hermonthica* alone (two-way ANOVA, Tukey's MC, $p < 0.01$) (Fig. 2.7h).

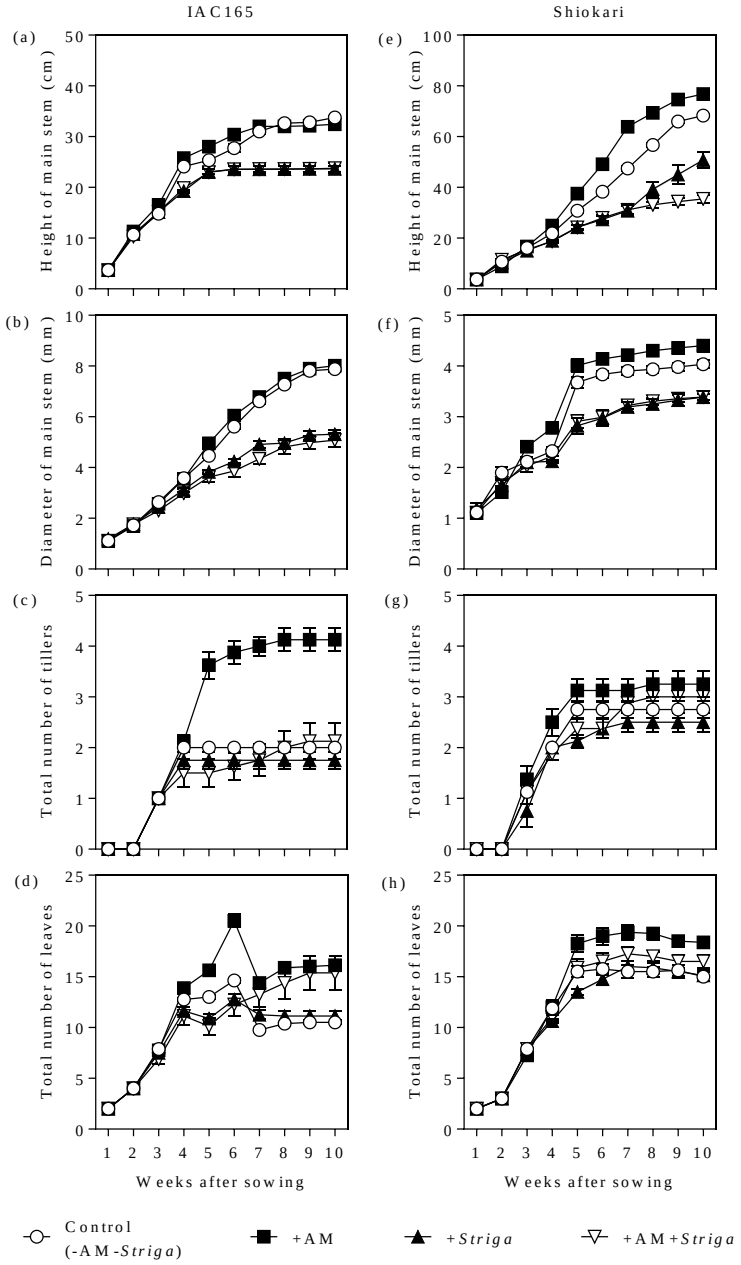


Fig. 2.7: Morphology of rice cultivars IAC 165 and Shiokari when grown with *R. irregularis* (+AM), *S. hermonthica* (+*Striga*), both *R. irregularis* and *S. hermonthica* together (+AM+*Striga*) or un-infected as control plants (-AM-*Striga*). (a) and (e) Height of main stem, (b) and (f) diameter of main stem, (c) and (g) total number of tillers, and (d) and (h) total number of leaves. Measurements were taken weekly beginning 1 WAS, and ending 10 WAS. Data shown is the mean for each parameter ± standard error (SE), n = 8.

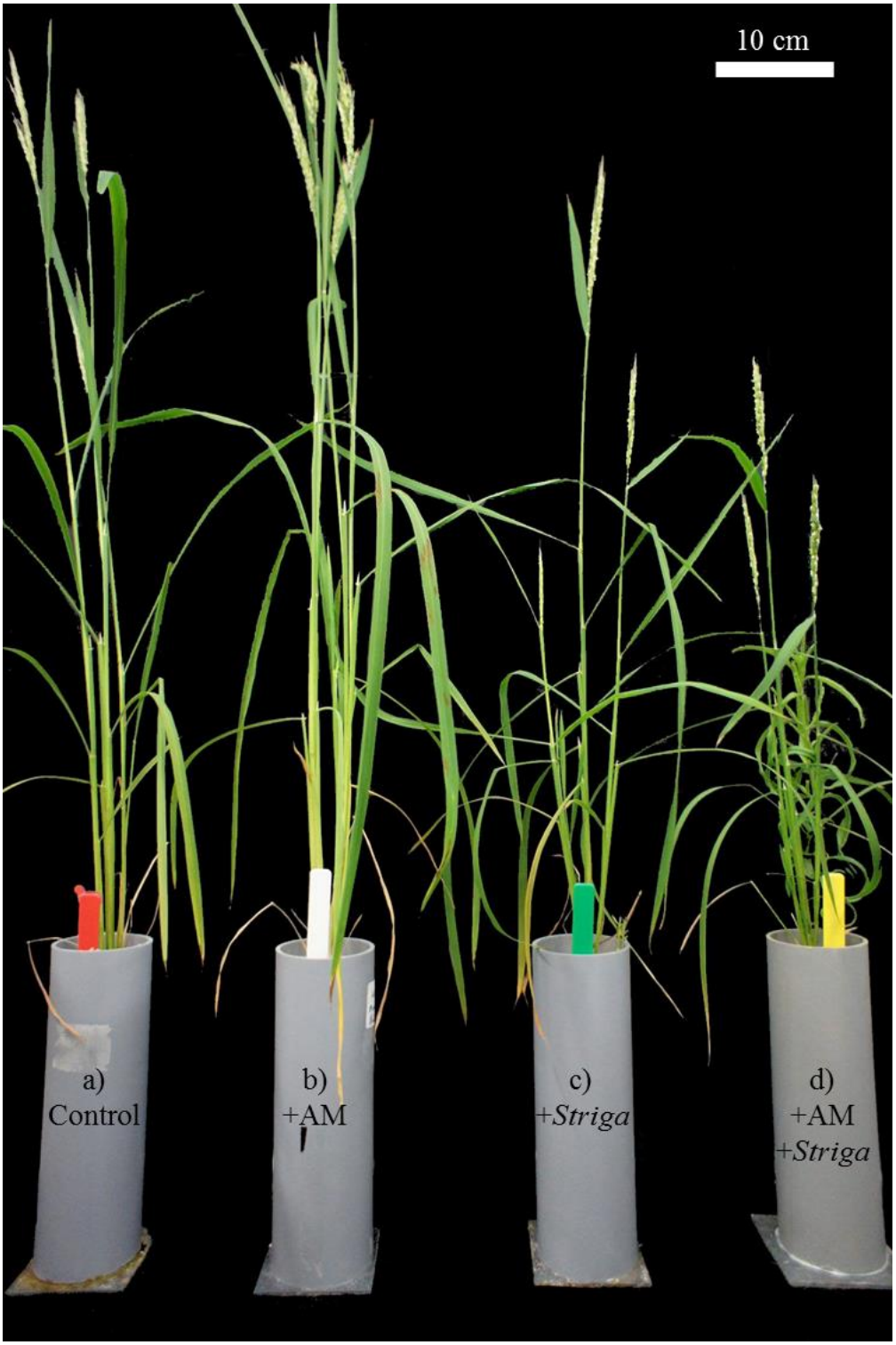


Fig. 2.8 Representative picture of rice (cultivar Shiokari) 10 weeks after sowing. (a) Rice grown alone (control, -AM-*Striga*). (b) Rice grown with *R. irregularis* alone (+AM). (c) Rice grown with *S. hermonthica* alone (+*Striga*). Rice grown with both *R. irregularis* and *S. hermonthica* together (+AM+*Striga*). Scale bar = 10 cm.

2.3.2 Above- and below-ground biomass and N and P concentration of rice cultivars IAC 165 and Shiokari in the presence of *R. irregularis* and *S. hermonthica*

Fig. 2.9 shows the effect of *R. irregularis* and *S. hermonthica,* alone or in combination, on the above ground and root biomass, and the N and P concentration of the leaves and roots of the rice cultivars IAC 165 and Shiokari after 10 weeks of growth. ANOVA tables for each factor and interaction showing model fit, F-values, degrees of freedom and significance are shown in appendix B. The following results are analysed and presented using additional post-hoc Tukey's multiple comparison (MC) testing where necessary.

When IAC 165 and Shiokari were grown with *R. irregularis* alone, the above ground biomass was significantly higher (two-way ANOVA, Tukey's MC, $p < 0.001$) compared to control plants, whereas the root biomass was unchanged (Fig. 2.9a and d). When IAC 165 and Shiokari were grown with *S. hermonthica* alone or in combination with *R. irregularis,* the above ground biomass was significantly lower (two-way ANOVA, Tukey's MC, $p < 0.05$) than control plants or plants grown with *R. irregularis* alone (Fig. 2.9a and d). Root biomass was also significantly lower (two-way ANOVA, Tukey's MC, $p < 0.05$) in IAC 165 but not in Shiokari (Fig. 2.9a and d).

There was no significant effect of any treatment on the N concentration of the leaves or roots of IAC 165 with the exception that the N concentration in roots colonised by *R. irregularis* was significantly lower (two-way ANOVA, Tukey's MC, $p < 0.05$) than in roots of all the other treatments (Fig. 2.9b). Similarly, there was no effect of any treatment on the N concentration of leaves and roots of Shiokari except for a slight decrease in the amount of N in leaves of plants grown with *R. irregularis* and *S. hermonthica* in combination, (two-way ANOVA, Tukey's MC, $p < 0.001$) (Fig. 2.9e).

When IAC 165 and Shiokari were grown with *R. irregularis* alone or in combination with *S. hermonthica,* both the leaf and root P concentration was significantly higher (two-way ANOVA, Tukey's MC, $p < 0.05$) than in control plants (Fig. 2.9c and f).

However, when IAC 165 and Shiokari were grown with *S. hermonthica* alone, P concentration in the leaves or roots did not differ significantly from control plants (Fig. 2.9c and f).

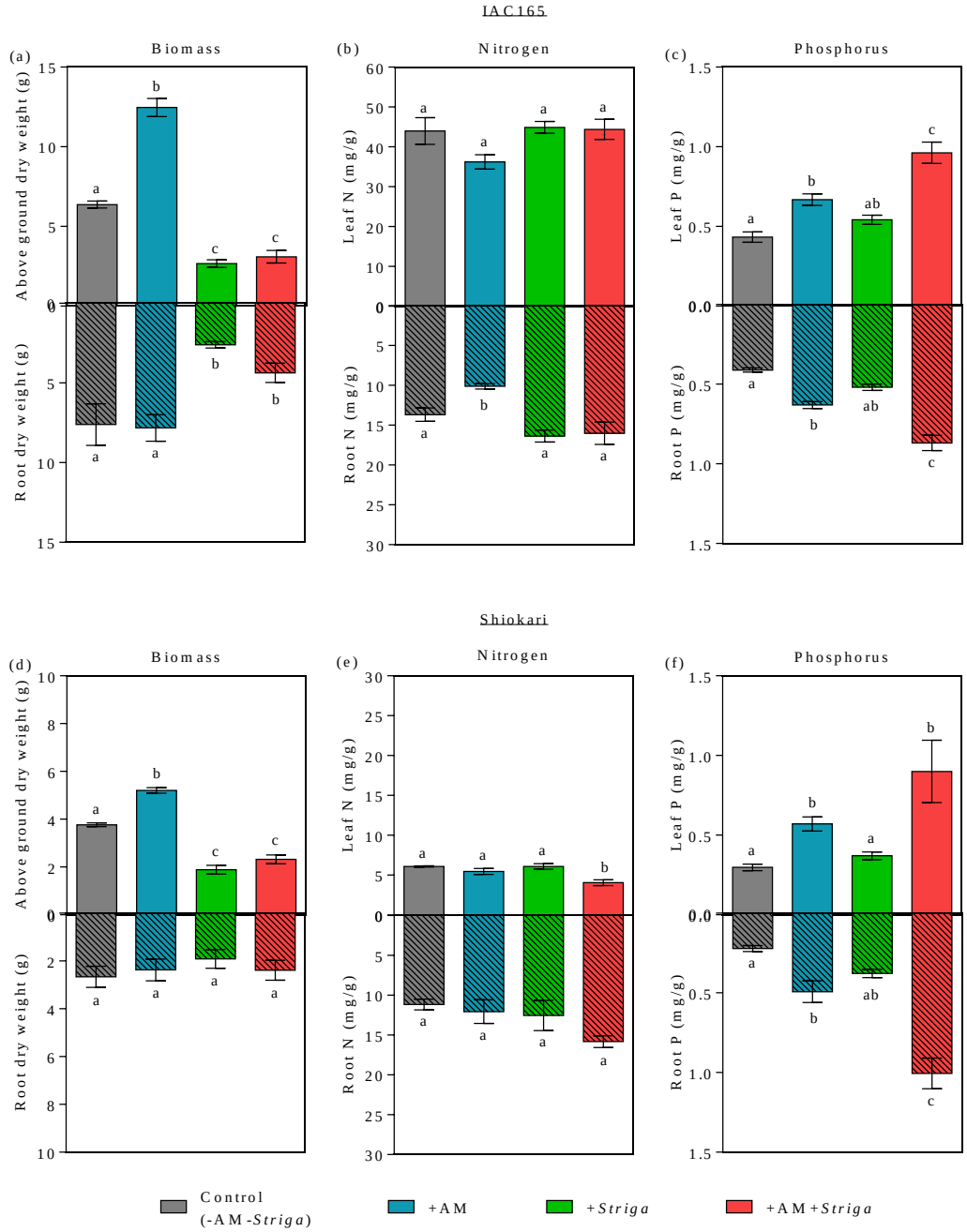


Fig. 2.9 Growth and nutrient status of cultivars IAC165 and Shiokari when grown with *R. irregularis* (+AM), *S. hermonthica* (+*Striga*), both *R. irregularis* and *S. hermonthica* (+AM+*Striga*), or un-infected as control plants (-AM-*Striga*). (a) and (d) Above ground (leaf and stem) and root dry weight. (b) and (e) Host leaf and root N concentration. (c) and (f) Host leaf and root P concentration. Data shown is the mean for each parameter ± SE. Columns sharing the same letters are not significantly different ($p > 0.05$, two way ANOVA, Tukey's MC), $n = 8$.

2.3.3 *Striga* infection and AM colonization of rice cultivars IAC165 and Shiokari grown with *R. irregularis* and *S. hermonthica*

Fig. 2.10 shows the effect of *S. hermonthica*, alone or in combination with *R. irregularis*, on the number and biomass of *S. hermonthica* individuals harvested from the roots of the rice cultivars IAC 165 and Shiokari after 10 weeks of growth. When either IAC 165 or Shiokari was grown with both *R. irregularis* and *S. hermonthica* together, the number and biomass of *S. hermonthica* was significantly greater compared to plants grown with *S. hermonthica* alone. In IAC 165, there was a 2.5-fold increase in both number (t-test, $p < 0.001$) and biomass (t-test, $p < 0.01$) (Fig. 2.10a and b) of attached *S. hermonthica* and in Shiokari, there was a 5.8-fold increase in number and a 60-fold increase in biomass (t-test, $p < 0.01$) (Fig. 2.10c and d). Also see Fig. 2.8d which shows *Striga* emerging from the co-colonised/infected plant.

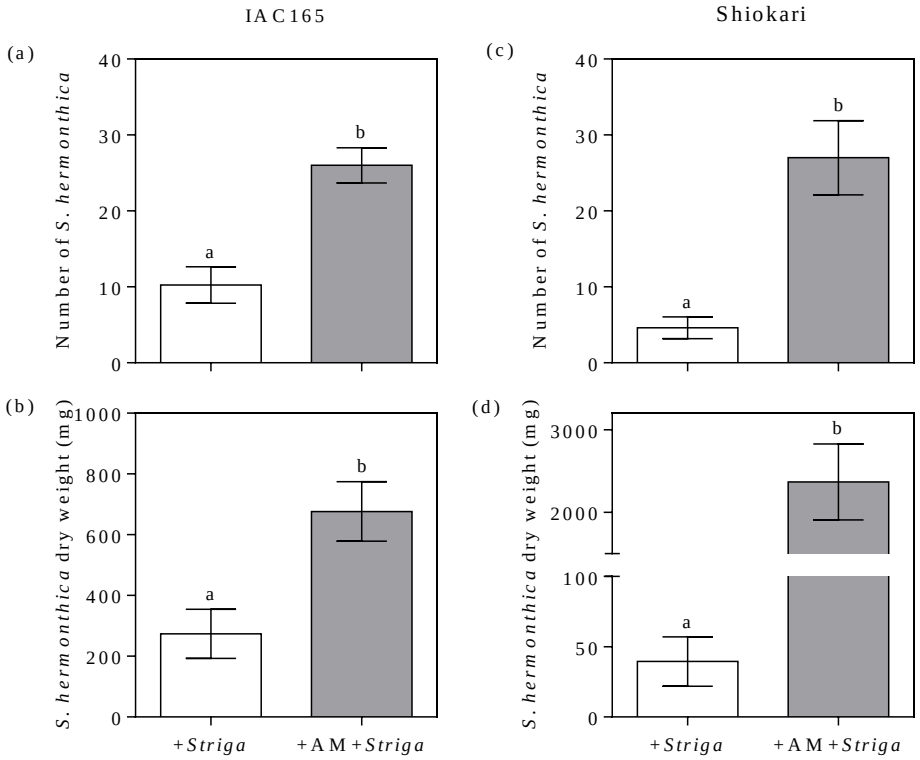


Fig. 2.10 *S. hermonthica* infection of cultivars IAC165 and Shiokari when grown with *S. hermonthica* (+*Striga*) and both *R. irregularis* and *S. hermonthica* (+AM+*Striga*). (a) and (c) *S. hermonthica* number. (b) and (d) *S. hermonthica* dry weight. Data shown is mean *S. hermonthica* number and mean *S. hermonthica* dry weight ± SE. Columns sharing the same letters are not significantly different ($p > 0.05$, student's t-test), $n = 8$.

Fig. 2.11 shows the effect of *R. irregularis*, alone or in combination with *S. hermonthica*, on the % root length colonised by *R. irregularis* for the roots of the rice cultivars IAC 165 and Shiokari after 10 weeks of growth. When either IAC 165 or Shiokari was grown with both *R. irregularis* and *S. hermonthica* together, % root length colonisation was significantly lower (t-test, $p < 0.01$) compared to plants grown with *R. irregularis* alone.

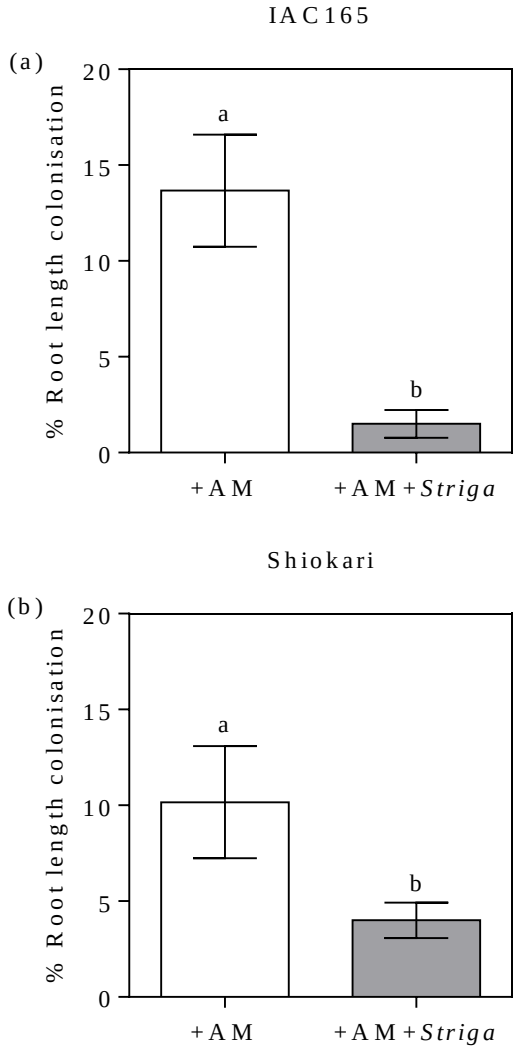


Fig. 2.11 *R. irregularis* colonisation of cultivars (a) IAC165 and (b) Shiokari when grown with *R. irregularis* (+AM) and both *R. irregularis* and *S. hermonthica* (+AM+*Striga*). Data shown is mean % root length colonisation ± SE. Columns sharing the same letters are not significantly different ($p > 0.05$, student's t-test), n = 8.

2.3.4 Root metabolome analysis of rice cultivar IAC165

To investigate the pathways underlying host defence metabolism, an untargeted metabolomic analysis was carried out on metabolite extracts from the roots of the rice cultivar IAC 165 grown without *S. hermonthica* or *R. irregularis* or with either symbiont alone or in combination. Fig. 2.12 shows the PCA for the IAC 165 m/z bin and %TIC root metabolome data for all treatments. The first component separated IAC 165 grown with *R. irregularis* (right, blue) from IAC 165 grown in combination with *R. irregularis* and *S. hermonthica* (left, red). The second component separated IAC 165 grown alone (bottom, grey) and IAC 165 grown with *S. hermonthica* alone (top, green) treatments. The widest spread is in the biological replicates for IAC 165 grown in combination with *R. irregularis* and *S. hermonthica*, biological replicates for IAC 165 grown with *R. irregularis* are the most tightly grouped. One biological replicate from IAC 165 grown alone and one from IAC 165 grown with *S. hermonthica* were spread away from the other biological replicates in their respective treatments. However, all biological replicates were included in subsequent analyses.

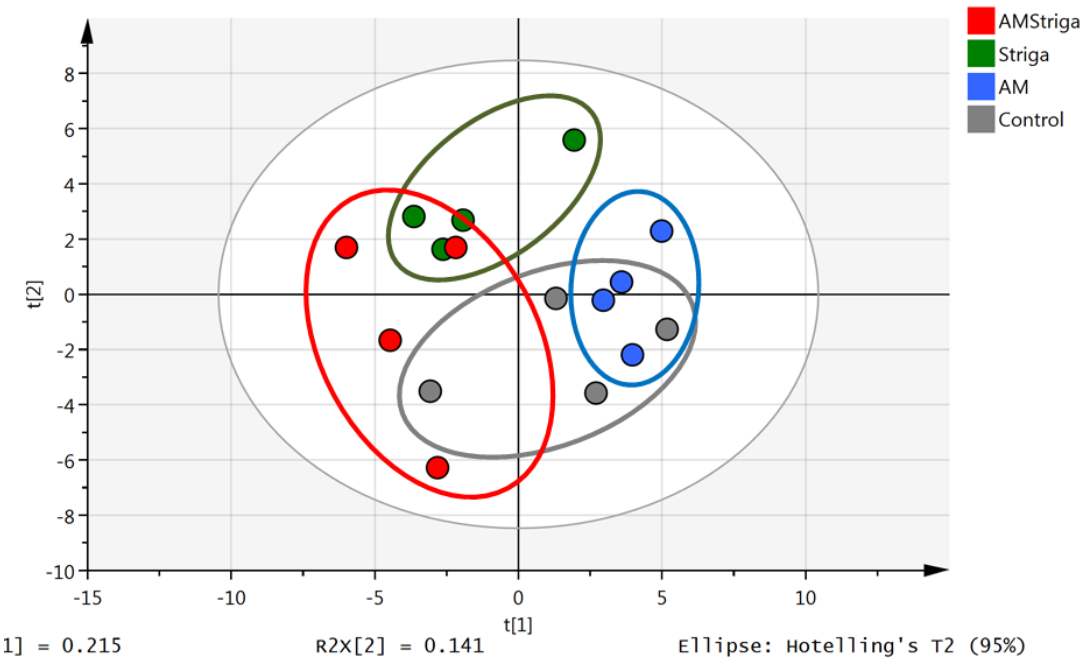


Fig. 2.12 Principle component analysis (PCA) scatter plot of the rice root 0.2 Da and %TIC binned data for the control (grey), +AM (AM, blue), +*Striga* (Striga, green) and +AM+*Striga* (AMStriga, red) treatments. Ovals coloured according to treatment highlight general grouping of the biological replicates within treatments. Data were Pareto scaled prior to PCA analysis, n = 4.

The putative metabolites which were up- and down-regulated in the roots of IAC 165 grown with *R. irregularis* and *S. hermonthica*, alone or in combination, compared to IAC 165 grown alone (control) were assigned to metabolic pathways based on KEGG Pathway metabolic maps. Many putatively identified metabolites which were differentially regulated (either up- or down-regulated) compared to the control were found to be present in two key pathways involved in plant defence; phenylpropanoid biosynthesis and flavonoid biosynthesis. The differential up- or down-regulation of these putative metabolites in IAC 165 grown with *R. irregularis* and *S. hermonthica*, individually or in combination, compared to IAC 165 grown alone (control) is shown in Table 2.1 and in Fig. 2.13.

Table 2.1 shows the data for the putative metabolites within these pathways which were confidently up- and down-regulated (as indicated by the OPLS-DA) in IAC 165 grown in the presence of *R. irregularis* and *S. hermonthica*, alone or in combination, compared to control plants. Exact mass and the total mass of the metabolite with its adduct are shown along with the corresponding m/z bin. For each m/z bin, mean % TIC is shown with standard error (SE) alongside. The differential up or down regulation of these putative metabolites in the different treatments is shown in Table 2.1 and on the phenylpropanoid and flavonoid biosynthetic maps (Fig 2.13). Growth of the host plant IAC 165 for 10 WAS with *R. irregularis* and *S. hermonthica*, alone or in combination, resulted in unique effects on the host root defence metabolome. Overall, there was an up-regulation of putative metabolites in IAC 165 colonised with *R. irregularis* alone, and a consistent down-regulation in IAC 165 when infected with *S. hermonthica* alone. The combination of the two symbionts resulted in a more variable response

In IAC 165 grown with *R. irregularis* alone, important precursor compounds of phenylpropanoid metabolism were up-regulated including L-phenylalanine, L-tyrosine, and trans-cinnamate. However, many of the down-stream metabolites leading to lignin biosynthesis were not differentially regulated in comparison to control plants. After the branching point at 4-coumarate towards p-coumaroyl-CoA and ferulate, two metabolites were up-regulated; coumaryl-acetate and ferulate. This suggests that lignin biosynthesis may have been up-regulated, although the change seen is fairly minimal.

In contrast to *R. irregularis*, the precursors L-phenylalanine and trans-cinnamate were down-regulated in IAC 165 grown with *S. hermonthica* alone. Consistent with this, down-stream metabolites of the phenylpropanoid biosynthesis pathway such as 4-coumryl alcohol (which leads directly into lignin biosynthesis), eugenol and methyleugenol and coumarin, were downregulated in the presence of *S. hermonthica*. The branch of the pathway leading from ferulate to sinapoyl aldehyde (which also leads into lignin biosynthesis) was not differentially regulated in the presence of *S. hermonthica*. This suggests active suppression of lignin biosynthesis.

The defence metabolism profile of IAC 165 gown with *R. irregularis* and *S. hermonthica* together showed a variable response in terms of up and down regulation of metabolites when compared to control plants. Unlike the individual symbioses, both up-and down-regulation of metabolites occurred. The metabolites L-phenylalanine, L-tyrosine and trans-cinnamate were not differentially regulated compared to control plants. The response of down-stream metabolites was variable; the branch leading to coumaryl acetate was unchanged, while coumarin, eugenol and sinapoyl aldehyde were down-regulated. However, ferulate and sinapate were up-regulated, indicating a differential response in specific branches of the phenylpropanoid pathway to growth with both symbionts.

The flavonoid biosynthesis pathway represents the other branch of defence-associated metabolism which was analysed in this study. Overall, the response of this pathway in IAC 165 grown with *R. irregularis* and *S. hermonthica*, alone or in combination compared to control plants, was similar to that seen for the phenylpropanoid biosynthesis pathway. However, an exception is found in 7,4'-Dihydroxyflavone (DHF) which, uniquely, was highly up-regulated in all three treatments, but particularly in IAC 165 grown with *R. irregularis* or *S. hermonthica* alone. In IAC 165 grown with *R. irregularis* alone, the rest of the flavonoid biosynthesis pathway led mainly to compounds which were unchanged in IAC 165 grown with *R. irregularis* alone compared to control plants, with the exceptions of dihydromyricetin and leucodelphinidin which were up-regulated. No metabolites of the flavonoid biosynthesis pathway were down-regulated in IAC 165 grown with *R. irregularis* alone. In contrast to IAC 165 grown with *R. irregularis* alone, many

metabolites of the flavonoid biosynthesis pathway were down-regulated in IAC 165 grown with *S. hermonthica* alone compared to control plants. The metabolites naringenin, diydrokaempferol, cis-3,4-leucopelargonidin (26-fold), pelargonidin, and dihydromyricetin were all down-regulated. IAC 165 grown with *r. irregularis* and *S. hermonthica* together again had a more variable defence-metabolite profile compared to control plants. Isoliquiritigenin, luteolin and quercetin were specifically up-regulated in IAC 165 grown with both symbionts. However, dihydrokaempferol and pelargonidin were both down-regulated, reflecting the effect of *S. hermonthica*. As with the phenylpropanoid pathway, this suggests differential regulation of specific branches of the flavonoid biosynthesis pathway.

In order to validate the identity of key metabolites tandem mass spectrometry was MS/MS was used to compare the spectra of standards for these metabolites to root extract spectra for control IAC 165 plants. Fig. 2.14 shows MS/MS spectra the trans-cinnamate standard (Fig. 2.14a) and for an IAC 165 control root extract (Fig. 2.14b). The metabolite trans-cinnamate (trans-cinnamic acid,) [M+H]; m/z = 149.0597) required a CE of 20 to obtain optimum fragmentation spectra. The metabolite was clearly identified in the root extracts of IAC 165 by comparison of the fragmentation pattern. Fig. 2.15 shows the MS/MS spectra for the luteolin standard (Fig. 2.15a) and for an IAC 165 control root extract (Fig. 2.15b). The metabolite luteolin ([M+H]; m/z = 287.0550) required a CE of 35 to obtain optimum fragmentation spectra and again validated the presence of this metabolite in root extracts from IAC 165. However, for four other selected metabolites; sinapaldehyde, sinapate, vitexin and chlorogenic acid, it was not possible to identify them in root extracts from IAC 165 via the spectra obtained. It is likely that the concentration of these metabolites was too low to confirm by fragmentation. Indeed, many intermediates within metabolic pathways are found at very low abundance in plants (Dunn et al., 2005).

Table 2.1 Summary table of metabolic responses of the IAC165 root metabolome to *R. irregularis* colonisation and *S. hermonthica* infection. Putative metabolite, exact mass, adduct, mass with adduct correction and m/z bin are shown. For each putative metabolite, mean %TIC and standard error (SE) are shown to four decimal places for all four treatments. %TIC fold change values are shown to two decimal places for m/z bins confidently up- and/or down-regulated in IAC 165 grown with *R. irregularis* and *S. hermonthica*, alone or in combination compared to the control. Where a bin was not confidently up- or down-regulated, a 0 value is shown. Compounds which are in bold have MS/MS data spectra as shown in Fig. 2.14 and Fig. 2.15.

					Control		+AM		+*Striga*		+AM+Striga		Fold change from control		
Compound	**Exact mass**	**Adduct**	**M+adduct**	**Bin**	**Mean %TIC**	**SE**	**Mean %TIC**	**SE**	**Mean %TIC**	**SE**	**Mean %TIC**	**SE**	**+AM**	**+*Striga***	**+AM+Striga**
Flavonoid biosynthesis															
Isoliquiritigenin	256.0736	+H	257.0809	257	1.7358	0.5207	1.1085	0.0584	2.3932	0.3815	3.2300	0.3269	0	0	1.86
Naringenin	272.0685	+H	273.0758	273	0.0367	0.0072	0.0363	0.0128	0.0173	0.0042	0.0258	0.0064	0	-2.12	0
Luteolin	286.0477	+H	287.0550	287	0.0141	0.0021	0.0207	0.0090	0.0150	0.0024	0.0230	0.0034	0	0	1.63
Dihydromyricetin	320.0532	+H	321.0605	321	0.0062	0.0016	0.0116	0.0027	0.0023	0.0010	0.0050	0.0020	1.86	-2.70	0
L-Phenylalanine	165.0790	+Na	188.0682	188	0.0285	0.0015	0.0490	0.0087	0.0173	0.0010	0.0360	0.0047	1.72	-1.65	0
7,4'-Dihydroxyflavone	254.0579	+Na	277.0471	277	0.0042	0.0019	0.0500	0.0185	0.0427	0.0125	0.0091	0.0015	12.03	10.26	2.18
Pelargonidin	271.0606	+Na	294.0498	294	0.2020	0.0379	0.1974	0.0289	0.1200	0.0092	0.1313	0.0300	0	-1.68	-1.54
Dihydrokaempferol	288.0634	+Na	311.0526	311	0.0674	0.0014	0.0553	0.0199	0.0321	0.0071	0.0429	0.0159	0	-2.10	-1.57
cis-3,4-Leucopelargonidin	290.0790	+Na	313.0682	313	0.0498	0.0180	0.0229	0.0076	0.0019	0.0019	0.0495	0.0304	0	-26.34	0
Quercetin	302.0427	+Na	325.0319	325	0.0111	0.0003	0.0117	0.0021	0.0083	0.0021	0.0152	0.0018	0	0	1.37
Leucodelphinidin	322.0689	+Na	345.0581	345	0.0487	0.0090	0.0821	0.0054	0.0411	0.0115	0.0620	0.0070	1.69	0	0
Phenylpropanoid biosynthesis															
trans-Cinnamate	148.0524	+H	149.0597	149	0.0123	0.0004	0.0241	0.0038	0.0050	0.0016	0.0108	0.0030	1.96	-2.45	0
Sinapoyl aldehyde	208.0736	+H	209.0809	209	0.0115	0.0006	0.0114	0.0003	0.0094	0.0031	0.0088	0.0018	0	0	-1.30
Sinapate	224.0685	+H	225.0758	225	0.0186	0.0072	0.0163	0.0039	0.0122	0.0026	0.0332	0.0025	0	0	1.78
Coumarin	146.0368	+Na	169.0260	169	0.0126	0.0013	0.0098	0.0022	0.0075	0.0021	0.0092	0.0018	0	-1.68	-1.37
Methyleugenol	178.0994	+Na	201.0886	201	0.0088	0.0004	0.0086	0.0021	0.0052	0.0018	0.0074	0.0011	0	-1.70	0
L-Tyrosine	181.0739	+Na	204.0631	204	0.0148	0.0039	0.0376	0.0066	0.0191	0.0040	0.0295	0.0060	2.53	0	0
Coumaryl acetate	192.0786	+Na	215.0678	215	0.0056	0.0010	0.0078	0.0005	0.0062	0.0019	0.0075	0.0013	1.40	0	0
4-Coumaryl alcohol	150.0681	+K	189.0313	189	0.0726	0.0115	0.0679	0.0274	0.0359	0.0131	0.0669	0.0178	0	-2.02	0
Eugenol	164.0837	+K	203.0469	203	0.0316	0.0013	0.0296	0.0061	0.0193	0.0056	0.0161	0.0014	0	-1.64	-1.96
5-Hydroxyferulic acid methyl ester	210.0528	+K	249.0160	249	0.0051	0.0013	0.0080	0.0010	0.0050	0.0014	0.0084	0.0008	1.55	0	1.63

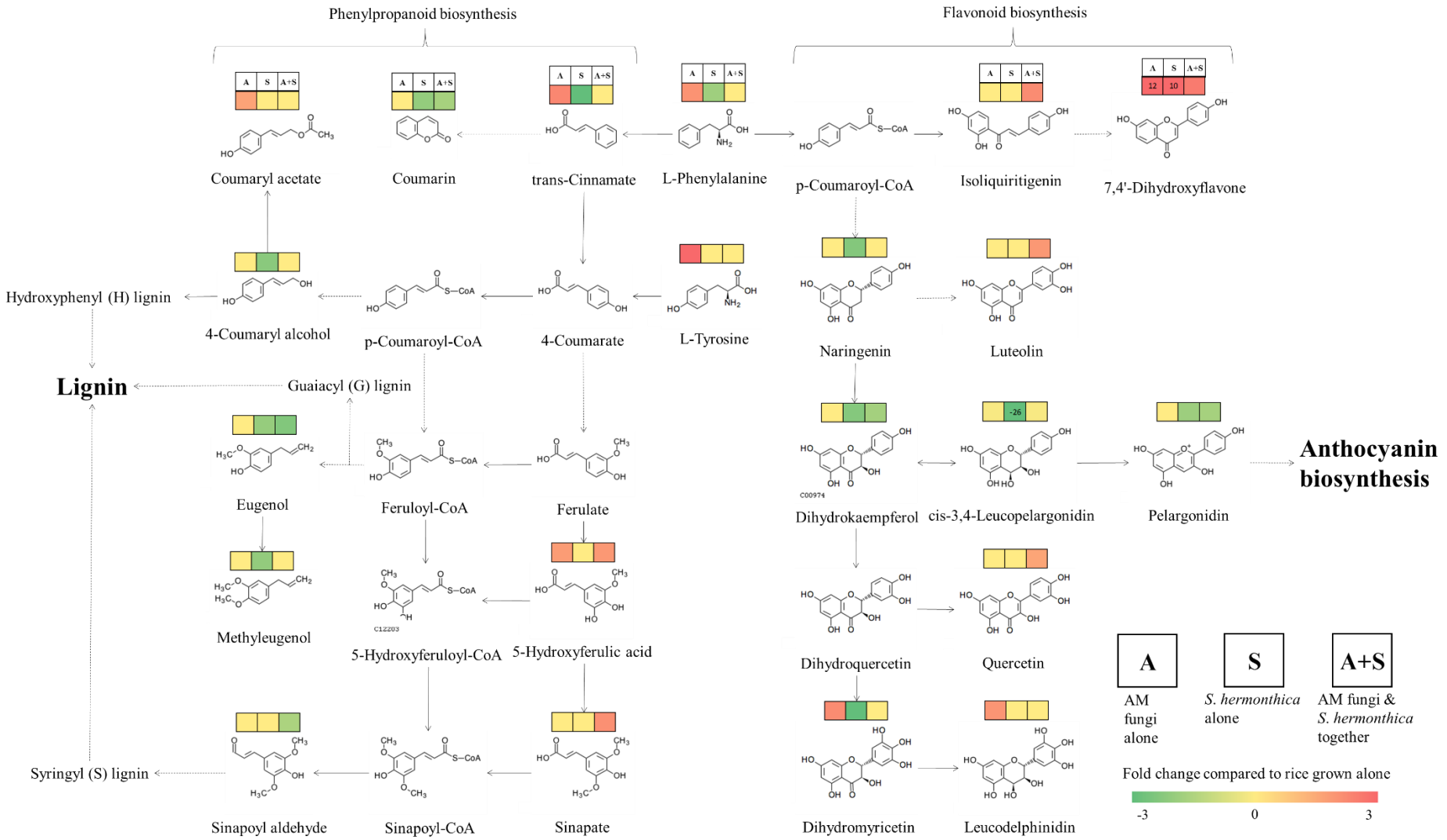
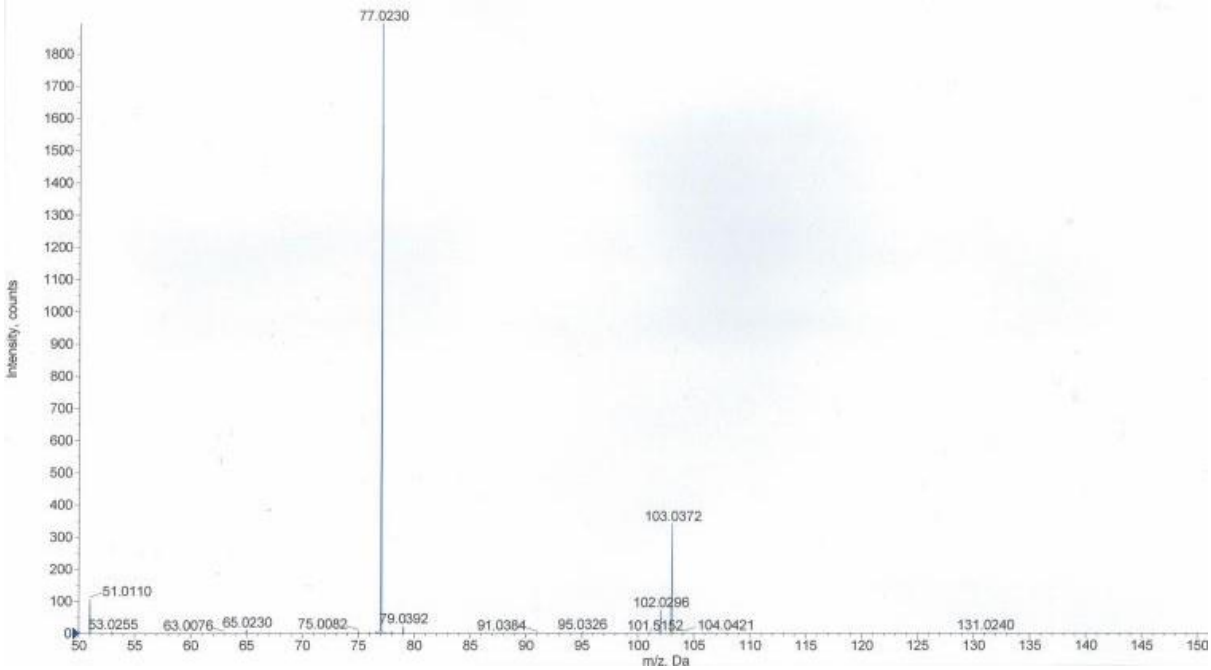


Fig. 2.13 Phenylpropanoid biosynthesis and flavonoid biosynthesis pathway map (also reference Table 2.1). Putative metabolites are shown with their fold regulation in colonised/infected treatments in comparison to un-infected control.

(a)



(b)

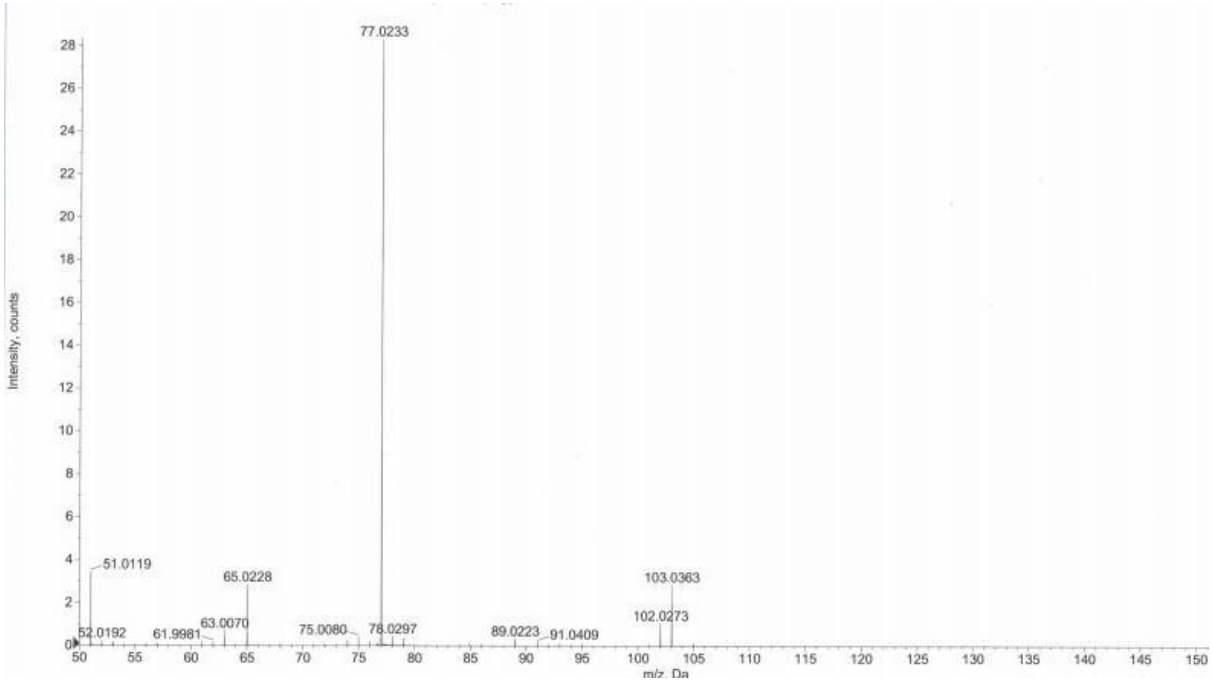
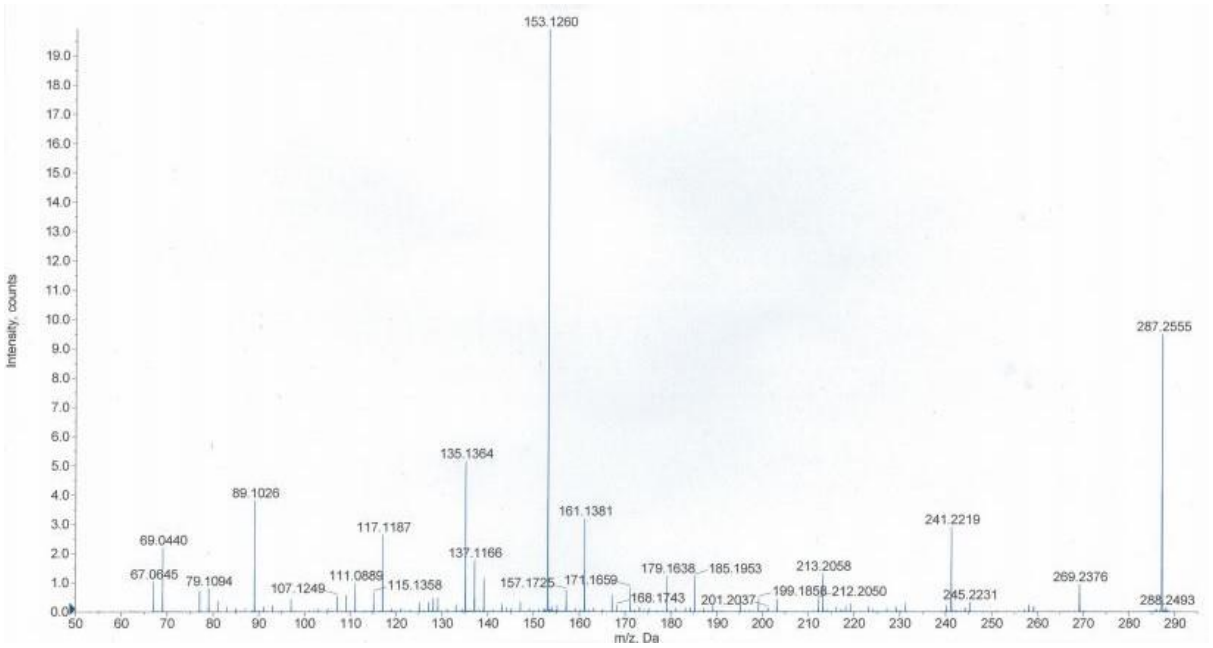


Fig. 2.14 MS/MS spectra for (a) the trans-cinnamate standard and (b) for a control IAC 165 sample extract under the same MS conditions. Visual comparison of the fragmentation patterns suggests the presence of trans-cinnamate in samples due to similar fragmentation peaks.

(a)



(b)

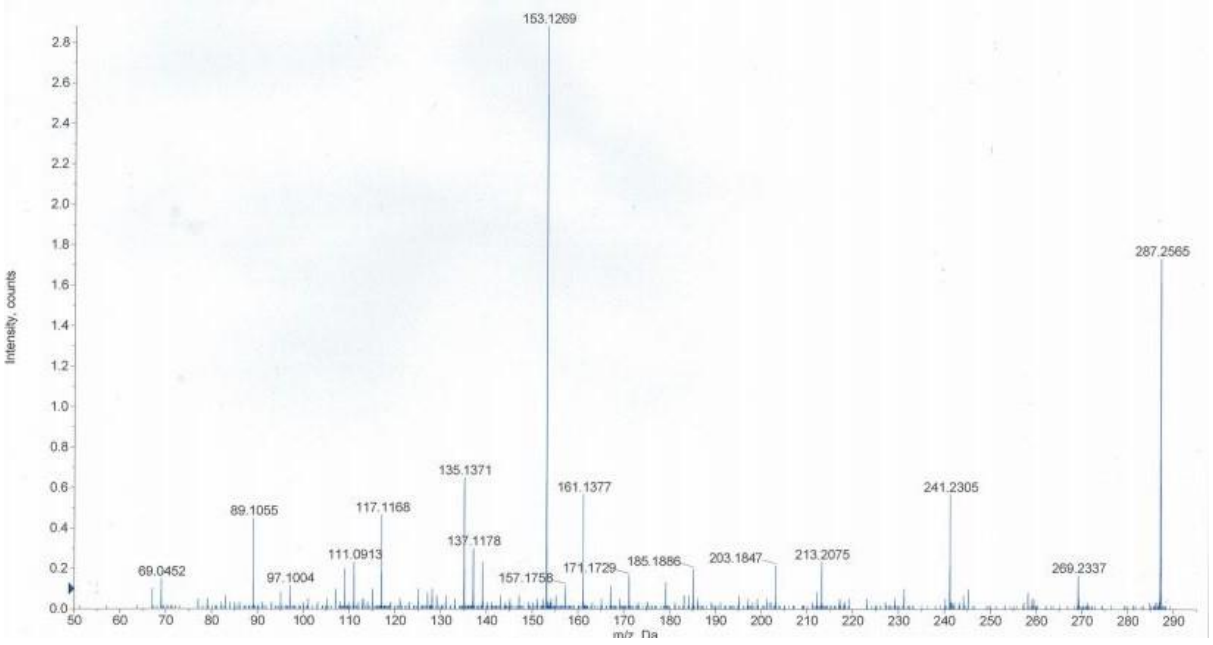


Fig. 2.15 MS/MS spectra for (a) the luteolin standard and (b) for a control IAC 165 sample extract under the same MS conditions. Visual comparison of the fragmentation patterns suggests the presence of luteolin in samples due to similar fragmentation peaks.

2.4 Discussion

This chapter investigated the effect of the interaction between *R. irregularis* and *S. hermonthica* on the growth, nutrient status and defence metabolome of the host plant rice, and the consequences for host development and symbiont success, measured by colonisation/infection levels. I hypothesized that, because AM fungi up-regulate host defence metabolism and/or improve host nutrient status, that they would decrease the amount of *S. hermonthica* infection and ameliorate the negative impacts of *Striga* on host growth and development. However, I have shown, for two different cultivars of rice, that the presence of the AM fungus *R. irregularis* significantly **increased** the number and biomass of *S. hermonthica* individuals on the rice cultivars in comparison to *Striga* alone. Furthermore, the negative impact of *S. hermonthica* on host growth and development was not alleviated by colonisation by the AM fungus.

2.4.1 Does nutrient status and/or alterations in host defence due to the presence of *R. irregularis* explain the increase in susceptibility to *S. hermonthica* infection?

This is the first time that colonisation by mycorrhizal fungi has been seen to increase the susceptibility to *S. hermonthica*. In contrast, Lendzemo et al. (2007) reported a reduction of *S. hermonthica* attachments in sorghum plants when grown in pots in combination with the AM fungi *Rhizophagus clarus* syn. *Glomus clarum* and *Gigaspora margarita* (Lendzemo et al., 2007). Another study by Lendzemo et al. (2005) reported a reduction of *S. hermonthica* emergence and dry weight on maize and sorghum grown in the field after inoculation of the soil with *Rhizophagus clarus* syn. *Glomus clarum* and *Gigaspora margarita*. More recently, Othira et al. (2012) also reported a reduction of *S. hermonthica* emergence, number and biomass in maize plants when grown in pots with three different AM fungal species (*Claroideoglomus etunicatum* syn. *Glomus etunicatum, Scutellospora fulgida, and Gigaspora margarita*). This protective effect of AM fungi has been attributed to a reduction in the exudation of germination stimulating strigolactones (SLs) following colonisation by AM fungi (Lopez-Raez et al., 2011). The reduction in exudation of germination stimulants correlates with the ability of AM fungi to improve host nutrient status, since SL release is increased under nutrient stress and down-regulated

by increasing N and/or P in the shoots of plants (Yoneyama et al., 2007a, Yoneyama et al., 2007b).

At the time plants were harvested in this study (10 WAS), *R. irregularis* enhanced host leaf P concentration in both IAC 165 and Shiokari in line with this well-known benefit of the symbiosis (Smith and Read, 2008). Interestingly, the nutrient status of rice plants grown with both *R. irregularis* and *S. hermonthica* together was also enhanced. However, *S. hermonthica* infection actually increased in these plants, so this improvement in nutrient status, and any subsequent down-regulation of germination stimulant release by the host cannot have occurred in time to reduce parasite germination. The order of colonisation/infection can therefore be hypothesised to be a critical determinant of the outcome of the interaction. *R. irregularis* must require a head start in order to colonise the host plant, provide it with nutrients, and down-regulate host SLs before they can be released into the rhizosphere. It is therefore likely that *R. irregularis* and *S. hermonthica* colonised/infected at a similar time earlier in the experiment, or even that *S. hermonthica* infected ahead of fungal colonisation. While the end result of the interaction in this experiment was observed at 10 WAS, the details of the early stages, such as the timing of colonisation/infection and at exactly at what point *S. hermonthica* infection was enhanced, are not clear from these data. The hypothesis that the order of colonisation/infection by the symbionts is crucial to the outcome of the interaction is tested in chapter 4.

Clearly, the AM-mediated improvement of host nutrient status did not reduce *S. hermonthica* infection. In fact, the increase in *S. hermonthica* biomass observed suggests that if anything, the parasite benefited from an enhanced nutrient source as a result of AM-mediated nutrient acquisition. The nutrient supply by the fungus appears to be at minimal C cost to the host, which is evident by the low levels of colonisation seen. This, in combination with the nutrient supply rate used in this experiment, clearly allowed the host to support this increase in *S. hermonthica* biomass. Nutrient partitioning is likely a critical contributing factor in determining the outcome of the interaction in this experiment. Because the susceptibility of the host increased due to the presence of *R. irregularis*, it could be hypothesised that the fungus actually down-regulated host defences in this study during the time that *S.*

hermonthica was invading the host root cortex. Again however, the fact that measurements were taken at 10 WAS means that earlier or highly localised events involving the suppression of host defence by AM fungi may not have been detected. Indeed, growth of IAC 165 with *R. irregularis* actually up-regulated many metabolites in the phenylpropanoid and flavonoid biosynthesis pathways, while none were down-regulated. Overall this suggests the induction of the host defence metabolome by *R. irregularis*. This observation agrees with that of Rivero et al. (2015), who found an increase in downstream metabolites of the phenylpropanoid pathway, including ferulate (also upregulated in the present study) *Solanum lycopersicum* (tomato) after 8 weeks of colonisation by *R. irregularis* and *Funneliformis mosseae* (syn. *Glomus mosseae*).

A number of metabolites in the flavonoid biosynthesis pathway were unchanged in IAC 165 grown with *R. irregularis*, although some metabolites were highly up-regulated. For example, 7,4'-Dihydroxyflavone, which is known to have antimicrobial properties in *Medicago truncatula* exposed to cotton root rot (*Phymatotrichopsis omnivora*) (Watson et al., 2015) was up-regulated 12-fold with *R. irregularis* and also was the only metabolite up-regulated with *S. hermonthica* alone (10-fold). However, apart from this, only two other metabolites were up-regulated, and to a lesser degree.

In contrast to IAC 165 grown with *R. irregularis* alone, growth of IAC 165 with S. *hermonthica* alone down-regulated phenylpropanoid biosynthesis and flavonoid biosynthesis. The downregulation of the phenylpropanoid biosynthesis pathway in particular suggests that lignin biosynthesis was suppressed by in IAC 165 infected by *S. hermonthica*. This result is consistent with previous studies that have shown a high susceptibility of this cultivar to *S. hermonthica* infection (Gurney et al., 2006, Swarbrick et al., 2008). Furthermore, transcriptomic analysis of rice infected by *S. hermonthica* has shown that gene expression is down-regulated to a greater extent in the susceptible cultivar IAC 165 compared to the less susceptible cultivar Nipponbare (Swarbrick et al., 2008). In the same study, the resistance response in Nipponbare was associated with the up-regulation of genes encoding enzymes involved in defence metabolism including *pal*. The down-regulation of the phenylpropanoid biosynthesis pathway by *S. hermonthica* in this study is also

consistent with the down-regulation of genes involved in defence pathways, lignin biosynthesis, and secondary cell wall modification observed in the cowpea cultivar B301 undergoing a compatible (susceptible) interaction with *S. gesnerioides* race SG4z (Huang et al., 2012). Lignification of host root tissues at the site of invading parasite structures is a key resistance mechanism which is used to prevent the establishment of vascular connections, as seen in histological studies. For example, Cameron et al. (2006) were able to show that resistance to *Rhinanthus minor* by the non-host forb *Leucanthemum vulgare* involves encapsulation of the invading parasite structures by highly lignified cells at the host-parasite interface, and that lignification did not occur at the parasite interface with the susceptible host plant *Cynosurus cristatus*. The flavonoid biosynthesis pathway was also widely down-regulated in IAC 165 grown with *S. hermonthica* alone. Particularly down-regulated was cis-3,4-leucopelargonidin (-26-fold), which leads to pelargonidin which in turn then leads to anthocyanin biosynthesis. Naringenin, which leads to the production of the rice phytoalexin sakuranetin, was also down-regulated. Sakuranetin has strong antimicrobial activity towards blast fungus (Ishihara et al., 2008), so it is feasible that downregulation of the pathway leading to its production in this study is symptomatic the susceptible interaction observed. Unlike with *R. irregularis*, the effect of *S. hermonthica* appears to have been long term.

In IAC 165 grown with *R. irregularis* in combination with *S. hermonthica*, metabolite quantities varied in a highly inconsistent manner in comparison the consistent down-regulation seen in IAC 165 grown with *S. hermonthica* alone. Results from the metabolite analysis have not given a clear indication as to why the presence *R. irregularis* increased *S. hermonthica* in rice. Instead, defence suppression which occurs in a transient, localised manner at the early stages of AM colonisation may have been involved. For example, the suppression of SA-dependent defence during the early stages of colonisation (possibly caused by an increase in ABA) is thought to be essential for the establishment of the AM symbiosis as part of mycorrhizal evasion of host defence responses (Herrera Medina et al., 2003, Herrera-Medina et al., 2007, Mohr and Cahill, 2007a). In another example of transient defence suppression by AM fungi, an effector protein (SP7) has been identified in *R. irregularis* during colonisation of *Medicago truncatula* roots by *R. irregularis*. This protein is secreted by the fungus and delivered to the host cell nucleus, where it binds

to and inhibits the pathogenesis-related transcription factor, ERF19 (Kloppholz et al., 2011). It can therefore be hypothesised that the transient, localised down-regulation of plant defences known to occur during the early stages of AM colonisation may be responsible for the increase in *S. hermonthica* observed here.

2.4.2 Does colonisation by *R. irregularis* alleviate the negative effects of *S. hermonthica*?

S. hermonthica clearly dominates the interaction in terms of its own infection success. However, it also dominates the interaction in terms of its effect on host biomass partitioning, despite there being a clear nutrient-enhancement effect of *R. irregularis,* alone and in combination with the parasite. In this study, there was a significant increase in the above ground dry weight of *R. irregularis*-colonised rice compared to rice grown alone. *R. irregularis* significantly enhanced host leaf and root P concentration in both IAC165 and Shiokari. These data are consistent with the known role of both AM fungi and sufficient nutrient supply in the down-regulation of SL biosynthesis in host plants (Lopez-Raez et al., 2011, Yoneyama et al., 2007a, Yoneyama et al., 2007b), and that SLs are involved in reductions in above-ground biomass partitioning under nutrient-limited conditions (Gomez-Roldan et al., 2008).

Growth of both cultivars with *S. hermonthica* significantly decreased above ground dry weight compared to the un-infected controls. Significantly decreased stem height and diameter were responsible for this effect. *S. hermonthica* had no effect on N or P concentration in rice. However, the highly negative impact of *S. hermonthica* on host growth shows that the total level of nutrients retained by the host was greatly reduced. Overall, the growth data in this study clearly shows that *S. hermonthica* has a strong parasitic effect on rice. This is consistent with previous studies, which have shown that loss of biomass manifests itself through stunting, decreased stem diameter, and decreased leaf area (Cechin and Press, 1994, Watling and Press, 2000). In this study there was no reduction in tiller number in plants infected with *S. hermonthica*, alone or in combination with *R. irregularis*. This differs from previous reports, which have shown that *S. hermonthica* infected rice plants also have fewer tillers than uninfected plants (Cechin and Press, 1994, Cissoko et al., 2011, Echegoyen-Nava, 2012b). However, both cultivars only produced two to four tillers, and IAC 165 in particular

is known to be a low-tillering cultivar, probably due to its high SL content (a major factor in its susceptibility to *S. hermonthica*) (Jamil et al., 2012).

Interestingly, plants co-infected with *R. irregularis* and *S. hermonthica* together exhibited the same biomass partitioning characteristics to those grown with *S. hermonthica* alone, despite enhanced P concentration. The enhanced biomass of *S. hermonthica* on plants grown with both symbionts shows that the parasite acted as a powerful nutrient sink, but that the nutrient supply to the host via nutrient solution, in combination with supply by the mutualistic fungus, enabled the host to support higher parasite demand without further reductions in growth. Nevertheless, as with plants infected with *S. hermonthica* alone, the reduction of host growth shows that the total level of N and P retained by the host was greatly reduced in comparison to un-infected controls. Studies regarding the effect of AM fungi on *S. hermonthica* infection and subsequent host growth have already been carried out, mainly on sorghum and maize (Lendzemo and Kuyper, 2001, Lendzemo et al., 2005, Lendzemo et al., 2007, Othira et al., 2012). Results have mainly indicated an alleviation of the *Striga* effect on host growth with some exceptions. For example, a pot experiment using a mixed AM inoculum and *S. hermonthica* to colonise/infect tolerant and sensitive sorghum cultivars resulted in alleviation of the *Striga*-effect on the tolerant cultivar but not the sensitive cultivar (Lendzemo and Kuyper, 2001). It is worth noting that this previous study by Lendzemo and Kuyper (2001) found a similar positive and negative effect of individual AM colonization and individual *S. hermonthica* respectively.

2.4.3 Conclusions

According to what has been seen in studies to date, AM fungi are expected to reduce infection by *S. hermonthica*. However, this study has shown that *S. hermonthica* dominates the interaction with *R. irregularis* in terms of effects on host morphology and growth, and even benefits in terms of infection success. On the other hand, while *R. irregularis* continued to provide nutrients to the host, this did not alleviate the effect of *S. hermonthica*, and colonization was reduced by the presence of the parasite. The provision of nutrients by *R. irregularis* while imposing a low C demand on the host, coupled with the nutrient supply via nutrient solution to the rice plants,

likely enabled them to support the enhanced number biomass in the presence of the parasite. It would be interesting to see if this ability is maintained with a low nutrient supply, and this is tested in chapter 3.

The global host defence metabolome reflected an opposing effect of the two symbionts singly. However, this did not help us to explain the overall dominance of *S. hermonthica* over *R. irregularis* when the symbionts were grown together. There are a few possible explanations for this observation when considering some of the underlying mechanisms of AM colonisation and *S. hermonthica* infection. Clearly, the SL signalling mechanism is not the only driving factor in the interaction between AM fungi and parasitic plants, despite previously observed decreases in *Striga* infection due to AM colonization (Lendzemo et al., 2005, Lendzemo et al., 2007, Othira et al., 2012). The outcome of the interaction may be determined by the timing and order of infection. In this experiment, roots entered a spore inoculum not a mycelial network. In the field, roots would likely enter a mycelial network which may result in faster colonisation.

Of course, timing is a factor which was not under control in this experiment. We could speculate that in this experiment, *S. hermonthica* infected before *R. irregularis*, preventing any AM-induced down-regulation of germination stimulant release from the host roots. Endogenous effects of AM colonization which favour the post-attachment stages of *Striga* infection are more likely to be behind the increase in parasite infection observed in this study. For example, AM root colonization is known to involve transient, localized defence suppression (Herrera Medina et al., 2003, Herrera-Medina et al., 2007). Control over the timing and order of infection, as well as localized analysis of the interaction at specific stages will allow the further dissection of the mechanisms behind this complex interaction. The effect of the order of colonisation/infection on the outcome of the interaction in terms of symbiont success is tested in chapter 4.

Chapter 3

How does altering nitrogen and phosphorus supply influence symbiont success and host growth during the interaction between *Rhizophagus irregularis* and *S*triga *hermonthica*?

3.1 Introduction

In chapter 2, it was shown that *R. irregularis* increased the susceptibility of two rice cultivars, IAC 165 and Shiokari, to *S. hermonthica*. This was shown as an increase in both the number and biomass of attached parasites in plants grown with both symbionts. At the time of harvest at 10 WAS, *R. irregularis* had conferred a nutrient benefit to its host plant, but this did not translate into a suppression of *S. hermonthica* infection. This was in stark contrast to the protective effect of AM fungi shown in other studies, which had been attributed to a decrease in host release of parasite germination stimulants (SLs) as a result of the nutrient benefit conferred by the fungus (Lendzemo et al., 2005, Lendzemo et al., 2007, Lopez-Raez et al., 2011). Immediately, this suggested that in chapter 2, *R. irregularis* enhanced host nutrient status after *Striga* germination, and not early enough to suppress germination stimulant release from the host roots. Thus the timing of colonisation/infection must be critical for determining the outcome of the interaction. It was therefore hypothesised that the increased number of *Striga* attachments was due to the suppression of plant defences known to occur at the early stages of AM colonisation, and that this would have occurred with both symbionts colonising/infecting the host at a similar time in the experiment.

As well as the number of attachments, the biomass of *Striga* also dramatically increased in the presence of *R. irregularis*, although this did not further decrease growth of the host in comparison to plants infected by *Striga* alone. The host plants in this experiment were clearly receiving enough nutrients to support this increased parasite demand, and the additional supply of nutrients from the fungus may have served to increase their value as a nutrient source for the parasite. The fungal supply of nutrients coupled with the low level of colonisation observed showed that the fungus was highly mutualistic with a low demand for host C in both the absence and presence of *S. hermonthica*. Therefore, the dominant sink for the re-allocation of host nutrient was *Striga*. The ability of host plants to support an increased biomass of *Striga* in the presence of *R. irregularis*, and the large size of plants grown in the absence of either symbiont and in the presence of *R. irregularis* alone shows that plants in the experiment in Chapter 2 received a plentiful supply of nutrients.

Based on the results of chapter 2, I now wish to test if different levels of nutrient supply to the host plant will alter *R. irregularis* and *S. hermonthica* colonisation/infection and the result of their interaction on host growth. By changing the nutrient supply, I may alter the ability of each symbiont to colonise/infect, and also the ability of the host plant to support symbiont demand. Both AM fungi and *S. hermonthica* impose significant carbons demands on their hosts (Jakobsen and Rosendahl, 1990, Irving and Cameron, 2009). Furthermore, it is already known that the level of nutrient supply to host plants alters their interaction with both AM fungi and *Striga* significantly.

In the case of *Striga*, infestation is most severe in nutrient poor conditions in sub-Saharan Africa (SSA) (Oswald, 2005). Low nutrient input practices such as those used in many areas of SSA by subsistence farmers generate nutrient poor fields, and this is where *Striga* infestation is a particular problem (Ejeta, 2007). However, high nutrient conditions are known to decrease emergence and biomass of *Striga* and reduce the negative impact on the host (Cechin and Press, 1993b, Cechin and Press, 1994). Increased nutrient supply (predominantly N but also P) can reduce parasite attachment, with examples of this seen in rice (Riches et al., 2005, Adagba et al., 2002, Jamil et al., 2011a), maize (Kamara et al., 2009, Ahonsi et al., 2002, Jamil et al., 2012), pearl millet (Jamil et al., 2014), and sorghum (Cechin and Press, 1993b, Showemimo et al., 2002, Jamil et al., 2013).

One of the main mechanisms for reduced parasite germination and therefore attachment is a reduction in germination stimulating compounds in host root exudates when fertilised with N/P. It is widely known that increasing N/P supply to plant roots suppresses SL exudation (Yoneyama et al., 2007a, Yoneyama et al., 2007b, Yoneyama et al., 2012, Lopez-Raez et al., 2008, Jamil et al., 2012). In accordance with this, studies have largely attributed the reductions in *Striga* infection under high nutrient conditions to a reduction in SLs released from host roots (Jamil et al., 2011a). For example, a glasshouse pot experiment by Jamil et al. (2011) showed that SL exudation and *Striga hermonthica* germination and attachment in the rice cultivar IAC 165 increased because of N/P deficiency, with SL exudation highest under P-deficient conditions (Jamil et al., 2011a). Jamil et al. (2012) also showed that under greenhouse conditions, the release of SLs from maize roots

increases with low N/P and increases *Striga* infection, and the effect of increasing N on reducing *Striga* infection was consistent in the field (Jamil et al., 2012). Nitrogen fertilisation can also have a direct toxic effect on *Striga* germination and shoot development (Cechin and Press, 1993a, Igbinnosa et al., 1996). For example, high ammonium nitrate concentrations have been shown to decrease *Striga* germination (Cechin and Press, 1993a), while high ammonium and urea concentrations can suppress *Striga* shoot development and elongation (Igbinnosa et al., 1996).

As with *Striga* infection, nutrient addition has also been shown to decrease AM colonisation. For example, Mader et al. (2000) measured AM colonisation in wheat, vetch-rye and grass clover grown in a long term field trial comparing low and high fertiliser input sites. It was found that % root length colonisation was significantly higher (by 30-60%) in plants grown in low input soils than in those grown in conventional soils (Mader et al., 2000). Similarly, a study by van der Gast et al. (2011) analysed AM fungal colonisation in plants grown in soils exposed to organic (low fertiliser input) and conventional (high fertiliser input) farm practices, and found that % root length colonisation was significantly higher in plants grown in organic soils (van der Gast et al., 2011). Again, in a similar manner to *Striga*, one of the main mechanisms which enhances AM colonisation is the exudation of SLs into the rhizosphere, which is enhanced by nutrient stress (Akiyama et al., 2010). SLs induce hyphal branching in AM fungi (Akiyama et al., 2005), increasing the likelihood of making contact with host roots (Parniske, 2008). This allows plants to enhance recruitment of AM fungi under nutrient limited conditions so as to exploit their hyphal network to scavenge for nutrients such as P (Akiyama et al., 2005, Foo et al., 2013).

In this chapter, I test the effect of lowering the N/P supply to host plants, and compare my results to those in chapter 2. Lowering the nutrient supply may affect the interaction between the host plant and the two symbionts, both singly and in combination, in two major ways. First, it may enhance colonisation/infection by increasing branching/germination stimulant release and thus the recruitment of the two symbionts to the host root. Secondly, it may alter the source-sink dynamics of the interaction and the ability of the host plant to support both symbionts due to their demands on the host for nutrients. In particular, this may alter the ability of host

plants to support enhanced *Striga* infection in the presence of *R. irregularis* as seen in chapter 2.

The aim of chapter 3 was to understand the effects of different amounts of substrate N/P levels on host colonisation/infection success of each symbiont, alone and in combination, and the subsequent effects on host growth and development. In this chapter, there are two main hypotheses that I will test:

Hypotheses

1) Decreasing N/P supply will enhance colonisation/infection of the host by both symbionts singly and in combination due to an increase in the host release of germination stimulants.
2) Decreasing N/P supply will limit the ability of the host to support a high level of *S. hermonthica* infection alone and/or in combination with *R. irregularis.*

Objectives

1) Grow the rice cultivar IAC 165 in factorial combination with *R. irregularis* and *S. hermonthica* alone and in combination in pots.
2) Supply the pots with two levels of N/P concentration; one in which plants are likely to receive a sufficient supply ('high'), and the other where the plants are likely to be deficient ('low') in reference to the clearly sufficient supply used in chapter 2.
3) Measure growth and development weekly over a 10-week period.
4) Carry out harvests at 5 and 10 weeks after sowing.
5) Measure nutrient status of the host.
6) Measure colonisation by *R. irregularis* and infection by *S. hermonthica*.
7) Collect root exudates from IAC 165 grown alone and grown with *R. irregularis* and perform a bioassay on *S. hermonthica* seeds to analyse germination and haustorium formation. This will test the effect of *R. irregularis* colonisation on host root germination stimulating activity on parasite seeds.

3.2 Materials and methods

3.2.1 Plant and fungal materials

Seeds of *Oryza sativa* ssp. *Japonica*, cultivar IAC 165 were obtained from the International Rice Research Institute (IRRI) Phillipines, *Striga hermonthica* seeds were collected from Kibos, 2013. The arbuscular mycorrhizal inoculum which contained *Rhizophagus irregularis* isolate 09 (Stockinger et al., 2009) was propagated on *Daucus carota* L. root organ culture (ROC). Roots and spores were cultured on modified Strulli-Romand (MSR) medium (Declerck et al., 1996, Declerck et al., 1998) in 150 mm diameter petri dishes and maintained in darkness at 24°C for five months. The culture was blended and mixed with sterile demineralised water to produce a suspension containing 400 spores ml^{-1}.

3.2.2 The effect of altering substrate N/P supply on host nutrient status, growth *R. irregularis* colonisation and *S. hermonthica* infection

The four treatments illustrated in section 2.2.2 (Fig. 2.1) were established for this experiment: they were: (1) rice plants alone (designated control (-AM-*Striga*)), (2) rice plants plus AM inoculum (designated +AM), (3) rice plants plus *S. hermonthica* seeds (designated +*Striga*), and (4) rice plants plus AM inoculum and *S. hermonthica* seeds (designated +AM+*Striga*). Plants were set up as described in section in 2.2.2 Fig. 2.1 except that the AM inoculum strain differed and two nutrient supplies were established. Pots containing mycorrhizal inoculum contained 10 ml of the *R. irregularis* suspension to make 4000 spores per pot in the top 10 cm of sand. Rice seeds of IAC 165 were introduced into pots as described in section 2.2.2. Eight biological replicates were established per treatment. Plants were grown in the same conditions described in section 2.2.2 during the months of July and August 2014 when the photoperiod was approximately 16h light and 8h dark.

Two levels of N/P were supplied to plants, while the supply of all other nutrients and the supply of water was equal. One N/P level (designated ‘high’ N/P) was intended to supply the plants with a sufficient level of N/P lower (to promote more mycorrhizal colonisation) but still comparable to the nutrient regime used in chapter

2. The other (designated ‘low’ N/P) was intended to make the plants deficient in both N/P via a level of N and P supply an order of magnitude lower than plants in chapter 2 and the ‘high’ level used in this chapter.

Plants in the high N/P treatment were watered once a day using an automatic dripper system delivering a total of 30 ml per day of 40 % Long Ashton solution containing 2 mM nitrogen and 20 µM phosphorus for 5 WAS, then 60 ml of the same solution until the end of the experiment at 10 WAS. Plants in the low N/P treatment were watered once a day using an automatic dripper system delivering a total of 30 ml per day of only distilled water for 2 WAS, then 30 ml of 40 % Long Ashton solution containing 0.25mM nitrogen and 2 µM phosphorus until 5 WAS, then 60 ml of the same solution until the end of the experiment at 10 WAS.

What this supply rate equates to in terms of cumulative N/P supply is shown in Fig. 3.1. Plants in the high nutrient treatment received a total of 177.24 mg N and 0.945 mg P by the end of the experiment at 10 WAS (Fig. 3.1a and b). Plants in the low nutrient treatment received a total of 19.201 mg N and 0.0819 mg P by the end of the experiment at 10 WAS (Fig. 3.1c and d). The total supply of N/P was therefore an order of magnitude lower for plants grown with low N/P than for plants grown with high N/P. Note that in chapter 2 a total of 236.32 mg N and 1.26 mg P was supplied by the end of the experiment (section 2.2.2, Fig. 2.3).

3.2.3 Measurements of plant morphology

Measurements of plant morphology (height and diameter of the main stem, and the total number of tillers and leaves) were carried out as detailed in section 2.2.3.

3.2.4 Harvesting of plant material

Harvesting of plant material was carried out at 5 and 10 weeks after sowing (WAS) as detailed in section 2.2.4, except that the roots systems were divided into create two sections, and all subsamples used for root analysis came from the top section.

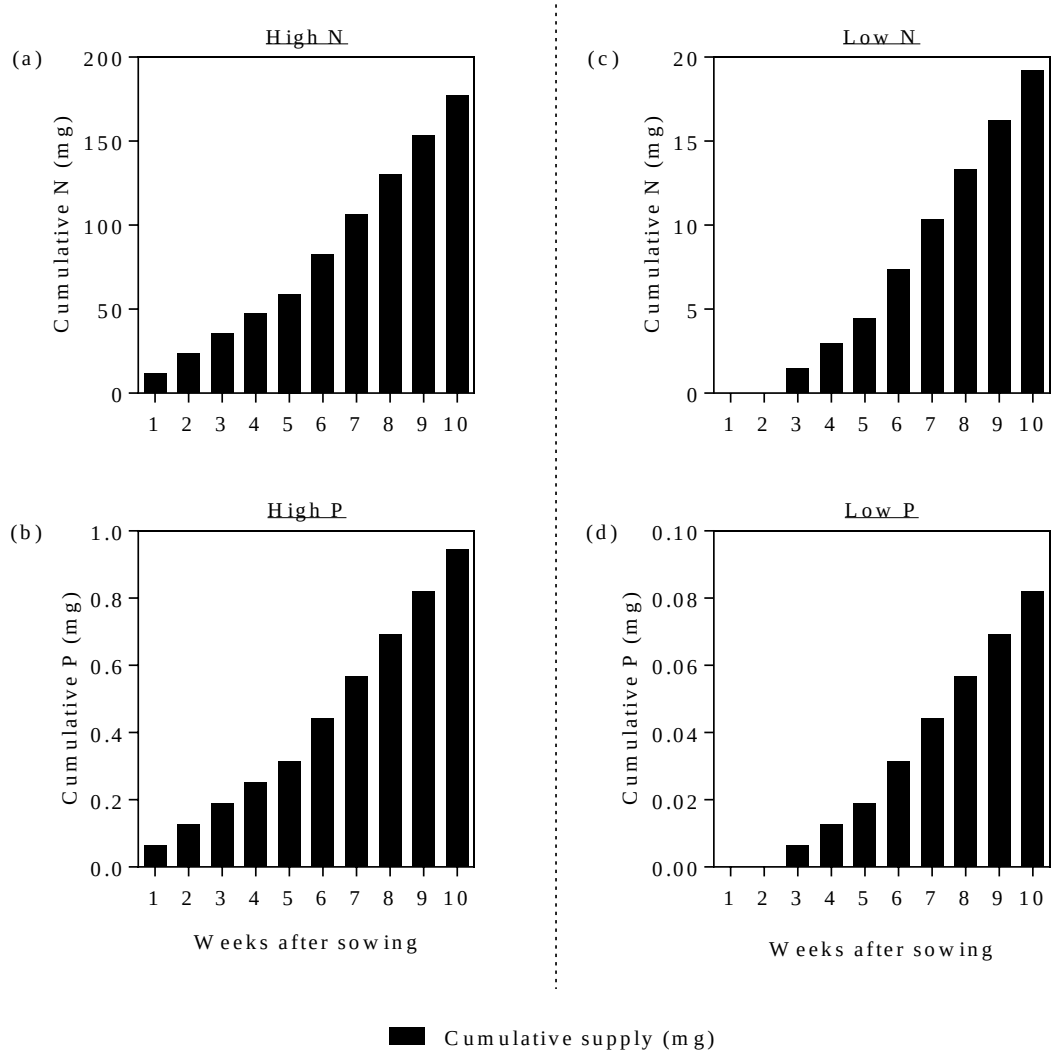


Fig. 3.1 The cumulative amount of N/P supplied to plants via nutrient solution during the 10 weeks of the experiment. Overall, plants supplied with 'low' N/P received an order of magnitude less N/P than plants supplied with 'high' N/P by the end of the experiment at 10 WAS.

3.2.5 Staining of roots and quantification of mycorrhizal colonisation

Mycorrhizal staining and quantification was carried out as detailed in section 2.2.5.

3.2.6 Nutrient determination

Nutrient concentration in the roots and leaves of each treatment was carried out as detailed in Chapter 2.2.6.

3.2.7 Root exudation collection

Root exudates were collected by adding a known amount of water to pots. Using test pots, it was calculated that 150 ml of water was required to drain the pots in order to collect 100 ml samples containing root exudates. Exudates from each plant were collected in two 40 ml and one 20 ml falcon tube and snap frozen in liquid nitrogen before storage at -80°C. Root exudates from rice grown with *R. irregularis* alone (+AM) and supplied with high N/P and root exudates from control (-AM-*Striga*) plants also supplied with high N/P were used in the subsequent analysis. Six biological replicates were used.

3.2.8 Germination bioassay

Root exudates from rice grown with and without *R. irregularis* alone (+AM and –AM-*Striga* respectively) and supplied with high N/P from the 5 and 10 WAS harvests were used to test the effect of *R. irregularis* colonisation on *S. hermonthica* seed germination and haustoria formation. Only these exudates were used because initial tests with all other root exudates showed no germination potential, probably because the root systems of all other plants were very small resulting in a lower total amount of exudates per pot, and due to dilution with water during collection.

S. hermonthica seeds were surface sterilised in 10% sodium hypochlorite solution for 4 min before being washed into a Ø90 mm glass-fibre filter paper disc (Whatman®) fashioned into a funnel and rinsed thoroughly with demineralized water. *S.* hermonthica seeds require preconditioning before germination (Matusova et al., 2004). Seeds were preconditioned on moistened glass-fibre filter paper in a Ø90 mm petri dish sealed with parafilm at 30 °C in darkness for 14 days.

After preconditioning, seeds were washed with deionised water into a 100 µm polyester mesh (Plastok Group, Birkenhead, UK) fashioned into a funnel. Seeds were transferred to a Schott (Duran®) bottle by rinsing them from the mesh with 0.1% agarose solution. The seed/agarose mixture was topped up to 75 ml (1.5x the mass of seeds used in mg) and mixed gently to give a uniform seed distribution. Half of this

seed mix was then decanted into a separate bottle. To one bottle, the synthetic germination stimulant GR24 was added to a final concentration of 0.1 ppm, and to the other the same volume of water was added. Thus seeds treated with and without GR24 were made (+GR24 and –GR24 respectively). One ml of the seed/agarose mix was then pipetted into 2 ml Eppendorf tubes using a cut off tip to allow seed transfer. One ml of root exudates was then added to the tubes individually. Six biological reps from each growth condition were used. These growth conditions were; two treatments (control and +AM), one N and P level (high), two time points (5 and 10 WAS). These were pipetted into the +GR24 and –GR24 tubes. To six +GR24 tubes and six –GR24 tubes were added the same volume of water to provide seeds not treated with root exudates. Three-hundred µl of these preparations was then pipetted into individual wells in 24-well multiwell plates. The plates were double bagged with zip lock bags and incubated at 30 °C in darkness for 28 hours.

At 28 hours post germination, the haustorial initiation factor 2,6-Dimethoxy-1,4-benzoquinone (DMBQ) was added to a final concentration of 10 µM, and the seeds were incubated for 18 hours to allow haustoria to develop. Seeds were observed under a dissecting microscope (Leica stereo, MZFLIII, Diagnostic Instruments Inc, USA) to measure % germination and % haustorium production. Subsamples were then mounted into 75 x 25 mm microscope slides, and representative photographs of radicles and haustorial were taken under a light microscope (Olympus BX51) with an attached digital camera (Olympus DP71).

3.2.9 Statistical analyses

Measurements of growth which were taken half way through the experiment at 5 weeks after sowing (WAS) and at the end of the experiment at 10 WAS were analysed using three-way ANOVA with Tukey's multiple comparisons (MC) post hoc analysis in Minitab 17 (version 17.2.1.0. Minitab Inc., Pennsylvania, USA). Presence and absence of *R. irregularis* was included in the analysis as one factor, presence and absence of *S. hermonthica* as another, and high and low N/P as another, with the interaction between the three factors then tested. For the weekly growth measurements, three-way ANOVA was used at the end of the experiment at 10 WAS, taking into account the fact that all seeds were treated equally before sowing, so any

effect on growth would be due to treatment conditions. Assumptions of normality and homogeneity of variances were met through $\log_{10}$, square root, or Box Cox transformations of variables when necessary, and confirmed by visual analysis of residual vs fitted values. Comparisons of AM colonisation were made using two-way ANOVA with presence of *R. irregularis* alone and presence of *S. hermonthica* in combination with *R. irregularis* as one factor, and high and low N/P as the other. Comparisons of *S. hermonthica* infection levels were made using two-way ANOVA with presence of *S. hermonthica* alone and presence of *S. hermonthica* in combination with *R. irregularis* as one factor, and high and low N/P as the other. Comparisons of *S. hermonthica* seed germination were made using two-way ANOVA with exudates from control and +AM plants as one factor, and presence and absence of GR24 as the other. The GR24 only treatment (which did not root any root exudates) was not included in the analysis and is only used as a visual reference. Graphs were generated using GraphPad Prism 6. ANOVA tables for each factor and interaction showing model fit, F-values, degrees of freedom and significance are shown in appendix B.

3.3 Results

3.3.1 The effect of high and low supply of N and P on the morphology of the rice cultivar IAC 165 grown in the presence of *R. irregularis* and *S. hermonthica* alone and in combination

Figures 3.2 and 3.3 show the effects of altering the supply of N and P on the growth and morphology of the rice cultivar IAC 165 when grown with *R. irregularis* or *S. hermonthica* alone and in combination compared to uninfected control plants. ANOVA tables for each factor and interaction showing model fit, F-values, degrees of freedom and significance are shown in appendix B. Control plants and plants colonised by *R. irregularis* showed almost identical main stem height and diameters over the 10-week period of the experiment when grown with a high or low supply of N and P. However, plants grown with a high nutrient supply were twice the size of those grown with a low nutrient supply (Fig. 3.2a, b, e and f; Fig. 3.3a – c). Thus there was no growth promoting effect of *R. irregularis* in this experiment. The height and stem diameter of plants grown in the presence of *S. hermonthica* alone or together with *R. irregularis* were also similar to each other, but severely reduced in comparison to uninfected plants and plants grown with *R. irregularis* alone (Fig. 3.2a, b, e and f; Fig. 3.3b and c). At 10 weeks after sowing the height and stem diameter of plants grown with *S. hermonthica* or *S. hermonthica* in combination with *R. irregularis* were 4 times lower than uninfected plants and plants grown with *R. irregularis* alone when grown at high nutrient supply and two times lower when grown at low nutrient supply (Fig. 3.2a, b, e and f; Fig. 3.2b and c).

The rice cultivar IAC 165 is a low tillering cultivar. On average between 1.0 – 2.0 tillers were produced on uninfected plants and plants grown with *R. irregularis* at high nutrient supply and this was significantly lower (> 1.0 tiller on average) in plants grown with *S. hermonthica* alone (Fig. 3.3c; Fig. 3.3b). As a consequence, uninfected plants and plants grown with *R. irregularis* alone had the same number of leaves (at 9 weeks after sowing before leaves senesced) but the number of leaves on plants grown with *S. hermonthica* alone or in combination with *R. irregularis* was significantly lower ($p < 0.001$) (Fig. 3.2d and Fig. 3.3b). When plants were grown

with a low supply of nutrients, no tillers were produced in any treatment and numbers of leaves on all host plants were similar (Fig. 3.2g and h and Fig. 3.3c).

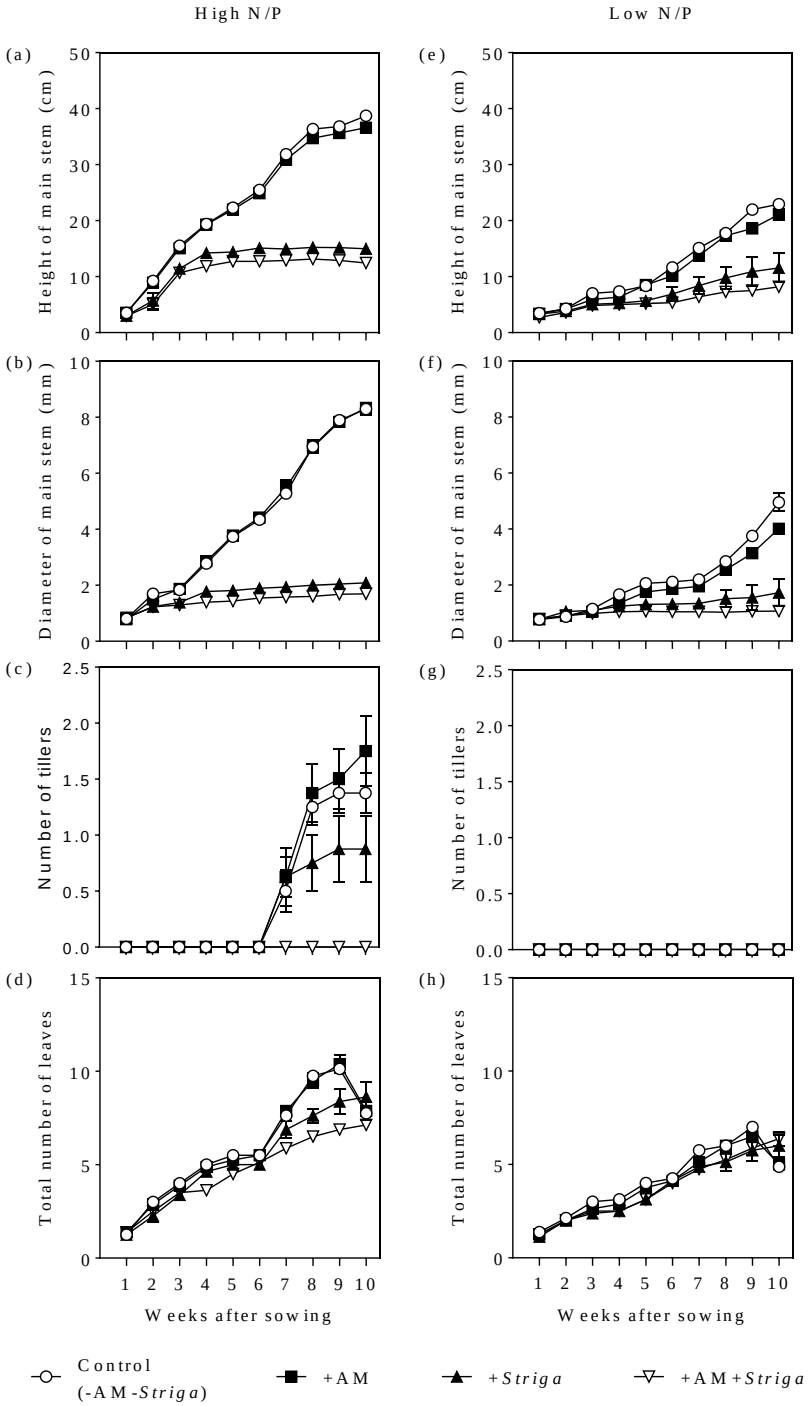


Fig. 3.2 Morphology of rice cultivar IAC 165 under high and low N/P when grown with *R. irregularis* (+AM), *S. hermonthica* (+*Striga*), and when grown with both *R. irregularis* and *S. hermonthica* together (+AM_*Striga*) or un-infected as control plants (-AM-*Striga*). (a) and (e) Height of main stem, (b) and (f) diameter of main stem, (c) and (g) total number of tillers, and (d) and (h) total number of leaves. Measurements were taken weekly beginning 1 WAS, and ending 10 WAS. Data shown is the mean for each parameter ± standard error (SE), n = 8.

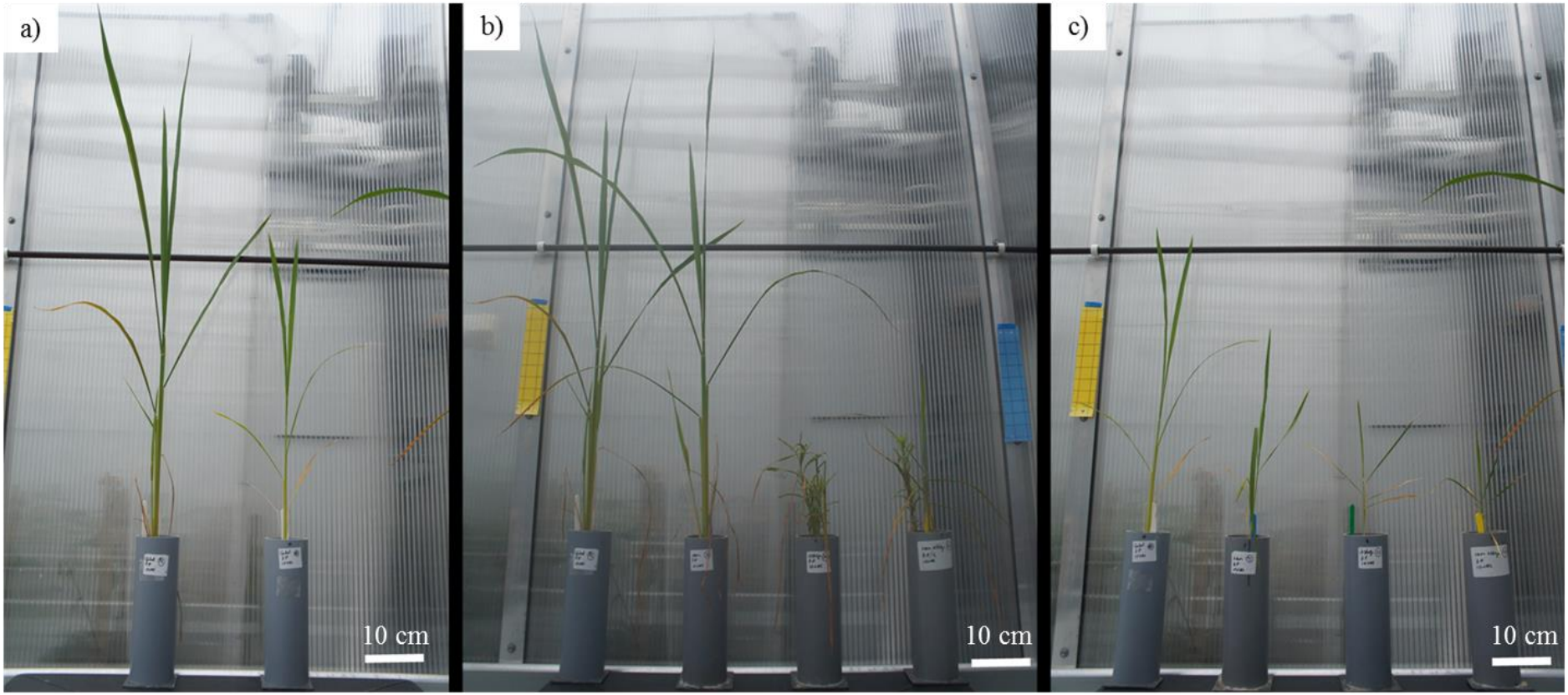


Fig. 3.3 Representative images of rice cultivar IAC 165 10 weeks after sowing. (a) Control plant supplied with high N/P (left) compared to control plant supplied with low N/P (right). (b) IAC 165 treatments supplied with high N/P from left to right: control, +AM, +*Striga*, +AM+*Striga*. (c) IAC 165 treatments supplied with low N/P from left to right: control, +AM, +*Striga*, +AM+*Striga*. Scale bars = 10 cm.

3.3.2 The effect of high and low N and P supply on the above- and below-ground biomass and N and P concentration of IAC 165 grown with *R. irregularis* and *S. hermonthica* alone or in combination

Fig. 3.4a and Fig. 3.4d show the effect of high and low N and P supply on the above- and below-ground biomass of IAC 165 grown with *R. irregularis* and *S. hermonthica* alone and in combination. ANOVA tables for each factor and interaction showing model fit, F-values, degrees of freedom and significance are shown in appendix B. The following results are analysed and presented using additional post-hoc Tukey's multiple comparison (MC) testing where necessary.

At both 5 and 10 WAS with high N/P there was a highly significant increase (three-way ANOVA, Tukey's MC, $p < 0.05$) in the biomass of roots and shoots of uninfected plants and plants grown with *R. irregularis* alone compared to plants grown with *S. hermonthica* alone or in combination with *R. irregularis* (Fig. 3.4a and d). A similar pattern was observed for the treatments grown with low nutrient supply at 5 WAS although root biomass was not altered by treatment, and the total biomass of all treatments was significantly lower (three-way ANOVA, Tukey's MC, $p < 0.05$) than for plants grown with a high nutrient supply (Fig. 3.4a and d). Again the pattern was similar at 10 WAS except that shoot biomass was not significantly lower in plants grown with *S. hermonthica* alone compared to plants grown with *R. irregularis* alone.

Fig. 3.4b and Fig. 3.4e show the effect of high and low N and P supply on the leaf and root N concentration of IAC 165 grown with *R. irregularis* and *S. hermonthica* and in combination. When plants were grown with a high nutrient supply the leaf N concentration was the same in all treatments (Fig. 3.4b and e). Similarly, root N concentration was barely affected by treatment at 5 WAS except for being higher (three-way ANOVA, Tukey's MC, $p < 0.05$) in plants grown with *S. hermonthica* alone than in plants grown with *R. irregularis* alone (Fig. 3.4b). By 10 WAS plants grown with *S. hermonthica* alone or in combination with *R. irregularis* had a higher (three-way ANOVA, Tukey's MC, $p < 0.05$) N concentration than uninfected plants

and plants grown with *R. irregularis* alone (Fig. 3.4e). When plants were grown with a low supply of N and P the shoot N concentration was not affected by treatment at either 5 or 10 WAS apart from at 10 WAS where it is higher (three-way ANOVA, Tukey's MC, $p < 0.05$) in plants grown with both symbionts than plants grown alone. The concentration of N in the roots of these plants followed a similar pattern to that seen at high nutrient supply i.e. at 10 WAS there was a small but significant increase (three-way ANOVA, Tukey's MC, $p < 0.05$) in N concentration in the treatments containing *S. hermonthica* (Fig. 3.4e).

Fig. 3.4c and Fig. 3.4f show the effect of high and low N and P supply on the leaf and root P concentration of IAC 165 grown with *R. irregularis* and *S. hermonthica* alone or in combination. When plants were grown with a high supply of nutrients, the phosphorus concentration of the leaves and roots of uninfected plants and plants grown with *R. irregularis* was lower (three-way ANOVA, Tukey's MC, $p < 0.05$) than in the leaves and roots of plants grown with *S. hermonthica* alone or in combination with *R. irregularis* at 10 WAS (Fig. 3.4f). When plants were grown with a low nutrient supply there was no significant difference in the P concentration of leaves and roots of all treatments at 5 WAS (fig. 3.4c). However, by 10 WAS the P concentration of leaves and roots was greater in the two treatments containing *S. hermonthica* than control plants (three-way ANOVA, Tukey's MC, $p < 0.05$) but not plants grown with *R. irregularis* alone (Fig. 3.4f).

3.3.3 The effect of high and low N/P supply on AM colonization of rice cultivar IAC165 grown with *R. irregularis* alone and in combination with *S. hermonthica*

Fig. 3.5 shows the effect of altering the supply of N and P on *R. irregularis* colonization of rice cultivar IAC165 grown with *R. irregularis*, alone and in combination with *S. hermonthica* after 5 and 10 WAS.

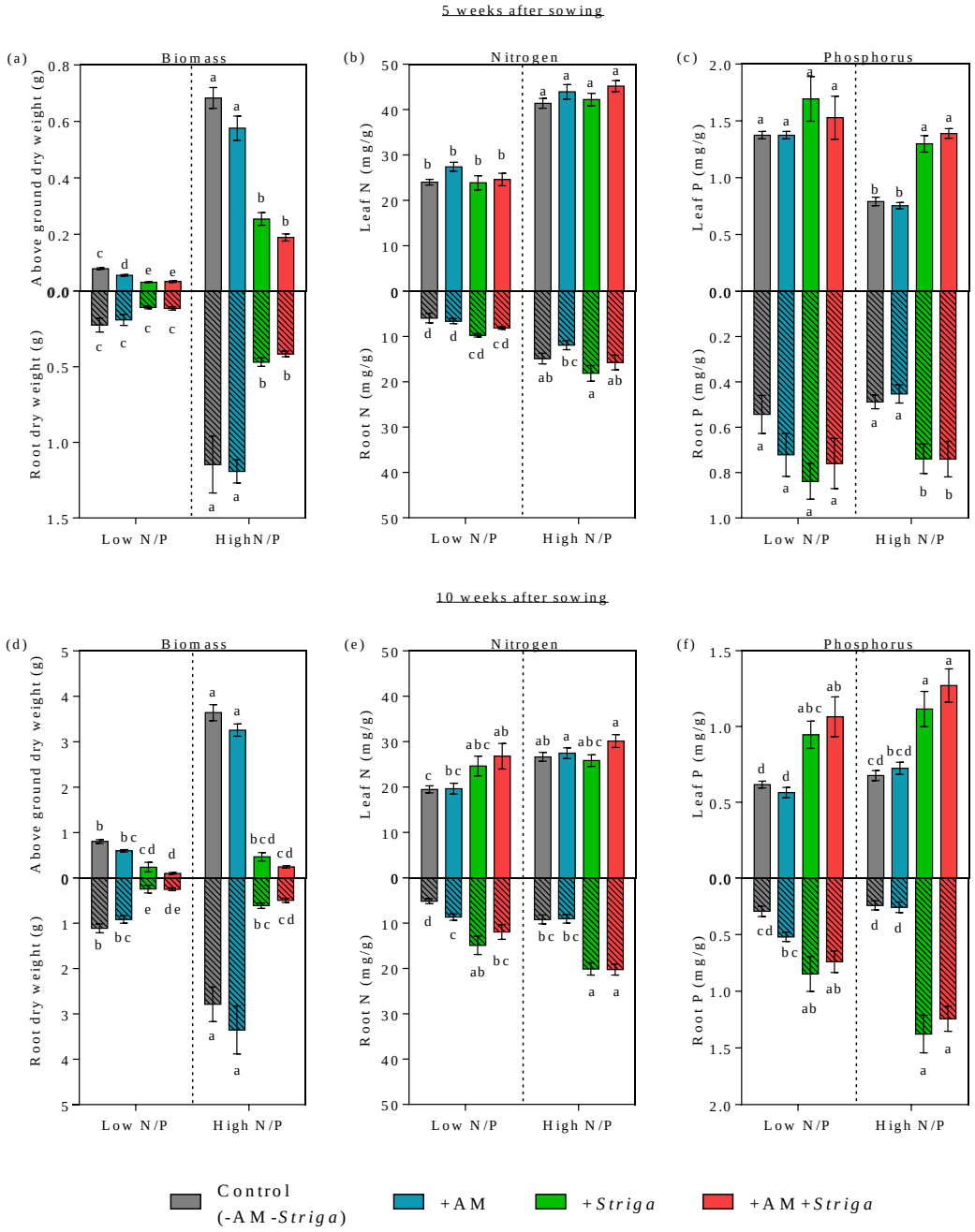


Fig. 3.4 Growth and nutrient status of the rice cultivar IAC165 under high and low N/P when grown with *R. irregularis* (+AM), *S. hermonthica* (+*Striga*), both *R. irregularis* and *S. hermonthica* (+AM+*Striga*), or un-infected as control plants (-AM-*Striga*) at 5 WAS (a-c) and 10 WAS (d-f). (a) and (d) Above ground (leaf and stem) and root dry weight. (b) and (e) Host leaf and root N concentration. (c) and (f) Host leaf and root P concentration. Data shown is mean dry weight ± SE. Columns sharing the same letters are not significantly different ($p > 0.05$, three-way ANOVA, Tukey's MC), n = 8.

At 5 WAS, % root colonisation of intraradical hyphae, vesicles and arbuscules in IAC 165 grown with *R. irregularis*, alone or in combination with *S. hermonthica*, was significantly lower (two-way ANOVA, Tukey's MC, $p < 0.001$) in plants supplied with low N/P compared to high N/P (Fig. 3.5a). By 10 WAS colonisation was equivalent in both nutrient treatments (Fig. 3.5b), where colonisation was characterised by a high abundance of arbuscules and vesicles (Fig. 3.6).

Colonisation was significantly decreased (two-way ANOVA, Tukey's MC, $p < 0.05$) by the presence of *S. hermonthica* at both low and high N/P supply levels by 10 WAS. Also of note is the fact that colonisation in plants supplied with high N/P was significantly lower (two-way ANOVA, Tukey's MC, $p < 0.05$) at 10 WAS compared to 5 WAS in plants colonised by *R. irregularis* in combination with *S. hermonthica*, perhaps representing a delayed suppressive effect of the parasite on colonisation.

3.3.4 The effect of high and low N/P supply on *S. hermonthica* infection of the rice cultivar IAC165 grown with *S. hermonthica* alone and in combination with *R. irregularis*

Fig. 3.7 shows the effect of high and low N/P supply on *S. hermonthica* infection of the rice cultivar IAC165 grown with *S. hermonthica* alone and in combination with *R. irregularis* at 5 and 10 WAS. The number and dry weight of parasites in IAC 165 grown with *S. hermonthica*, alone or in combination with *R. irregularis*, was significantly higher (two-way ANOVA, Tukey's MC, $p < 0.05$) in plants supplied with high N/P compared to low N/P (Fig. 3.7a, b, g and f). However, when normalising this data per unit of root biomass, then at low N/P there is a significantly higher (two-way ANOVA, Tukey's MC, $p < 0.05$) number of *S. hermonthica*, with the exception of co-colonised/infected plants at 10 WAS (Fig. 3.7c and d). Throughout the experiment, the presence of *R. irregularis* did not alter *S. hermonthica* number or dry weight with the only exception at the high N/P level at 5 WAS, where *S. hermonthica* dry weight was significantly lower (two-way ANOVA, Tukey's MC, $p < 0.05$) in the presence of *R. irregularis* (Fig. 3.7e and g).

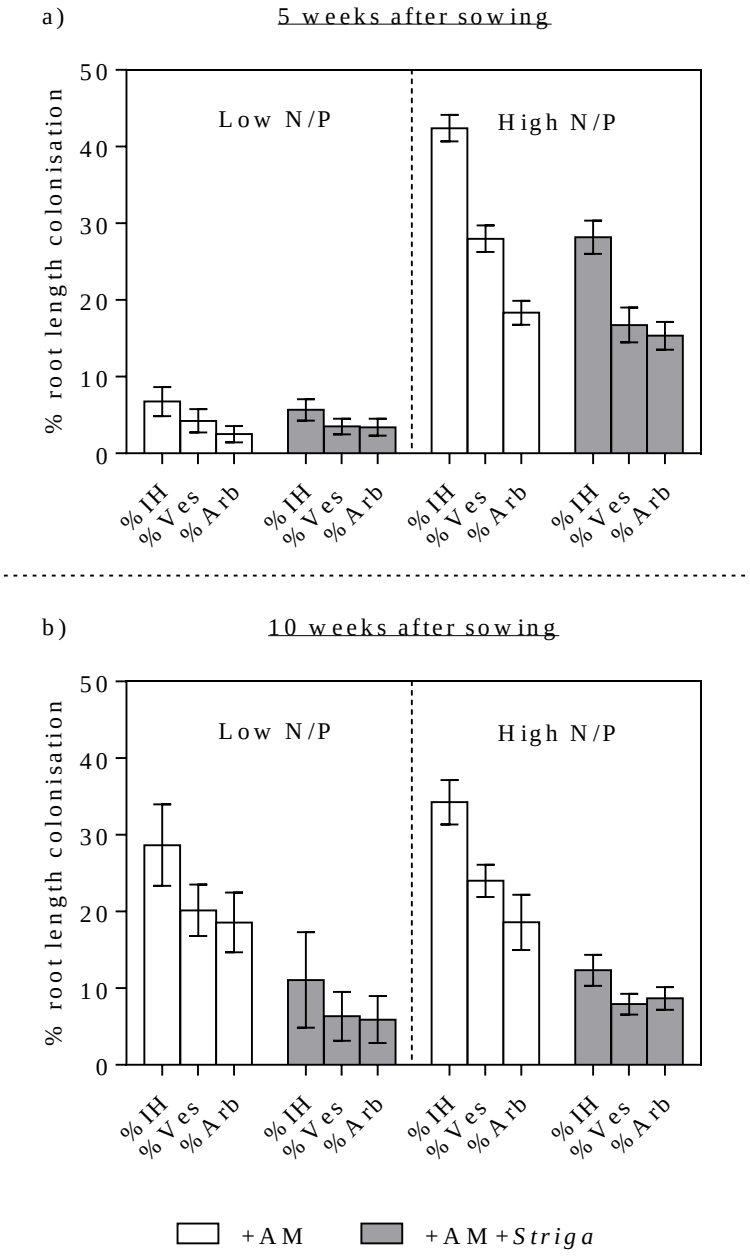


Fig. 3.5 *R. irregularis* colonisation of IAC165 at (a) 5 and (b) 10 WAS under high and low N/P supply. Intraradical hyphae (IH), vesicle (Ves) and arbuscules (Arb) are shown. Data shown is mean % root length colonisation ± SE, n = 8. Comparisons of results are outlined in section 3.3.3.

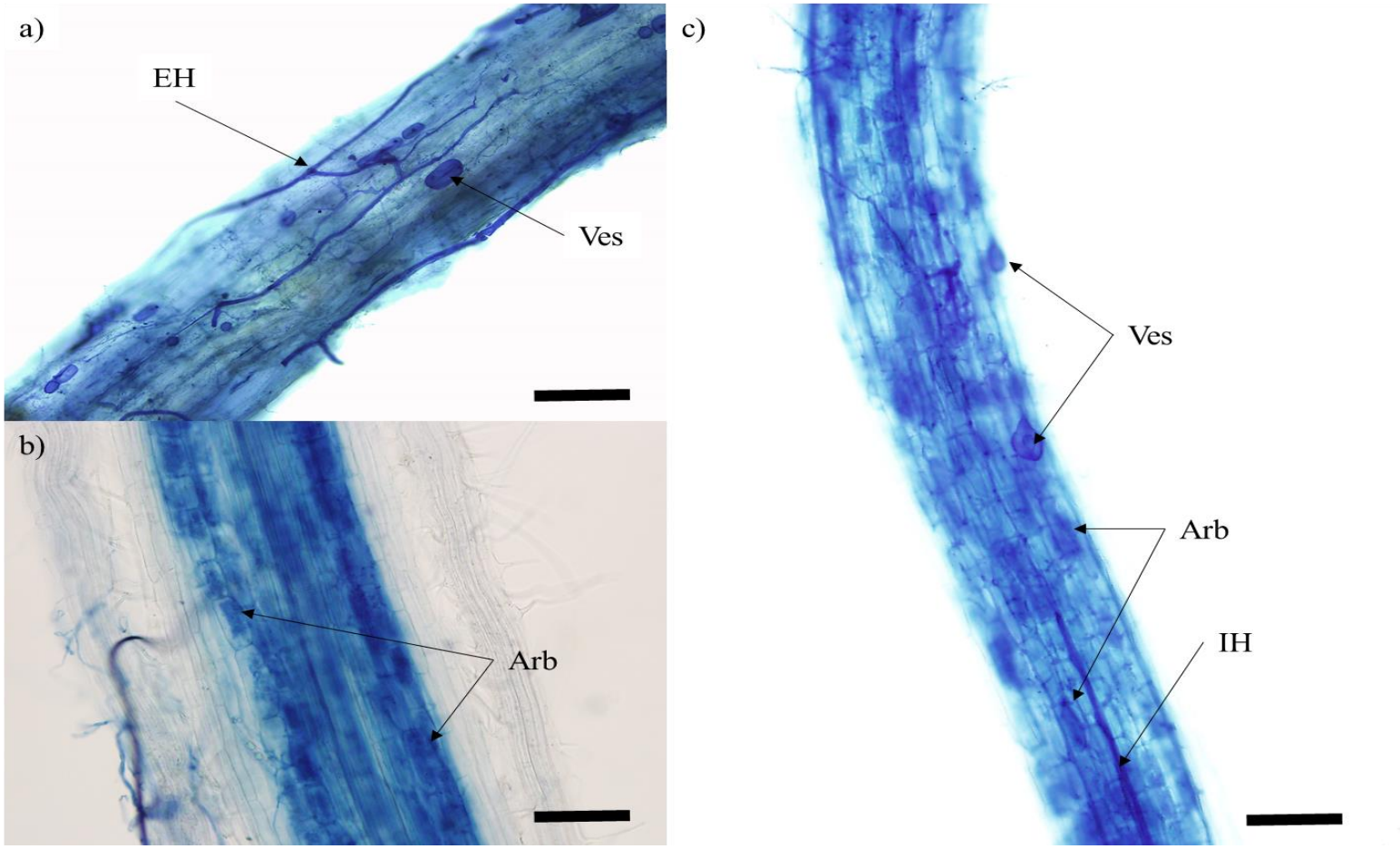


Fig. 3.6 Images of roots from IAC 165 colonised by *R. irregularis* and supplied with 'high' N/P harvested at 10 WAS. (a) Extraradical colonisation of the root shown by extraradical hyphae (EH) and an intraradical vesicle (Ves) scale bar = 100 µm. (b) Intraradical colonisation of the root showing empty cells surrounding arbuscule-filled (Arb) cells of the root cortex, scale bar = 50 µm. (c) Intraradical colonisation of the root taken with a brighter background to show intraradical structures: vesicles, arbuscules and intraradical hyphae (IH), scale bar = 50 µm.

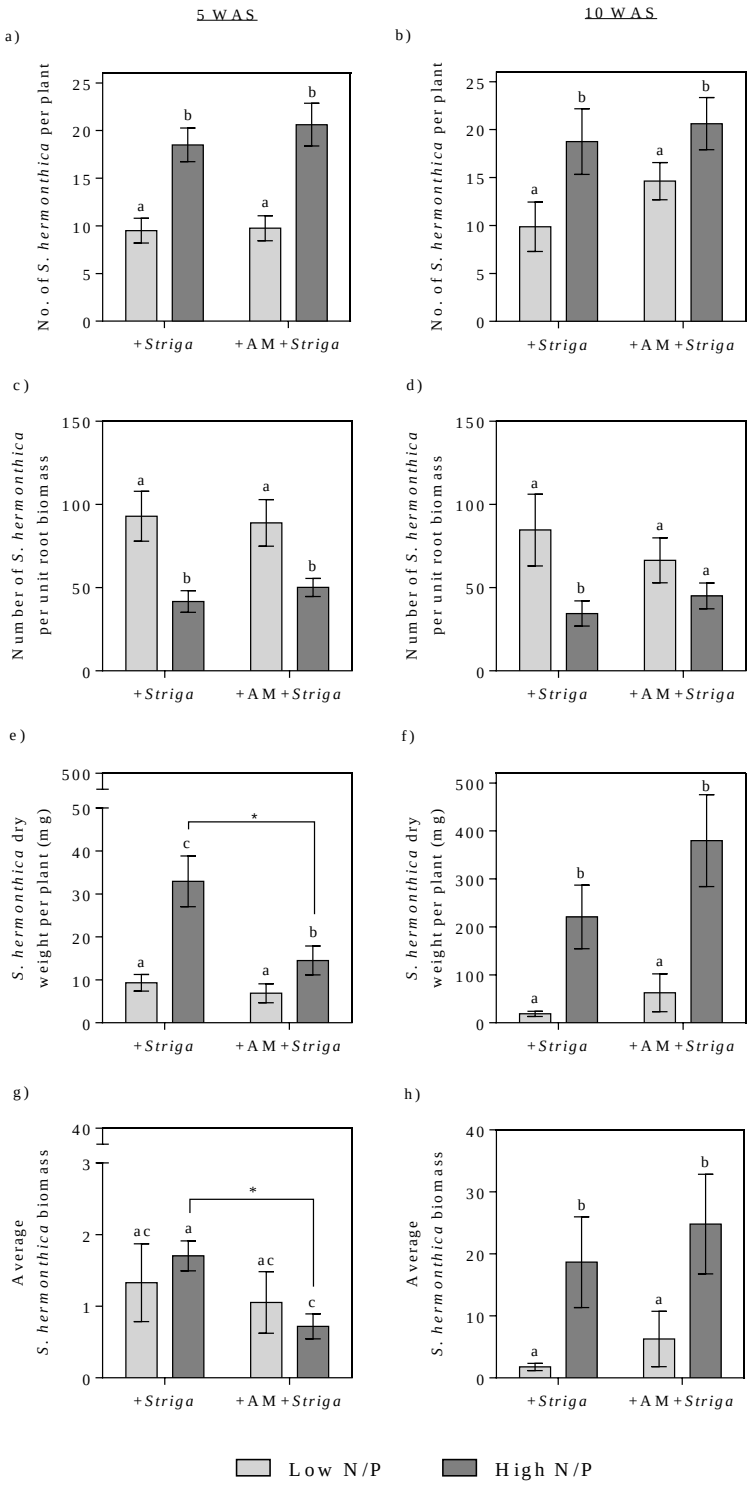


Fig. 3.7 Number and dry weight of *S. hermonthica* attached to IAC165 roots when grown with *S. hermonthica*, alone and in combination with *R. irregularis* under high and low N/P conditions at 5 WAS (left column) and 10 WAS (right column). (a)/(b) Number of *S. hermonthica*. (c)/(d) Number of *S. hermonthica* per gram of root dry weight. (e)/(f) *S. hermonthica* dry weight. (g)/(h) Average dry weight of *S. hermonthica*. Data shown are mean values ± SE. Columns sharing the same letters are not significantly different ($p > 0.05$, two-way ANOVA), $n = 8$. Stars on (e) and (g) indicate significant parasite biomass reduction in co-colonised/infected plants.

3.3.5 Ability of roots exudates from the rice cultivar IAC 165 grown with high N/P supply in the absence or presence of *R. irregularis* to germinate *S. hermonthica* seeds and cause the development of haustoria

Fig. 3.8 and 3.9 show the effect of exudates collected from IAC 165 grown with and without *R. irregularis* on the seed germinating potential and haustorium formation *S. hermonthica*. Only exudates from control and +AM plants supplied with high N/P collected at 10 WAS were used because initial tests with all other root exudates showed no germination potential, probably because the root systems of all other plants were very small resulting in a lower total amount of exudates per pot.

Without the addition of GR24, there was no significant effect of root exudates collected from plants grown with *R. irregularis* on germination or haustorial development of *S. hermonthica* seeds compared to plants grown alone (Fig. 3.8a and b; Fig. 3.9a and b). The germinating activity of the root exudates alone was low (~20%) compared to the synthetic germination stimulant GR24 (~50%, Fig. 3.8a), perhaps due to the collection method resulting in their dilution. Spiking with GR24 was used to overcome the low germination from exudates alone. With the addition of GR24, germination of *S. hermonthica* seeds exposed to root exudates collected from IAC 165 grown with *R. irregularis* was significantly higher than when GR24 was not added to root exudates (two-way ANOVA, Tukey's MC, $p < 0.001$). Furthermore, when root exudates were spiked with GR24, germination was significantly lower in root exudates from plants grown with *R. irregularis* (two-way ANOVA, Tukey's MC, $p < 0.001$) than in root exudates collected from IAC 165 grown alone (Fig. 3.8a). However, there was still no effect of root exudate source on haustorial formation (Fig. 3.8b).

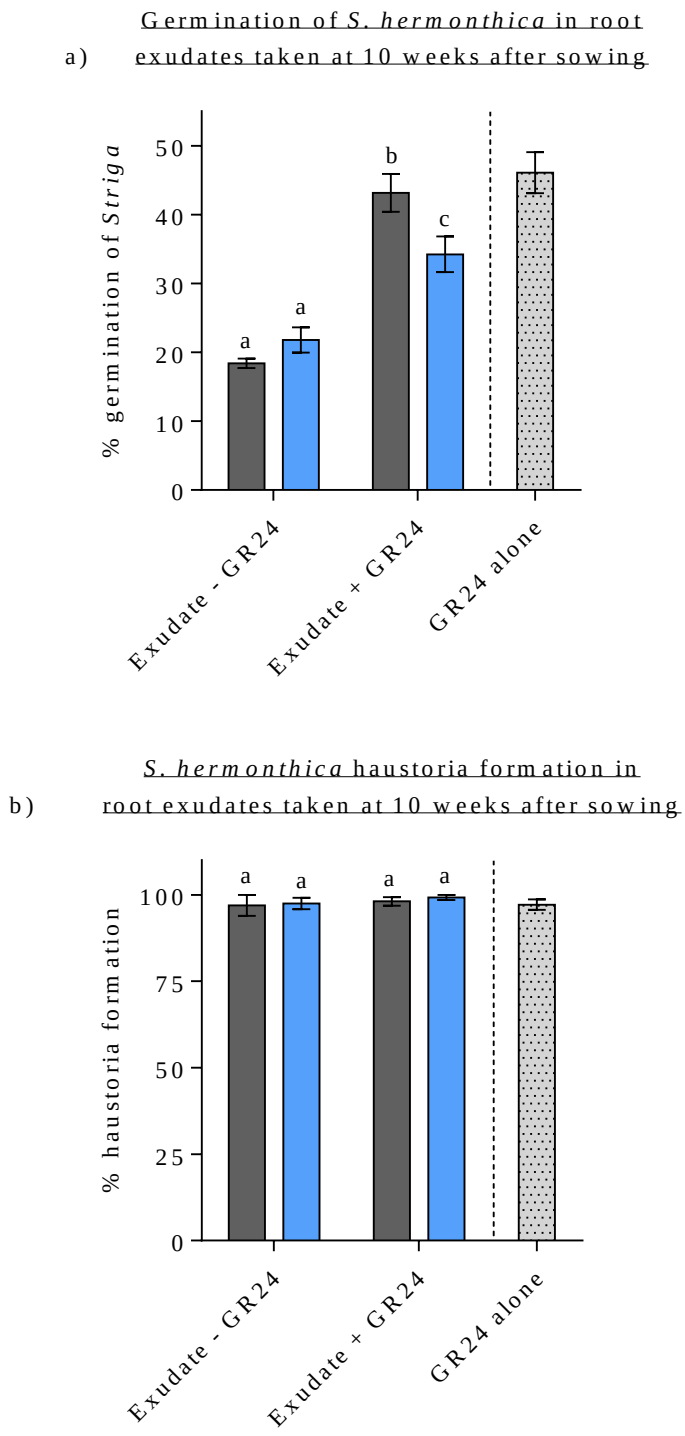


Fig. 3.8 Germination and haustorial development of *S. hermonthica* exposed to root exudates from the rice cultivar IAC 165 grown with and without *R. irregularis* supplied with high N/P. (a) and (c) Percentage germination without GR24 addition and with GR24 addition respectively. (b) and (d) Percentage haustoria formation without GR24 addition and with GR24 addition respectively. Data shown is the mean for each parameter ± SE. Columns sharing the same letters are not significantly different ($p > 0.05$, two-way ANOVA), n = 6.

a)

b)

Fig. 3.9 Representative images of germination and haustoria formation in *S. hermonthica* seeds treated with root exudates from IAC 165 grown. As shown, there was no difference in haustoria formation when treated with the two root exudate sources; a) alone b) in the presence of *R. irregularis*.

3.4 Discussion

This chapter investigated the effect of different amounts of substrate N/P levels on host colonisation and infection levels by *R. irregularis* and *S. hermonthica*, alone and in combination, and subsequent effects on host nutrient status, growth and development. Firstly, I hypothesised that decreasing N/P supply would enhance colonisation/infection of both symbionts because both are known to be enhanced by hosts under nutrient-limited, primarily via the release of hyphal branching / germination signals. Secondly, I hypothesised that decreasing N/P supply would limit the ability of the host plant to support an enhanced level of *S. hermonthica* infection in the presence of *R. irregularis* as was shown in chapter 2. My results did not show an increase in the recruitment of AM fungi, but there was evidence of an increase in the number of *S. hermonthica* after taking into account differences in root biomass. Furthermore, numerous contrasts between this experiment and the results of chapter 2 have shown the importance of symbiont competition for host resources, and the ability of hosts to meet these demands. Unlike in chapter 2, *R. irregularis* did not enhance host nutrient status or growth under either nutrient regime. Furthermore, the fungus showed some ability to reduce parasite growth, but did not alter *S. hermonthica* number or alleviate the negative effects of *S. hermonthica* on host growth and development.

In this chapter, I first hypothesised that decreasing N/P supply would enhance colonisation/infection, based on the fact that plants release more branching/germination stimulants from their roots under nutrient-limited conditions (Yoneyama et al., 2007a, Yoneyama et al., 2007b, Yoneyama et al., 2012, Lopez-Raez et al., 2008, Jamil et al., 2012) and that increased colonisation/infection by AM fungi and *Striga* is associated with low nutrient conditions in the field (Mader et al., 2000, van der Gast et al., 2011, Oswald, 2005). However, colonisation by *R. irregularis* was not enhanced under the low N/P treatment. Colonisation was lower in plants supplied with low N/P at the first harvest, but then it was equivalent in both nutrient treatments by the second harvest. This is probably because the low N/P nutrient supply was so low that the plants were so nutrients stressed that they could not dedicate resources towards the recruitment and maintenance of mycorrhizal

colonisation. Table 3.1 shows that the low N/P treatment in this chapter supplied plants with an order of magnitude less N/P than plants in the high N/P treatment and plants in chapter 2, whereas supply to plants in the high N/P treatment and plants in chapter 2 is more comparable. If a less extreme decrease in nutrient supply was used in the low N/P treatment, then this may have resulted in an increase in colonisation because the plant would still be healthy enough to dedicate resources towards establishing and maintaining colonisation.

Table 3.1 Summary table of the total cumulative nutrient supply and tissue content of the rice cultivar IAC 165 grown alone (control), and the colonisation/infection of IAC 165 grown with *R. irregularis* or *S. hermonthica* alone and in combination after 10 weeks in chapters 2 and 3.

	Chapter 2 (+AM+*Striga*)	**Chapter 3 High N/P (+AM+*Striga*)**	**Chapter 3 Low N/P (+AM+*Striga*)**
Total N supply (mg)	236.32	177.24	19.20
Total P supply (mg)	1.26	0.95	0.08
Leaf N (mg/g)	43.99	26.66	24.04
Root N (mg/g)	13.68	9.17	5.13
Leaf P (mg/g)	0.43	0.68	0.62
Root P (mg/g)	0.41	0.24	0.29
% AM colonisation	13.67 (1.5*)	34.25 (12.33*)	28.67 (11.08*)
Number of *S.hermonthica*	10.25 (26*)	18.75 (20.63)	9.875 (14.63)
S. hermonthica dry weight (mg)	273.63 (676.63*)	220.67 (379.84)	18.66 (62.54)

Similar to AM colonisation, decreased N/P supply did not increase the number and biomass of *S. hermonthica* parasites. Indeed, the number was lower in plants in the low N/P treatment. This is probably due to the low root growth of the highly nutrient stressed plants in the low N/P treatment resulting in few areas of root-parasite contact. Furthermore, plants supplied with low N/P were not able to support the growth of the parasite. Normalisation of *S. hermonthica* number per unit of root mass resulted in a higher number (but not biomass) of parasites in plants supplied with low N/P. This normalisation should be treated with caution because it assumes that the number of *S. hermonthica* increases with root mass alone regardless of root morphological characteristics. For example, resistance to *Striga* in maize and sorghum has been correlated with root systems with a less branched architecture (Cherifari et al., 1990, Amusan et al., 2008, Rich and Ejeta, 2008). However, this may suggest that the plants grown with low N/P did recruit more parasites due to an increase in

germination stimulant exudation as predicted, but the lack of roots prevented adequate contact with the parasite seeds to allow attachment.

3.4.1 How did mycorrhizal colonisation and host response to *R. irregularis* differ between chapter 2 and chapter 3?

Interestingly, under both nutrient regimes in this chapter, mycorrhizal colonisation was much higher in general (up to ~34%) than in chapter 2 (up to ~14%). The mycorrhizal symbiosis in chapter 2 was characterised as highly mutualistic due to the growth promotion and nutrient status enhancement of the host, which occurred despite a low level of colonisation, indicating a low C demand by the fungus. However, in this chapter, the very high level of colonisation was not accompanied by any host growth or nutrient status enhancement. In fact, growth of the highly stressed plants in the low N/P treatment was actually decreased by the fungus.

In contrast to chapter 2, these results suggest a high C demand by the fungus which, in the high N/P treatment, is being met by the host without adverse growth consequences. Between 4% and 20% of plant-fixed C can be allocated to mycorrhizal fungi alone, so AM fungi impose a significant C demand (Johnson et al., 1997). It is likely that photosynthesis in colonised plants was increased in order to meet this demand. For example, rice plants colonised by the AM fungus *Claroideoglomus etunicatum* (syn. *Glomus etunicatum*) exhibit higher net photosynthetic rate, stomatal conductance and transpiration rate than un-colonised plants (Porcel et al., 2015). However, the demand is clearly too high for plants grown with low N/P and their growth is reduced. Therefore, in contrast to chapter 2, the mycorrhizal symbiosis in chapter 3 can be characterised as commensal. This is not unusual, because previous studies have already shown that the effect of AM colonisation on host growth is known to vary on a spectrum between mutualism and parasitism (Klironomos, 2003).

Although it was not the original intention of this chapter to test the effect of a different fungal isolate on the interaction, it appears that the isolate of *R. irregularis* used (isolate 09) behaves very differently to the commercial isolate used in chapter 2. Indeed, AM fungi are known to be highly variable between species and even

between different isolates (Munkvold et al., 2004, Kivlin et al., 2011). For example, Mensah et al. (2015) investigated the effect of 31 isolates from 10 species of AM fungi on P and N nutrition in *Medicago sativa* and identified a range of performances in terms of host growth and N/P benefits from isolates which had no effect to highly beneficial isolates. Furthermore, it is known that *R. irregularis* genotypes vary between low-nutrient grassland environments and arable fields possible representing different adaptations to these environments (Borstler et al., 2010). This difference in isolate performance appears to be true for the isolates of *R. irregularis* used in chapter 2 and this chapter. Clearly, this isolate enforced trade on the host plants in this chapter even when the host was severely nutrient-deficient.

The isolate of *R. irregularis* used in this chapter, 09, is widely used in mycorrhizal studies because it is well established in axenic cultures, colonises to a high degree, and is known to transfer large amounts nutrients in return for large amounts of C from the plant compared to other species (Engelmoer et al., 2014). Indeed, *R. irregularis* isolate 09 has previously been shown to transfer high amounts of P and C and develop large numbers of arbuscules in comparison to the less competitive *G. aggregatum*, which reflects its preference for nutrient exchange (Kiers et al., 2011). Furthermore, Knegt et al. (2016) showed that *R. irregularis* isolate 09 outcompetes *G. aggregatum* for colonisation and aggressively colonises plants of both low (shaded) and high (non-shaded) quality. Overall, the interaction between the host and the isolate of *R. irregularis* used in this chapter is very different to that seen in chapter 2 as discussed above. As a consequence of this, the interaction of this isolate with the parasite also differed.

3.4.2 How did competition for host nutrients by the competitive AM fungus affect the interaction with S. *hermonthica*?

Growth of IAC 165 with *R. irregularis* in combination with *S. hermonthica* did not lead to an increase the number of *S. hermonthica* attachments as was seen in chapter 2. Furthermore, parasite biomass was significantly lower in plants grown with both symbionts at the first harvest. Previous studies which showed a reduction in *S. hermonthica* infection in mycorrhizal plants (Lendzemo et al., 2005, Lendzemo et al., 2007, Othira et al., 2012) attributed their effect to a reduction in germination

stimulant release from host roots as a result of enhanced nutrient status conferred by the fungus (Lopez-Raez et al., 2011). However, the reduction in biomass seen in this experiment is probably due to the competitive acquisition of C by the fungus limiting the growth of the parasite.

Parasite number was not altered by the presence of *R. irregularis* in this chapter, and mycorrhizal root exudates used in this chapter had little effect on *S. hermonthica* germination, which is supported by the fact that the fungus did not confer a nutrient benefit to the host. Interestingly, there did appear to be a slight decrease in the germination activity of mycorrhizal root exudates when the parasite seeds were induced to germinate by GR24. This may have been caused by a fungus-derived compound, which has been shown previously by the suppressive effect of germinated spore exudates collected from *R. irregularis* and *G. rosea* on *Orobanche cumana* seed germination (Louarn et al., 2012).

In chapter 2, I hypothesised that the colonisation of the host and nutrient supply by AM fungi needs to happen before *S. hermonthica* germination in order to reduce infection, and that down-regulation of host defences known to occur at the early stages of AM colonisation resulted in increased susceptibility to *S. hermonthica* seen in chapter 2. In this chapter however, it could be hypothesised that this more competitive isolate of AM fungi may colonise earlier, but since it does not improve host nutrient status, the release of germination stimulants from host roots is not altered. Furthermore, by colonising more aggressively and more quickly, the down-regulation of host defences which only occur at the early stages of AM colonisation may have occurred before infection by *S. hermonthica*, thus not enhancing the ability of *S. hermonthica* to infect. Indeed, successful AM colonisation can involve the systemic priming of JA-dependent defences (Jung et al., 2012, Van der Ent et al., 2009, Van Wees et al., 2008). Recent RNA expression analysis of *Striga hermonthica*-infected rice roots suggests the importance of a rapid JA-dependent response for resistance which is followed by the induction of SA pathways (Mutuku et al., 2015). However, since *R. irregularis* did not alter *S. hermonthica* infection in this chapter, it is unlikely that there is any host defenced-based interaction between the two symbionts. Instead, this chapter has shown that *R. irregularis* can effectively

compete with *S. hermonthica* for host resources, potentially decreasing the ability of the host to support the parasite under extremely nutrient-deficient conditions.

3.4.3 Conclusions

In this chapter, the severe reduction of growth in plants supplied with low N/P ultimately hampered the investigation of the effect of nutrient deficiency on AM colonisation and *Striga* infection individually and the effect on the interaction. However, the contrasting result for the effect of AM colonisation on *Striga* infection compared to chapter 2 has highlighted the importance of understanding the genotype and context-dependent nature of the AM symbiosis, and how this ultimately influences the ability of AM fungi to alter *Striga* infection. This matter is discussed further in the general discussion. It is unusual that, so far in this thesis, AM fungi have not been shown to suppress *Striga* infection, and this begs the question of what genotypes, environmental conditions, and symbiont arrival times at the host root are required to produce this effect. Critically, both chapters 2 and 3 have not yet explored the role that the order and timing of colonisation/infection plays in determining the result of the interaction. This will be explored in chapter 4.

Chapter 4

How does altering the order of infection influence the outcome of the competitive interaction between *Striga hermonthica* and *Rhizophagus irregularis*

4.1 Introduction

Chapters 2 and 3 both involved pot experiments in which *R. irregularis* and *S. hermonthica* were added to the pots together and left to colonise/infect as the host roots grew down through the substrate. In this setting, the timing of colonisation/infection depends on a number of factors; the rate of root growth through the substrate to make contact with the parasite seeds, the release of hyphal branching and germination ques from the host roots, the rate of hyphal branching by *R. irregularis*, the rate of germination by *S. hermonthica*, and then the ability of both symbionts to colonise/infect the host. The parasite seeds used in the pot experiments in chapters 2 and 3 were not pre-conditioned or pre-germinated. *Striga* seed conditioning can take days, with optimal germination occurring after about 10 days (Matusova et al., 2004), so it would have been reasonable to assume that the fungus would colonise first or at least with roughly equal timing to the parasite. Despite this knowledge, the order of colonisation/infection was the major unknown factor in the previous two chapters. However, based on their effects on each other and on the host plant, I made hypotheses regarding the order of symbiont arrival and their effects on host physiology. Knowing more about how the order of symbiont arrival alters their success will tell me more about the physiological mechanisms underlying the interaction. Thus, in this chapter, I will manipulate the order of colonisation/infection and measure symbiont success and distribution on the rice root system.

4.1.1 Why is the order of colonisation/infection important in the interaction between AM fungi and *Striga*?

In chapter 2, I hypothesised that the transient, localised down-regulation of host defences known to occur during the early stages of colonisation by AM fungi was responsible for increasing *S. hermonthica* infection, and that for this to occur, both symbionts would need to be colonising/infecting the host plant at similar times. In chapter 3, it was hypothesised that *R. irregularis* colonised before *S. hermonthica* and thus did not enhance *S. hermonthica* infection because the transient, localised suppression of plant defences had already occurred. The competitive nature of the *R. irregularis* isolate (09) used in chapter 3 was evident due to its high colonisation rate in comparison to the commercial isolate used in chapter 2. Furthermore, the

commensal effect of the 09 isolate, and the fact that it appeared to have some suppressive effect on *S. hermonthica* biomass early in the experiment, demonstrated that it imposed a high C demand on rice. Overall, the evidence from the previous chapters in this thesis suggests that the order of arrival is critical for determining the outcome of the interaction. Ultimately, the effect of early arrival of one symbiont on the success of the other is difficult to predict because of the numerous individual effects that both symbionts have on host physiology. These vary both spatially and temporally, and are briefly summarised for each symbiont below.

For AM fungi, spores close to host roots can germinate and extraradical hyphae can interact with host roots in a matter of days (Fig. 4.1a). The extraradical presence of AM fungi is well known to induce multiple changes in the host plants even before hyphal contact is made. For example, fungal signalling factors; mycorrhizal (Myc) factors, lipo-chitooligosaccharides (LCOs) and chito-oligosaccharides (COs) induce a range of host responses including calcium spiking (Kosuta et al., 2003, Genre et al., 2013) and the expression of symbiosis-related genes (Czaja et al., 2012).

As the fungus makes contact with the root, detection of fungal MAMPs such as chitin by the host plant can initiate SA-dependent defences (Blilou et al., 1999, Blilou et al., 2000a, Blilou et al., 2000b). However, the early stages of successful AM colonisation involve the transient, localised suppression of the initial host defence response (Herrera Medina et al., 2003, Herrera-Medina et al., 2007). For example, an effector protein (SP7) has been identified in *R. irregularis* during colonisation of *Medicago truncatula* roots which is secreted by the fungus and delivered to the host cell nucleus, where it binds to and inhibits the pathogenesis-related transcription factor, ERF19 (Kloppholz et al., 2011). Furthermore, AM fungi may induce abscisic acid (ABA) synthesis in the host to suppress the initiation of SA-dependent defences (Herrera Medina et al., 2003, Mohr and Cahill, 2007a). However, although ABA can suppress SA-dependent defences, it can also promote defence mechanisms systemically including cell-wall strengthening (Ton et al., 2009).

Successful colonisation and the formation of arbuscules by *R. irregularis* can take 2 – 3 weeks in rice (Fig. 4.1c). Successful colonization of the host by AM fungi can then result in long term systemic priming of host JA- and ethylene-dependent

defences (Jung et al., 2012, Van der Ent et al., 2009, Van Wees et al., 2008). Successful colonisation leads to the exchange of nutrients (N and P) provided by the fungus in return for plant C. While this can lead to enhancement of host nutrient status in mutualistic interactions (as seen in chapter 2), the C demand, which can be as much as 20% of host photosynthate (Jakobsen and Rosendahl, 1990), can lead to commensal effects on host nutrient status and growth (as seen in chapter 3).

For *Striga*, a major factor in the length of time taken to infect can be conditioning time (Matusova et al., 2004). However, to bypass this variable, and to investigate the post-attachment mechanisms involved in *Striga* infection, seeds can be pre-germinated using the artificial germination simulant GR24 (Gurney et al., 2006). As shown in Fig. 4.1c, after the application of germinated *S. hermonthica* seeds to a susceptible cultivar such as the one used in this thesis, IAC 165, it takes less than a day for the parasite to attach (Gurney et al., 2006),

Effects of *Striga* on host defences have been shown to involve both SA and JA induction (Hiraoka and Sugimoto, 2008, Swarbrick et al., 2008, Mutuku et al., 2015). A recent study has suggested that resistance involves a rapid induction of JA 3 days after infection, which is accompanied by gradual accumulation of SA (Mutuku et al., 2015). As with AM fungi, successful infection by *S. hermonthica* of susceptible hosts like the rice cultivar IAC 165 requires the suppression of host defences, as shown in chapter 2 and for example by Swarbrick *et al*., (2008). Suppression of host defence responses may include the suppression of hypersensitive response (HR), the deposition of cell wall physical barriers such as lignin, and the accumulation of toxic phenolic compounds derived from phenylpropanoid metabolism which can prevent parasite ingress at the root cortex (Perez-De-Luque et al., 2008) and endodermis (Perez-De-Luque et al., 2005).

Localised defence suppression by *Striga* may be mediated by effector secretion in a similar manner to successful AM colonisation and compatible interactions between host plants and pathogens. For example, as the *Striga* haustorium penetrates the host root cortex, the parasite uses plant cell wall degrading enzymes (PCWDEs) such as pectate lyases and expansin proteins to degrade and loosen host cell walls (Yoshida et al., 2016b, Yoshida et al., 2016a). This results in the presence of host apoplastic

material in the host symplast, where it can act as a powerful elicitor, signalling to the host that it is being wounded (Mitsumasu et al., 2015, Yoshida et al., 2016a). Plant-parasitic nematodes also use PCWDEs coupled with effector secretion to suppress subsequent host immune responses (Lozano-Torres et al., 2014, Mitsumasu et al., 2015). Therefore it is possible that *Striga* also uses effectors in a similar way (Li and Timko, 2009, Huang et al., 2012, Mitsumasu et al., 2015). After penetrating the host root, it takes about three days for a compatible parasite to establish vascular connections and begin to grow by exploiting host resources, as shown in Fig. 4.1d (Gurney et al., 2006).

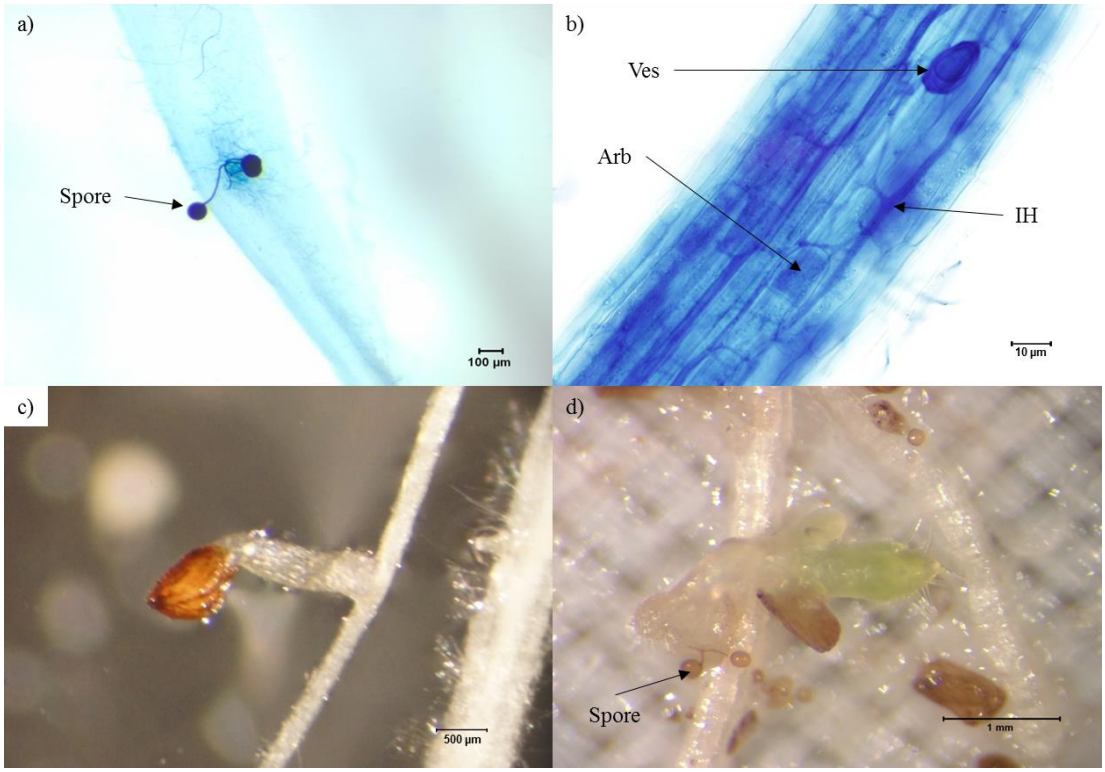


Fig. 4.1 (a) *R. irregularis* isolate 09 Spores germinating near roots 3 days after colonisation (DAC). Intraradical colonisation is not seen at this time point. (b) Root length intraradical colonisation reaches a level of about 25% after 2 weeks, and includes extensive vesicle and arbuscule formation (c) Attached *S. hermonthica* 3 days after infection (DAI). (d) Attached and growing *S. hermonthica* 6 DAI. For comparison, 6 days of colonisation by *R. irregularis* only produces at most one or two roots in a whole root system with intraradical hyphae but no arbuscules or vesicles. In this particular image, *R. irregularis* was also applied to the roots; spores of the fungus can be seen on the same root as the parasite.

The effects of both AM fungi and *Striga* infection are summarised in Table 4.1. In summary, both *R. irregularis* and *S. hermonthica* can have almost immediate effects on host root physiology, so early arrival by one could have a significant effect on the outcome of the interaction. Both need to suppress host defences local to the point of entry in order to successfully colonise/infect, so a synergistic relationship could be expected upon co-colonisation/infection at similar areas of the host root. However, both can also induce and prime plant defences, potentially at both local and systemic levels, so an antagonistic effect could also be predicted in the case of early arrival by one of the symbionts. Finally, both are obligate symbionts that require resources from the host plant. Thus, effective competition for resources by one symbiont, particularly if it arrives early and establishes symbiosis, could restrict the growth of the invader.

Table 4.1 Summary table of the colonisation/infection process by AM fungi and *Striga* respectively. Overlap between symbiont effects on host physiology including defence regulation and nutrient demand present the possibility of both synergistic or antagonistic effects of the interaction on symbiont success. This could be dependent on depending on the order and timing of symbiont arrival.

Time

AM colonisation	*Striga* infection
Host N/P deficiency	
SL exudation	
AM hyphal branching	*Striga* seed germination
Pre-symbiotic signalling	
Fungal Myc factor, CO and LCO release	*Striga* H_2O_2 and peroxidase release
Host symbiosis-specific gene expression	HIF production
Fungal hyphopodium differentiation	*Striga* haustoria formation
Contact with and penetration of the host root	
Host detection of fungal MAMPs	Host detection of wounding by *Striga*
Transient SA accumulation	Early JA defence induction in less-susceptible hosts
Induction of ABA synthesis	High ABA in parasite and host
Localised suppression of plant defences	
Established colonisation/infection	
Systemic signalling - ABA/JA?	Host stomatal closure - ABA
Systemic priming of JA & ET	Sustained suppression/induction of defences
Sugars mobilised to AMs	Water/nutrients towards parasite

4.1.2 Could a priority effect be established by early arrival of either AM fungi or *Striga*?

Judging from what I have observed so far in this thesis, and also from observations made in the literature, both *S. hermonthica* and *R. irregularis* isolate 09 clearly enter into a highly compatible and competitive interaction with the host plant and each other. Both symbionts are therefore highly adept at altering host physiology to their own ends, which includes suppressing host defences, and also obtaining host resources once established. Because of this, in the present chapter I hypothesise that the first symbiont to arrive at the host will establish a priority effect thus suppressing the success of the invading symbiont.

Priority effects describe the impact of the early arrival of one species on the success of late-arriving species (invaders). Some research on priority effects due to the order of host interaction between symbionts has already been carried out. For example, Werner and Kiers (2015) investigated the effect of order and timing of arrival on colonisation between two AM fungi, *R. irregularis* and *G. aggregatum*. It was found that the pre-colonising fungus suppressed the invading species after a 4-week head start, but *R. irregularis* only need 2 weeks to suppress *G. aggregatum*. Also it was found that the invading species did not suppress the established species, but upon co-colonisation, *R. irregularis* outcompeted *G. aggregatum*. The authors hypothesised that the host plant may suppress the second invader (Werner and Kiers, 2015). This hypothesis is supported by results from split-root experiments, where colonisation of one half of a root system suppresses colonisation of the other half by AM fungi colonised after (Vierheilig, 2004). Split-root experiments also show a systemic effect of AM colonisation on other areas of the host root. Furthermore, it appeared that a colonisation threshold needs to be achieved in order to suppress colonisation by a late arrival (Vierheilig, 2004, Werner and Kiers, 2015). In another study, Chavez-Calvillo et al. (2016) investigated the effect of order of infection on synergism and antagonism between papaya ringspot virus (PRSV) and papaya mosaic virus (PapMV) on papaya (*Carica papaya*) host plants. It was found that synergism occurred when PRS infects first or at the same time as PapMV, whereas antagonism occurred when PapMV infects first. The study also showed that PapMV activates

host defences against PRSV resulting in protection from PRSV infection (Chavez-Calvillo et al., 2016).

For me to study priority effects using AM fungi and *Striga* in this chapter, symbiont arrival time needs to be controlled by having easy, non-destructive access to the host root system. Rhizotron systems (Fig. 4.2) are ideal for this because they allow plants to be grown in a soil-free environment and for the roots to be freely observed (Gurney et al., 2006). For parasitic plant research, this is particularly useful because the development of infection can be observed non-destructively over time and also allows the harvesting of root and parasite material rapidly and without damage due to the removal of soil. Rhizotron systems like the one shown in Fig 4.2 have been developed and used extensively to investigate *Striga* parasitism at the University of Sheffield (Gurney et al., 2002, Gurney et al., 2003, Gurney et al., 2006). However, they have not yet been used to provide soil free root systems to study the interaction between *R. irregularis* and *S. hermonthica*.

Fig. 4.2 Rice growing in a rhizotron 33 days after rice seed germination and 21 days after infection with pre-germinated *S. hermonthica* seeds.

The aim of chapter 4 was to determine whether the order of colonisation/infection affects the success and spatial distribution of either symbiont.

Hypothesis:

The first symbiont to colonise/infect the host plant will suppress infection by the other because early arrival will establish a priority effect via competition for space, resources and effects on host defences which may act antagonistically on the invading symbiont.

Objectives:

1) Grow, colonise and infect the rice cultivar IAC 165 with *R. irregularis* isolate 09 and pre-germinated *S. hermonthica* in rhizotrons.
2) Co-colonise/infect with *R. irregularis* and *S. hermonthica* at the same time.
3) Pre-colonise with *R. irregularis* for five days before infecting with *S. hermonthica* for 14 days.
4) Pre-infect with *S. hermonthica* for five days before colonising with *R. irregularis* for 14 days.
5) In a separate experiment, pre-colonise with *R. irregularis* 21 days before infecting with *S. hermonthica* to investigate the effect of well-established colonisation on parasite infection.
6) Measure *S. hermonthica* infection of roots colonised by *R. irregularis* and roots not colonised by *R. irregularis* separately.
7) Measure colonisation of roots infected with *S. hermonthica* and roots not infected by *S. hermonthica* separately.

4.2 Materials and methods

4.2.1 Plant and fungal materials

Plant and fungal materials were the same as those described in section 3.2.1.

4.2.2 Growth of the rice cultivar IAC 165 in rhizotrons

Rice seeds of cultivar IAC 165 were surface sterilised with 10% (v/v) bleach for 15 min followed by extensive washing with water. IAC 165 seeds were placed 1 cm below the top long edge between two wet (with de-ionised H_2O) 20 x 7 cm strips of glass-fibre filter paper (Whatman®), which themselves were then placed between two wet blocks of horticultural rockwool (growndan®Vital, UK) of equal size. The rockwool blocks were placed in a seed tray containing ~1 cm deionised water and covered with a propagator lid. IAC 165 seeds were then germinated for 5 days in a controlled environment chamber with a day/night temperature of 28 / 24°C with a 16 h photoperiod and 60% relative humidity, and an irradiance of 450 µmol s^{-1} m^{-2} at plant height.

After 5 days of germination, IAC 165 seeds were transferred to a root observation chamber (rhizotron) as described by Gurney et al. (2006). A rhizotron consisted of a modified 25 x 25 x 2 cm Perspex tissue culture plate with a hole in the top to allow for growth of the rice stem and access of nutrient solution and a hole in the bottom to allow for drainage of excess nutrient solution. The rhizotron was packed with moist vermiculite with a rockwool block at the base to prevent the loss of vermiculite. A dampened square polyester mesh (Plastok Group, Birkenhead, UK) with a 100 µm pore size was placed on top of the vermiculite to provide a surface for root growth, *R. irregularis* inoculum and *S. hermonthica* seed application. After transferring germinated IAC 165 seeds to the rhizotron, the rhizotron cover was taped in place, and the rhizotron was covered in aluminium foil to prevent root exposure to light. Rhizotrons were then returned to the controlled environment chamber. Each rhizotron was watered two times a day using an automatic dripper system delivering a total of 30 ml per day of modified 40% Long Ashton solution containing 35 µM P and 0.5 mM of ammonium nitrate (Fig. 4.3).

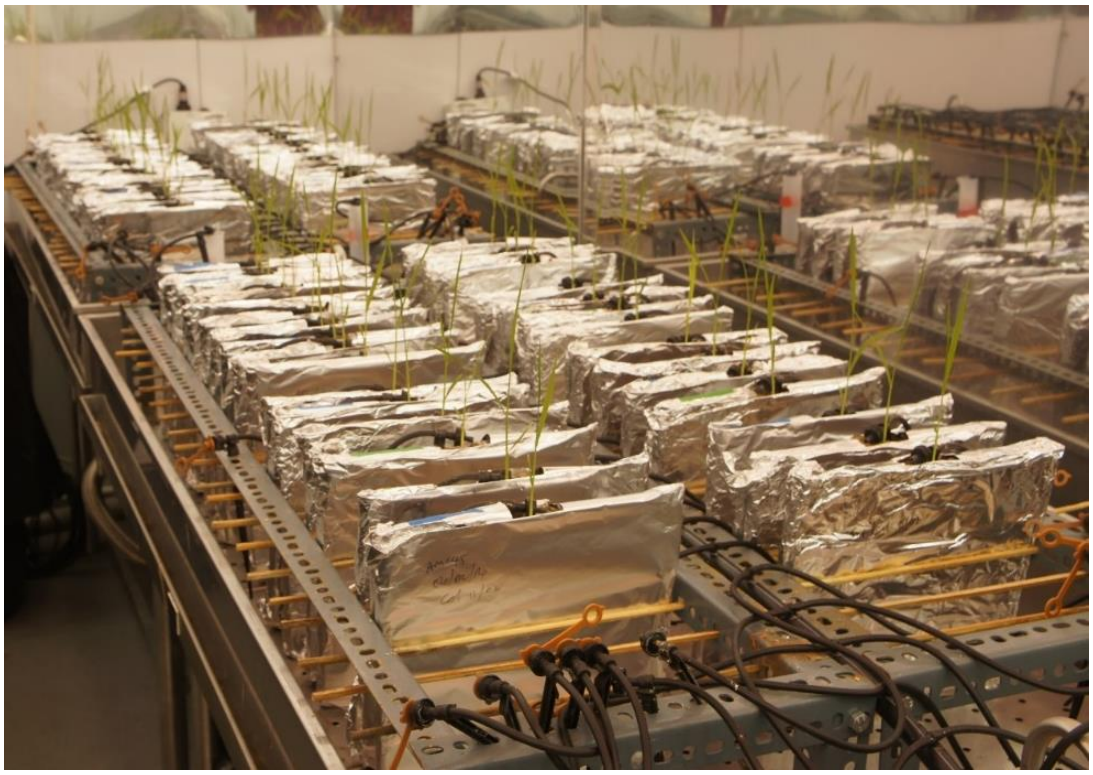


Fig. 4.3 Rice cultivar IAC 165 growing in rhizotrons in the controlled environment chamber. Nutrients were supplied by the automatic drippers inserted into the top of each rhizotron.

4.2.3 Colonisation/infection of IAC 165 in rhizotrons with *R. irregularis* and *S. hermonthica*

The *R. irregularis* isolate 09 inoculum was prepared as described in section 3.2.1. On the day of colonisation, 10 ml of the spore suspension was pipetted onto the roots resulting in 4000 spores per rhizotron. *S. hermonthica* seeds were surface sterilised in 10% sodium hypochlorite solution for 4 min before being washed into a Ø90 mm glass-fibre filter paper disc (Whatman®) fashioned into a funnel and rinsed thoroughly with demineralized water. *S. hermonthica* seeds require preconditioning before germination (Matusova et al., 2004). Seeds were preconditioned on moistened glass-fibre filter paper in a 90 mm diameter petri dish sealed with parafilm at 30 °C in darkness for 14 days. After preconditioning, *S. hermonthica* seeds were germinated in petri dishes using 3 ml a 0.1 mg L^{-1} solution of the artificial germination simulant GR24 or 16 hours overnight in order to promote synchronous

germination and attachment to host roots (Gurney et al., 2006). Germination rates were between 60 and 70%. On the day of infection, plants were infected with 12.5 mg of seeds by aligning them along the roots using a paint brush. The rice root system is made up of three main types of root; crown roots, large lateral roots and fine lateral roots (Hochholdinger et al., 2004, Rebouillat et al., 2009). Crown roots are the thickest and grow straight down in rhizotrons, large lateral roots grow horizontally and downwards from the crown roots, and fine lateral roots grow from both crown and large lateral roots agravitropically (Hochholdinger et al., 2004, Rebouillat et al., 2009). AM fungi predominantly colonise the large lateral roots but also colonise crown roots, whereas fine lateral roots are not colonised (Gutjahr et al., 2009). To encourage interaction between the two symbionts, large lateral roots were preferentially infected with *S. hermonthica* and fine lateral roots were avoided.

4.2.4 Experimental timeline for co-colonisation/infection and invasion after 5 days.

Roots of IAC 165 were colonised/infected either at the same time, or by giving a 5-day head start to either *R. irregularis* or *S. hermonthica* as shown in Fig. 4.4. At the point of pre-colonisation and pre-infection with the first symbiont (day 19), a subset of plants was also colonised or infected and were left until the end of the experiment to act as controls for either *R. irregularis* colonisation without the later addition of *S. hermonthica* or as a control for *S. hermonthica* infection without the later addition of *R. irregularis*. Co-colonised/infected plants were colonised/infected on the same day as the invading symbionts were added to the pre-colonised/infected plants (day 24). At this point, another subset of plants either colonised with *R. irregularis* alone or *S. hermonthica* alone were established and left until the end of the experiment to provide aged-matched plants for the point of colonisation/infection at day 24. Plants were then left for 14 d before harvest. Five biological replicates were established for each treatment.

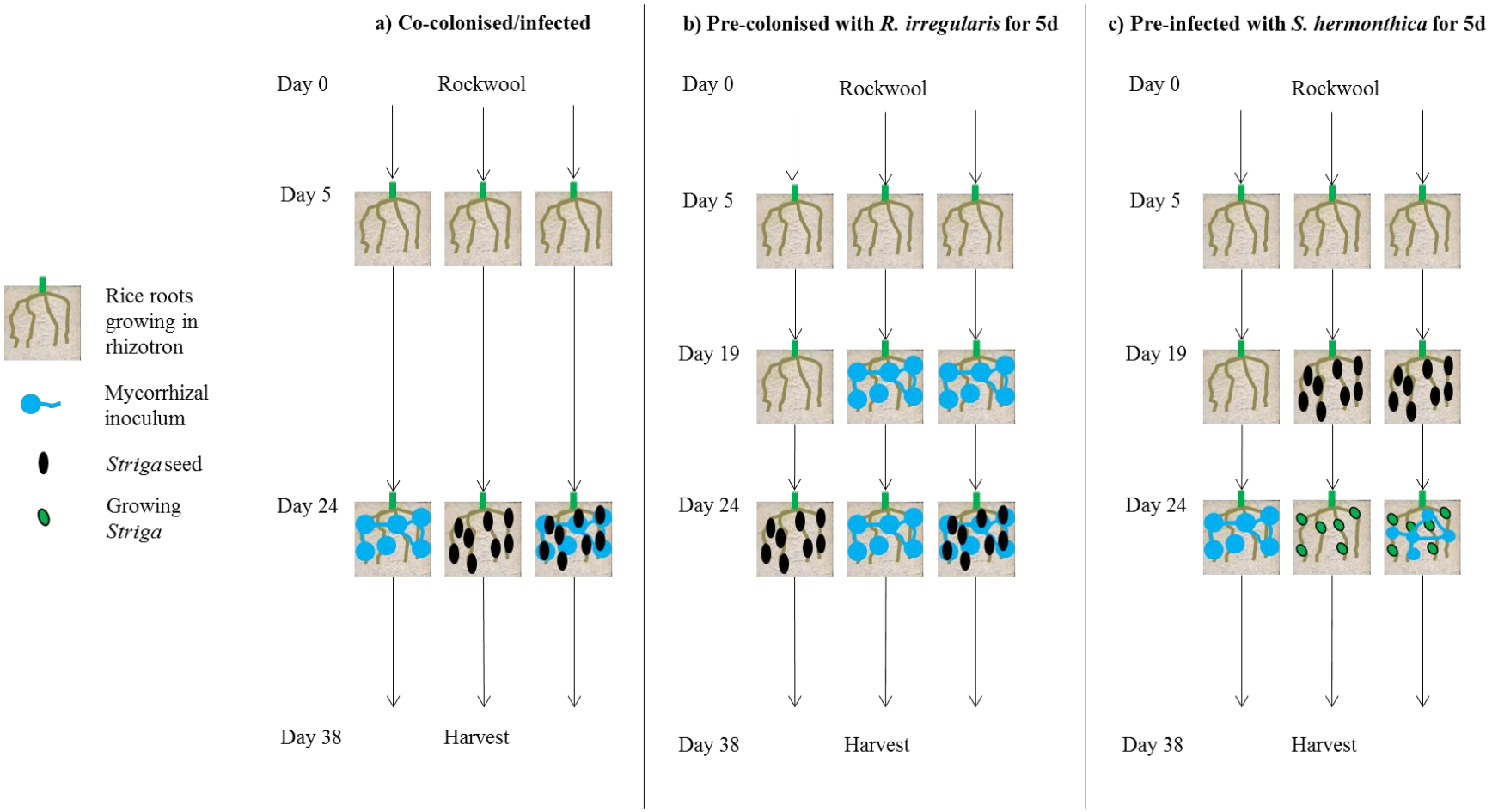


Fig. 4.4 Timeline of the 5-day pre-colonisation/infection experiment showing the times of colonisation with *R. irregularis* and infection with *S. hermonthica* in (a) IAC 165 co-colonised/infected with both symbionts simultaneously (b) IAC 165 pre-colonised with *R. irregularis* 5 days before infection with *S. hermonthica* and (c) IAC 165 pre-infected with *S. hermonthica* 5 days before colonisation with *R. irregularis*.

4.2.5 Experimental timeline for pre-colonisation with *R. irregularis* for 21 days before *S. hermonthica* invasion.

In a separate experiment, *R. irregularis* was left to colonise host roots for 21 days before *S. hermonthica* infection. After *S. hermonthica* infection, the parasite was left to infect and grow for 21 days before harvest (Fig. 4.5). Six biological reps were established for each treatment.

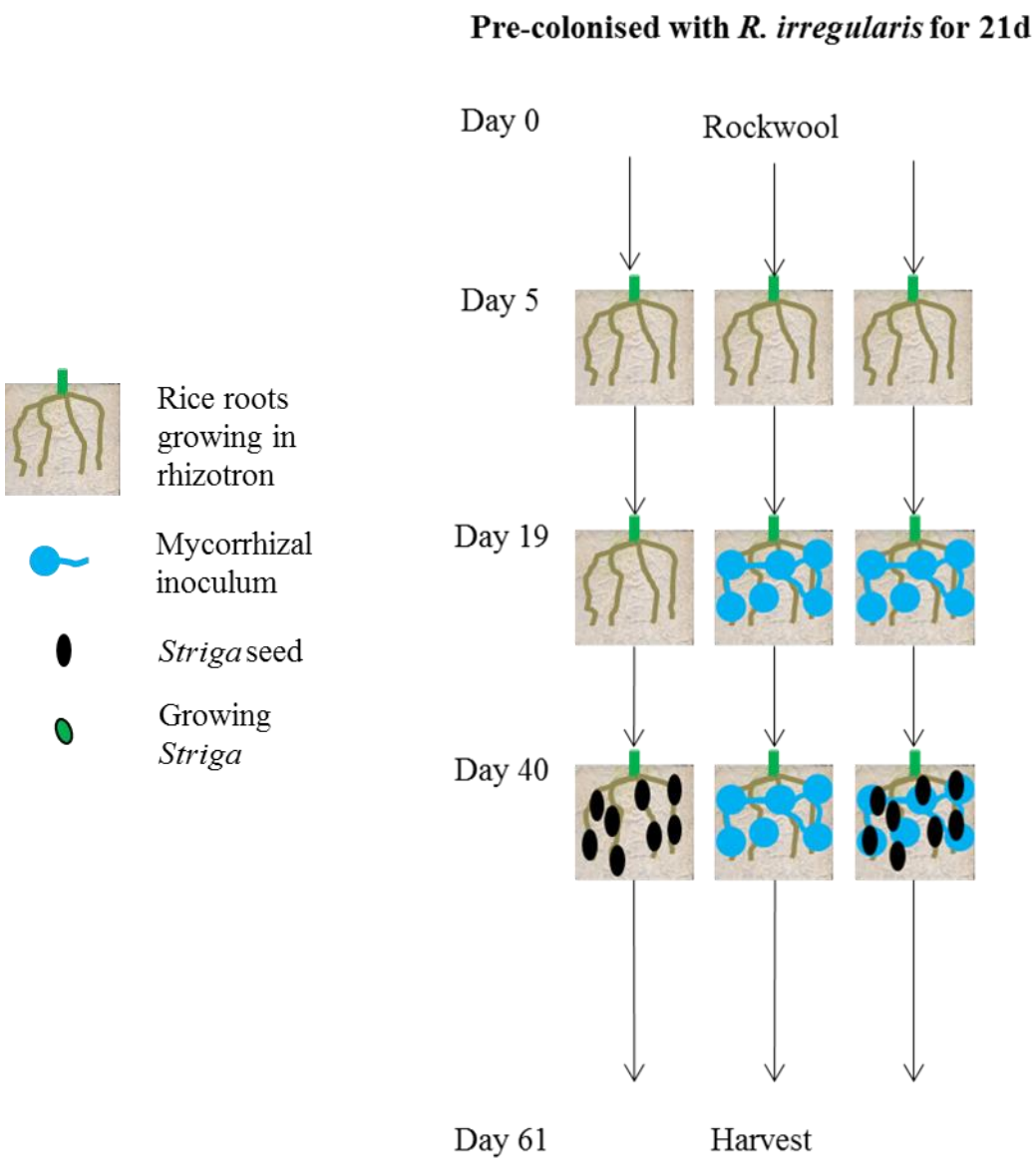


Fig. 4.5 Timeline of the 21-day pre-colonisation experiment showing the time of colonisation with *R. irregularis* and infection with *S. hermonthica*. Plants were harvested 21 days after *S. hermonthica* infection.

4.2.6 Quantification of *S. hermonthica* infection

Fourteen days after the final colonisation/infection stage, individual *S. hermonthica* parasites were harvested from the roots of *S. hermonthica*-infected plants, placed in petri dishes and photographed using a Canon EOS 300D digital camera. The number and length of *S. hermonthica* from each plant was determined from the petri dish photographs using ImageJ 1.45s (1.4.3.67) software. *S. hermonthica* plants were then oven dried at 48 ^{o}C for 3 days for the determination of dry weight per host plant.

4.2.7 Staining of roots and quantification of mycorrhizal colonisation

From plants which were colonised by *R. irregularis* and infected by *S. hermonthica*, roots from which *S. hermonthica* individuals were harvested were kept separate from roots which did not have *S. hermonthica* attachments and each subsample was preserved separately in 50 % ethanol and stained for quantification of mycorrhizal colonisation via microscopy (Fig. 4.6). Roots from plants which were not infected with *S. hermonthica* at any point in the experiment were harvested and preserved in the same way. Therefore, mycorrhizal colonisation was quantified in three different types of roots (1) Roots which were colonised by *R. irregularis* alone throughout the experiment (2) Roots which were inoculated with *R. irregularis* but which also had *S. hermonthica* attached to them (3) Roots from the same plants as (2) which did not have *S. hermonthica* attached to them at the time of harvest.

Mycorrhizal staining and quantification was carried out as detailed in section 2.2.5, except that 50 intersections between roots and grid lines were counted as the root systems were relatively small compared to those from the pot experiments in chapters 2 and 3.

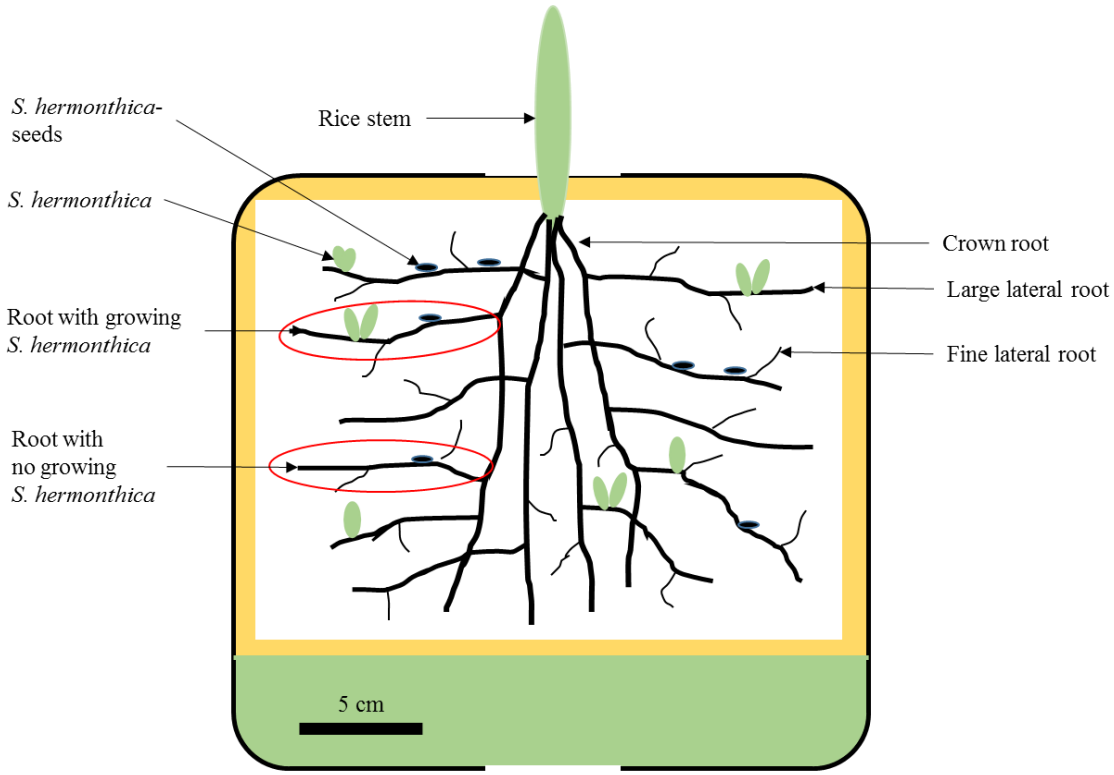


Fig. 4.6 Rhizotron system used to colonise/infect rice with *R. irregularis* and *S. hermonthica*. At harvest, rice roots which were infected by *S. hermonthica* were harvested, stained and analysed for mycorrhizal colonisation separately from root which did not have any *S. hermonthica* growing on them (red circles) in order to investigate spatial distribution trends of the two symbionts.

4.2.8 Statistical analyses

Comparisons of AM colonisation and *S. hermonthica* infection levels were made using Student's t-test in Excel. Graphs were generated using GraphPad Prism 6.

4.3 Results

4.3.1 *R. irregularis* colonisation and *S. hermonthica* infection after co-colonisation/infection and after arrival 5 days after pre-colonisation/infection.

At harvest, roots that were infected by *S. hermonthica* and uninfected roots were collected separately and the colonisation of these roots by the AM fungus assessed independently of roots that had not been infected by the parasitic plant. I observed very different colonisation patterns in these two sub sets of roots. Of the roots infected by the parasite, only a single root (representing 1 of approximately 45 roots studied that were infected with *S. hermonthica*) showed the presence of both intraradical AM structures on the same root as an attached S. hermonthica parasite (Fig.4.7d). In the rest of my samples, roots infected with S. hermonthica did not contain any fungal material (Fig 4.7 a-c). The mycorrhizal colonisation data presented in Fig. 4.8a-d therefore shows the colonisation of roots which did not have *S. hermonthica* attached to them (NB because AM colonisation of roots infected by *S. hermonthica* within the root system was essentially zero).

Fig. 4.8 shows both the *R. irregularis* colonisation and *S. hermonthica* infection of plants grown with each symbiont alone, plants co-colonised/infected with both, and plants pre-colonised/pre-infected with each symbiont for 5 days (NB these data refer to the entire root system). Fig. 4.9 shows representative pictures of rhizotrons for plants co-colonised/infected by both symbionts, plants pre-colonised by *R. irregularis* for 5 days before *S. hermonthica* infection, and plants grown with *S. hermonthica* alone.

Simultaneous co-colonisation/infection with *R. irregularis* and *S. hermonthica* and pre-colonisation with *R. irregularis* for 5 days before infection with *S. hermonthica* significantly reduced *S. hermonthica* number ($p < 0.01$), dry weight ($p < 0.01$) and total length ($p < 0.05$) compared to plants infected with *S. hermonthica* alone (Fig. 4.8a and b; Fig. a – c). Colonisation by *R. irregularis* 5 days after infection with *S. hermonthica* did not alter *S. hermonthica* number or dry weight, but did significantly reduce ($p < 0.05$) the total length of the parasite (Fig. 4.8c).

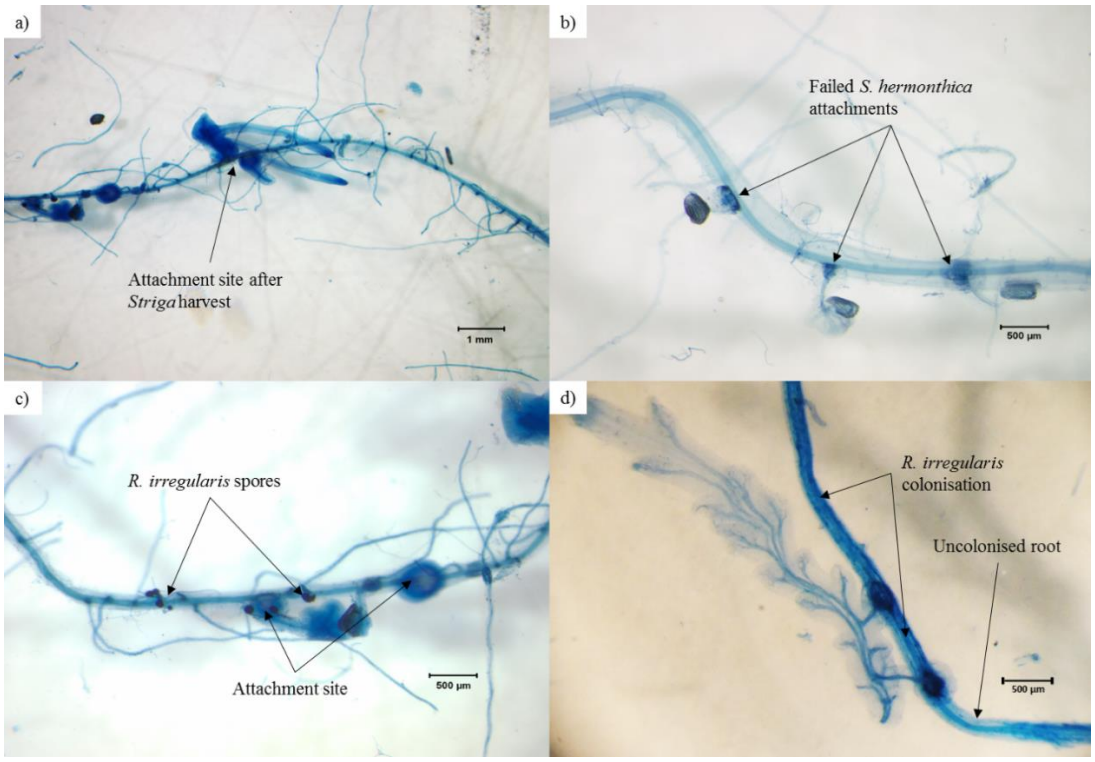


Fig. 4.7 Example images of roots from which *S. hermonthica* were harvested and which were subsequently stained for mycorrhizal colonisation. (a) The base of a successful attachment site left behind after harvesting an *S. hermonthica* parasite. (b) Failed *S. hermonthica* attachments. (c) *S. hermonthica* attachments sites and spores on the host root. None of the roots in (a) to (c) show any intraradical colonisation. (d) Only one example of was found of a root with both an *S. hermonthica* attachment site and intraradical colonisation by *R. irregularis*. This was seen in a root from a plant which had been colonised/infected simultaneously with both symbionts (co-colonised/infected). The parasite had made two attachments to the host root, and intraradical colonisation including arbuscules formation was observed in the root in between and upstream of the attachment sites away from the root tip, whereas there was no colonisation past the attachment sites and towards the root tip.

Mycorrhizal colonisation was also influenced by the experimental treatments with simultaneous co-colonisation/infection with *R. irregularis* and *S. hermonthica* and pre-infection with *S. hermonthica* 5 days before colonisation by *R. irregularis* significantly reducing *R. irregularis* % root length colonisation ($p < 0.01$), % vesicle ($p < 0.01$) and % arbuscule ($p < 0.01$) presence (Fig. 4.7a and c). However, infection with *S. hermonthica* 5 days after colonisation by R. irregularis did not alter *R. irregularis* colonisation (Fig. 4.8b). Mycorrhizal roots had a high abundance of arbuscules similar to those seen in chapter 3 (section 3.3.3 Fig. 3.6 in chapter 3 and Fig. 4.10 in this section).

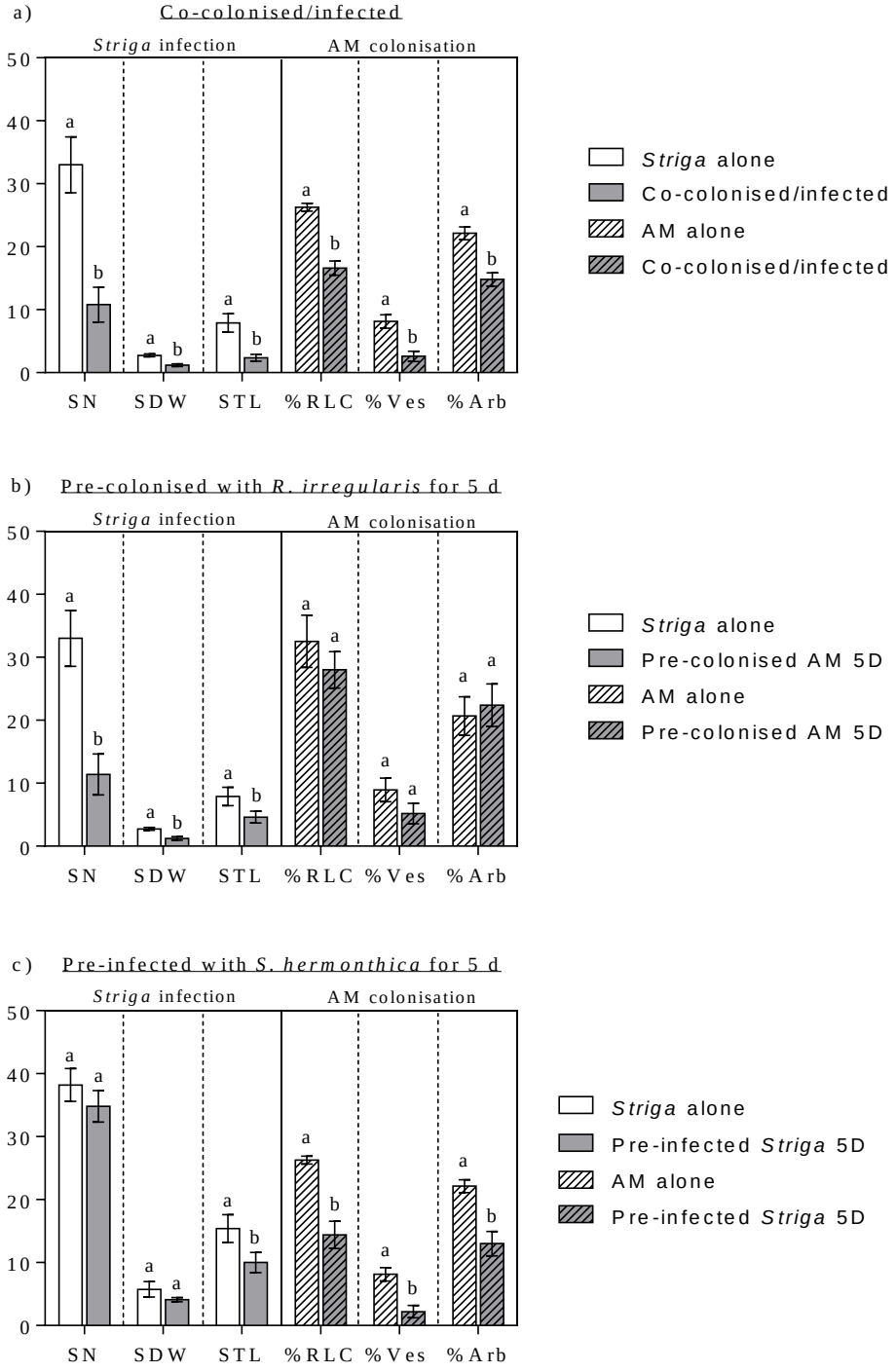


Fig. 4.8 *R. irregularis* colonisation and *S. hermonthica* infection in the three orders tested. (a) Simultaneous colonisation/infection (co-colonised/infected) (b) Pre-colonisation with *R. irregularis* for 5 days before *S. hermonthica* infection (c) Pre-infection with *S. hermonthica* for 5 days before *R. irregularis* colonisation. SN = *S. hermonthica* number, SDW = *S. hermonthica* dry weight, STL = *S. hermonthica* total length, RLC = root length colonisation (total), Ves = vesicles, Arb = arbuscules. Data shown is mean *S. hermonthica* number, dry weight and total length, and mean total root length, vesicle, and arbuscule colonisation ± SE. Columns sharing the same letters are not significantly different ($p > 0.05$, Student's t-test), n = 5.

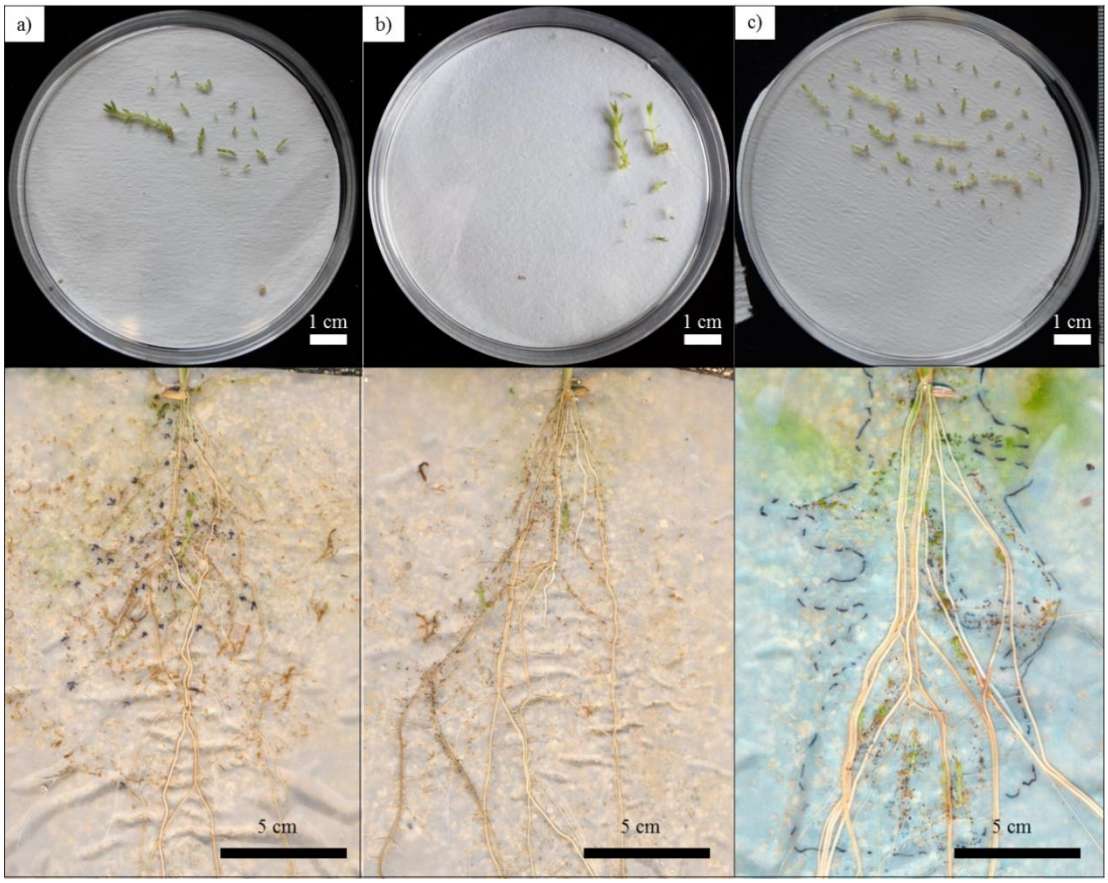


Fig. 4.9 *S. hermonthica* harvested from the roots of IAC 165 after 14 days when (a) co-infected with *R. irregularis* (b) infected 5 days after colonisation by *R. irregularis* (c) infected without the addition of *R. irregularis*.

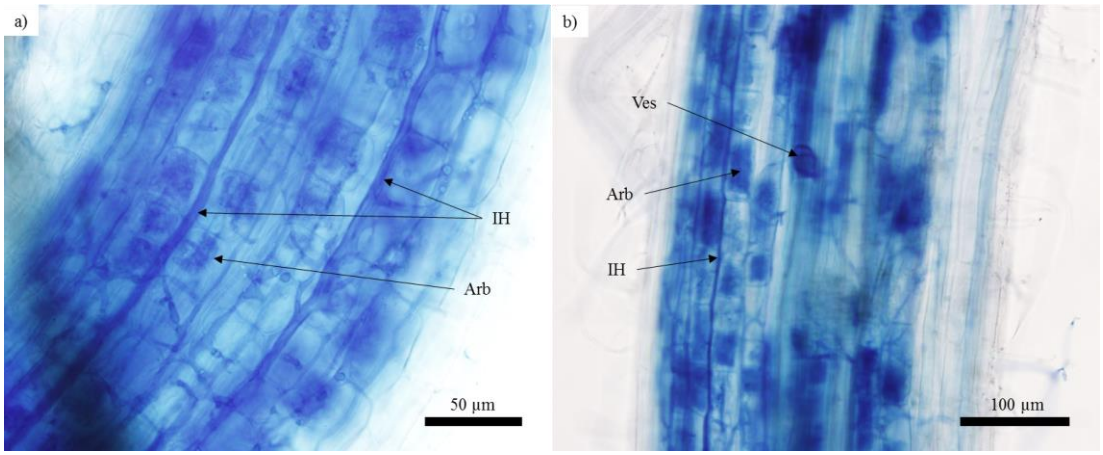


Fig. 4.10 Mycorrhizal colonisation 2 weeks after inoculation was 25-30% in the absence of *S. hermonthica* or if roots were colonised by *R. irregularis* 5 days before *S. hermonthica* infection. Colonisation was characterised by a high level or arbuscules in comparison to vesicles.

In summary of the results above, when *S. hermonthica* arrives first, it excludes mycorrhizal colonisation in roots that it has infected and also suppresses colonisation in un-infected roots. When *R. irregularis* arrives first, it excludes *S. hermonthica* infection of roots in which the fungus forms intraradical structures. Late arrival (invasion) by either symbiont does not lead to the suppression of the first to arrive. When both arrive at the same time, they suppress each other.

4.3.2 Pre-colonisation with *R. irregularis* for 21 days before *S. hermonthica* invasion.

In order to investigate the effect of well-established mycorrhizal colonisation on *S. hermonthica* infection, an experiment was performed where *R. irregularis* was allowed to colonise for 21 days before *S. hermonthica* was introduced to the roots.

As with the experiment in section 4.3.1 above, roots that were infected by *S. hermonthica* and roots that were not infected by *S. hermonthica* were collected separately from each plant and the colonisation of these roots by *R. irregularis* was assessed independently in these two sub sets of roots. Similar to section 4.3.1, only 2 roots from approximately 60 root pieces studied showed the presence of both intraradical AM structures on the same root as an attached *S. hermonthica* parasite. In the rest of the roots which were infected by *S. hermonthica*, there was no intraradical fungal material. Again, because of this, the mycorrhizal colonisation data presented in Fig. 4.11 shows the colonisation of roots which did not have *S. hermonthica* attachments on them (NB because AM colonisation of roots infected by *S. hermonthica* was essentially zero).

Fig. 4.11 shows *R. irregularis* colonisation and *S. hermonthica* infection of roots colonised/infected with *R. irregularis* and *S. hermonthica* alone and roots pre-colonised by *R. irregularis* for 21 days before *S. hermonthica* invasion. Infection by *S. hermonthica* 21 days after *R. irregularis* colonisation did not alter *S. hermonthica* number, dry weight or total length compared to when IAC 165 was infected by *S. hermonthica* without pre-colonisation by *R. irregularis* (Fig. 4.11). However, total % root length colonisation by the fungus ($p < 0.01$), and the presence of arbuscules ($p <$

0.01) was significantly reduced compared to when *R. irregularis* colonised without the later addition of *S. hermonthica* (Fig.4.11).

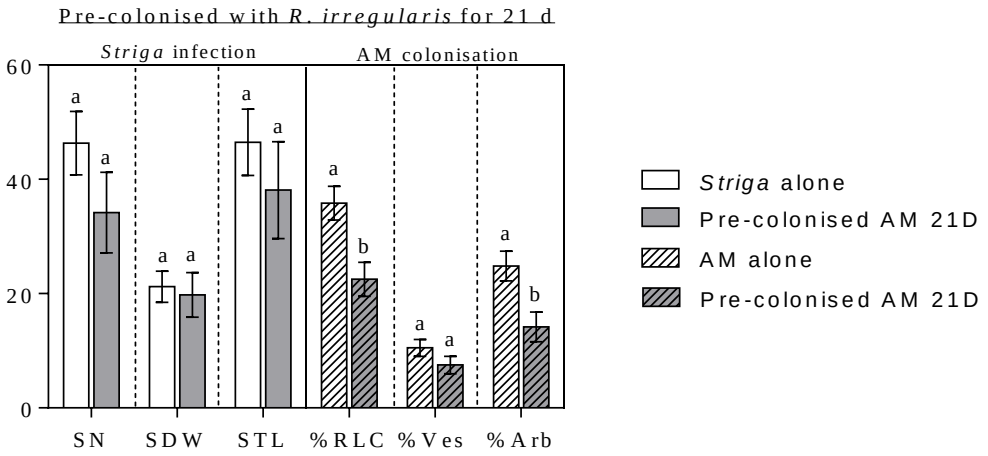


Fig. 4.11 *R. irregularis* colonisation and *S. hermonthica* infection in IAC 165 pre-colonised with *R. irregularis* for 21 days before infection by *S. hermonthica*, which was left to infect and grow on the host for 21 days before harvest. SN = *S. hermonthica* number, SDW = *S. hermonthica* dry weight, STL = *S. hermonthica* total length, RLC = root length colonisation (total), Ves = vesicles, Arb = arbuscules. Data shown is mean *S. hermonthica* number, dry weight and total length, and mean total root length, vesicle, and arbuscule colonisation ± SE. Columns sharing the same letters are not significantly different ($p > 0.05$, Student's t-test), n = 5.

4.4 Discussion

In chapters 2 and 3 of this thesis, the result of the interaction between AM fungi and *Striga* was hypothesised to be determined by the order of arrival and the downstream consequences of this on host defences and nutrient status. In this chapter I investigated how the order of infection by *R. irregularis* and *S. hermonthica* alters the relative success and spatial distribution of both symbionts by alternating their arrival order in rhizotrons and by measuring *R. irregularis* colonisation in roots with and without *S. hermonthica* attachments. Priority effects describe the impact of the early arrival of one species on the success of later arrivals. I hypothesised that the first symbiont to colonise/infect the host plant will suppress infection by the other because early arrival will establish a priority effect via competition for space, resources and effects on host defences, which may act antagonistically on the invading symbiont. In agreement with this hypothesis, pre-colonisation/infection for 5 days resulted in a clear priority effect, suppressing colonisation/infection by the invading symbiont, while simultaneous co-colonisation/infection resulted in the suppression of both symbionts. The occurrence of intraradical fungal structures and parasite attachments on the same root was exceedingly rare, showing a strong localised effect of early arrival on the incoming symbiont (Fig. 4.12).

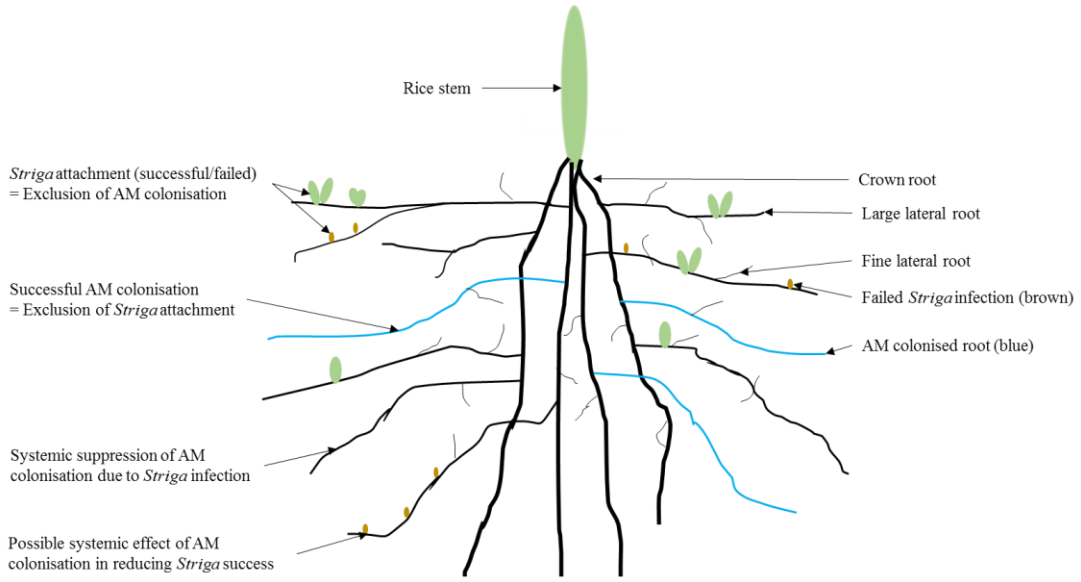


Fig. 4.12 Summary of the result of pre-colonisation with AM fungi, pre-infection with *Striga*, and co-colonisation/infection with both symbionts. The exclusion of AM fungi from *Striga*-infected roots, and *Striga* from AM-colonised roots demonstrates that there is a strong localised effect of each symbiont on host physiology. Decreased colonisation in roots not infected by *Striga* suggests a systemic effect of *Striga* infection across the root system.

4.4.1 What is the mechanistic basis for the priority effect observed for *R. irregularis*?

R. irregularis pre-colonisation for 5 days resulted in a clear priority effect which suppressed infection by the invading *S. hermonthica* parasite, but also meant that the later arrival and infection by *S. hermonthica* did not alter the established level of AM colonisation (Fig. 4.13). This is a particularly interesting result because very few roots showed any intraradical colonisation at 5 days. This suggests that pre-symbiotic signalling by the fungus suppressed *Striga* infection at the pre-attachment stages of the parasite lifecycle, which may have included adverse effect of radicle growth, haustoria formation and attachment. The parasite seeds used in this experiment were pre-germinated, so any adverse effect on parasite seed germination can be ruled out. Therefore, the mycorrhizal signalling effect may have happened in two main ways; by direct fungal effects or by indirect effects of AM fungi on host physiology.

First, *Striga* may have been suppressed directly by fungal chemicals produced during the pre-symbiotic life cycle of *R. irregularis*. The ability of AM fungi to directly suppress *Striga* after germination but before root invasion has not been demonstrated. However, compounds produced by AM fungi are thought to have a suppressive effect on germination, so there is potential for this idea. For example, Louarn *et al*. (2012) reported reduced *Orobanche cumana* seed germination specifically in the presence of germinated spore exudates from *R. irregularis* and *Gigaspora rosea*, and suggested that this was partly responsible for a moderate reduction in parasite attachments in mycorrhizal sunflower co-inoculated with both symbionts. Furthermore, fungal toxins such as phyllostictine A produced by *Phyllosticta cirsii* have been shown to reduce germination and germ tube elongation in *Orobanche ramose* and *Cuscuta campestris* (Vurro et al., 2009).

Second, *Striga* may have been suppressed by host-produced chemicals which were produced as a result of *R. irregularis* signalling. Pre-symbiotic signalling by AM fungi involves the synthesis and release of mycorrhizal (Myc) factors, chito-oligosaccharides (COs) and lipo-chitooligosaccharides (LCOs) (Kosuta et al., 2003, Genre et al., 2013, Maillet et al., 2011).

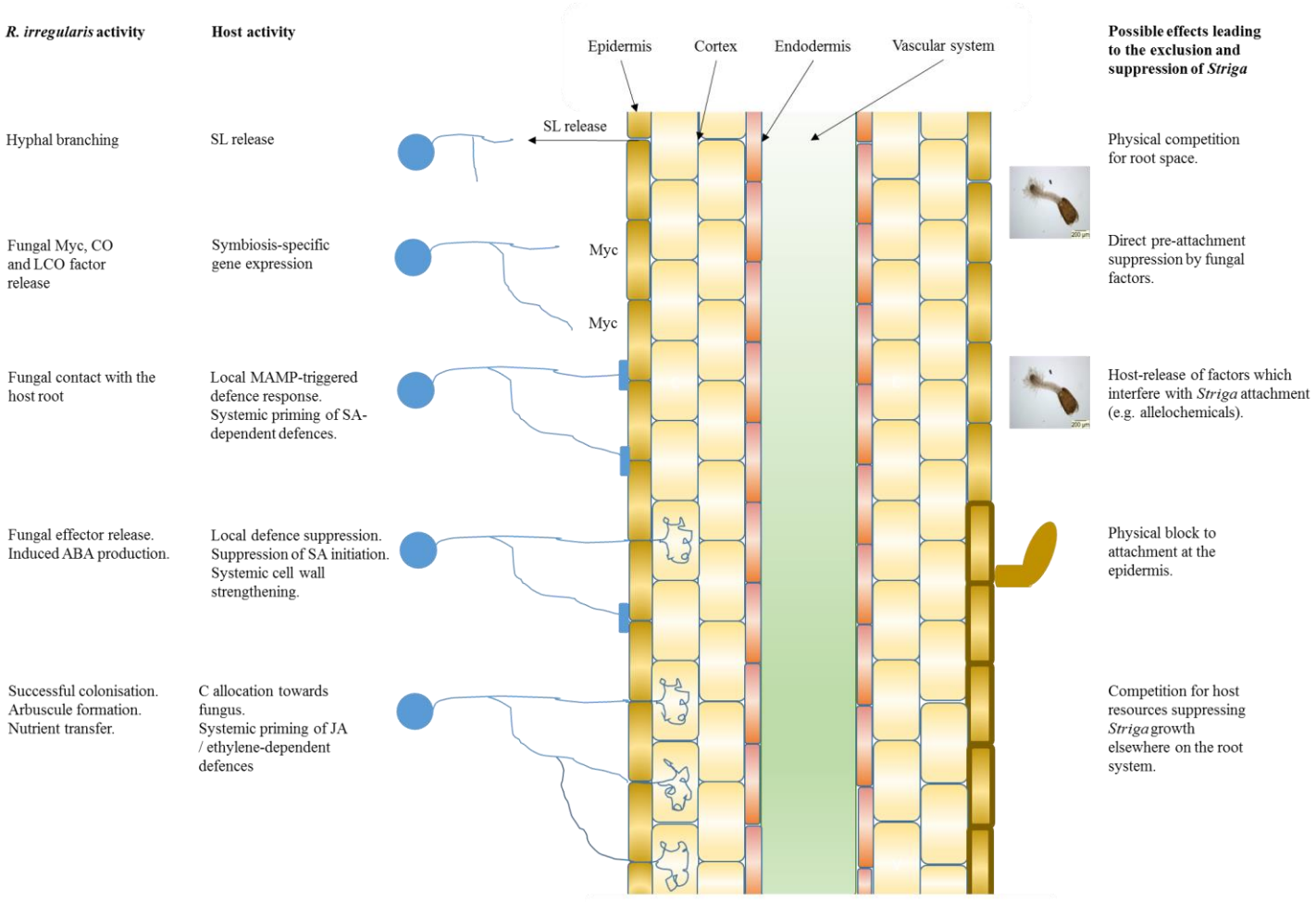


Fig. 4.13 Possible effects of colonisation by AM fungi on *Striga* infection. In the current experiment, *Striga* was excluded from attaching to mycorrhizal roots. Possible indirect effects of AM fungi on *Striga* via changes to host physiology, and possible direct effects of AM fungi on the *Striga* parasite before attachment are shown.

Myc factors, COs and LCOs trigger a range of host responses including calcium spiking and the expression of symbiosis-related genes (Kosuta et al., 2008, Czaja et al., 2012, Genre et al., 2013). As well as these known changes to root physiology, pre-symbiotic signalling may also lead to the release of host chemicals which suppressed the ability of *S. hermonthica* to attach to the root. Host-released chemicals have previously been shown to interfere with parasite radicle elongation and haustoria formation (Vurro et al., 2009, Fernandez-Aparicio et al., 2013). For example, the flavonoid isoschaftoside isolated from *Desmodium uncinatum* root exudates has been identified as an allelochemical which inhibits growth of the *S. hermonthica* radicle (Hooper et al., 2010). Furthermore, germination and radicle development of *Orobanche crenata* is inhibited by a number of cereal-produced allelochemicals (Fernandez-Aparicio et al., 2013). However, the induction of host release of allelochemicals due to pre-symbiotic AM signalling has not been shown. Pre-symbiotic AM signals can also induce morphological changes in host roots. For example, Olah *et al*. (2005) reported that a diffusible signal release by AM fungi before root contact induced lateral root formation in *M. truncatula*. It may be possible that diffusible signals involved in changes in host root anatomy can also influence the parasite haustorium (Olah et al., 2005).

The apparent exclusion of *S. hermonthica* from specific roots demonstrates a strong localised effect of AM colonisation, which again with only a 5-day head start for the fungus suggests that pre-symbiotic signalling was involved in this. Although pre-symbiotic signalling has not been shown to have such a tightly restricted, root level effect on host physiology, it is interesting to note the work of Gutjahr et al., (2009), who demonstrated that, of the three main orders of roots produced by rice, AM fungi show highly preferentially colonisation of one. This suggests that only specific roots may be amenable to AM colonisation at the pre-symbiotic signalling stage. After the detection of AM Myc factors, it is known that plant-derived cutin monomers are released from the root surface, which may be involved in the stimulation of hyphopodium differentiation in the fungus and a switch to a penetration stage of the symbiosis, forming a specialised type of appressoria called the hyphopodium (Wang et al., 2012, Murray et al., 2013). The prepenetration apparatus (PPA) is then formed by the plant to determine the path of the infecting fungal hyphae through the

epidermis and outer cortical cells (Genre et al., 2005). It makes sense that this complex re-organising of the host root in preparation for AM colonisation would only occur in roots which the fungus will eventually colonise. This idea provides a basis for a root-specific effect of AM colonisation on the host, and therefore for root-specific effects on the *Striga* parasite. The fact that there was even a lack of *Striga* attachments on mycorrhizal roots is both striking and puzzling. Overall, the mechanisms by which this could have occurred are elusive, but the suggestions above may provide some clues.

As well as the strong localised effect of AM colonisation on *Striga* at the pre-attachment stages of the parasite lifecycle, it is also possible that *R. irregularis* could have had systemic effects on *Striga* success, suppressing its attachment and infection elsewhere in the root system. After only 5 days on the host root system, host defences could have been altered by the presence of fungal MAMPs such as chitin, which can initiate SA-dependent defences when plants first detect fungi (Blilou et al., 1999, Blilou et al., 2000a, Blilou et al., 2000b). However, successful AM colonisation involves suppression of the SA-response (Herrera Medina et al., 2003, Herrera-Medina et al., 2007, Mohr and Cahill, 2007a), so it seems unlikely that in such a well colonised plant, SA-dependent defences would be activated by the fungus. Systemic suppression of the initial host defence response may occur via increased host ABA production and transport (Cameron et al., 2013). However, it has been suggested that the induction of ABA synthesis in the host by AM fungi may also promote systemic cell-wall strengthening (Ton et al., 2009, Cameron et al., 2013). Cell wall strengthening, for example by lignification, is a well-known resistance response against a range of pathogens (Xu et al., 2011, Miedes et al., 2014) and parasitic plants (Perez-De-Luque et al., 2005). It is unlikely that *R. irregularis* caused cell wall strengthening in the same root that the fungus then colonised. For example, Harrison and Dixon (1994) observed a lack of lignification specifically in cells of *M. truncatula* colonised by *Diversispora epigaea* (syn. *Glomus versiforme*). However, systemic cell wall strengthening may have reduced the ability of *S. hermonthica* to infect the host globally. For example, prevention of parasitic plant development at the cortex is associated with the deposition of cell wall physical barriers such as lignin (Perez-De-Luque et al., 2008), and lignification of cell walls at

the root endodermis can be seen at the host-parasite interface between resistant legume hosts and *O. crenata* (Perez-De-Luque et al., 2005).

Successful colonisation by AM fungi can result in the systemic priming of JA- and ethylene- dependent defences (Jung et al., 2012, Van der Ent et al., 2009, Van Wees et al., 2008). However, it is highly unlikely that this occurred when *R. irregularis* was only allowed to colonise for 5 days before the application of *S. hermonthica*. In this chapter, a separate experiment was set up to analyse the long-term effect of fully established mycorrhizal colonisation by applying *S. hermonthica* after 21 days after AM application. In this experiment, *S. hermonthica* infection was not altered, while mycorrhizal colonisation was suppressed. Clearly, the priority effect of the early arrival of the fungus was not sustained after the establishment of AM colonisation. After successful colonisation AM fungi are known to prime host defences, and colonisation is often associated with protective effects against pathogens (Jung et al., 2012). However, since the fungus did not alter *S. hermonthica* infection and was suppressed itself, this suggests that resource competition was more likely responsible. Obligate hemiparasites parasites such as *Striga* spp. receive about 30% of their carbon from their host plant (Irving and Cameron, 2009), while AM fungi can obtain as much as 20% of host photosynthate (Jakobsen and Rosendahl, 1990). It is possible that in order to cope with the demand imposed by late the invading parasite, the host plant could not sustain previous levels of AM colonisation.

Throughout this thesis, *S. hermonthica* has been the more dominant of the two symbionts both in terms of its own success and its effects on the host plant. The fact that *S. hermonthica* can override the priority effect of *R. irregularis* at such a late stage further correlates with this observation. In chapter 3, where the same isolate of *R. irregularis* was used, *S. hermonthica* reduced AM colonisation. By comparison with the results in this chapter, it is possible to speculate that *R. irregularis* could have colonised the host long before *S. hermonthica* infection, as was suggested in chapter 3. However, it is also possible that in chapter 3 *S. hermonthica* infected before *R. irregularis* colonisation resulting in suppression of AM colonisation. The next section will now discuss how early arrival of *S. hermonthica* in the present chapter led to a decrease in AM colonisation.

4.4.2 What is the mechanistic basis for the priority effect observed for *S. hermonthica*?

When the order of arrival was reversed, and *S. hermonthica* was applied 5 days before *R. irregularis*, the priority effect was reversed, and the parasite excluded the fungus from roots that it attached to, and also suppressed fungal colonisation elsewhere in the root system. Furthermore, the late arrival of the fungus did not reduce subsequent parasite biomass by competing for host nutrients. Unlike *R. irregularis*, 5 days is enough time for pre-germinated *S. hermonthica* to reach the late stages of symbiosis by penetrating the host root, establishing xylem continuity, and extracting host nutrients. The rice cultivar used in this study, IAC 165, is known to be highly susceptible to *Striga* (Swarbrick et al., 2008). Susceptible interactions with *Striga* have been shown to involve large-scale down-regulations in gene expression compared to resistant interactions (Swarbrick et al., 2008), and this thesis demonstrated sustained down-regulation of phenylpropanoid biosynthesis due to parasite infection (chapter 2). However, the exclusion of *R. irregularis* likely involved an antagonistic up-regulation of specific plant defences by the parasite.

Localised effects of *Striga* on susceptible host defences have been shown to involve both SA and JA induction. For example, Hiraoka and Sugimoto (2008) analysed gene expression in root sections next to the site of parasite attachment during the early stages of *S. hermonthica* parasitism (when attachment and tubercle formation was observed). Susceptible interactions with *S. hermonthica* involved the induction of JA- and the suppression of SA-induced genes. However, Mutuku et al., 2015 showed that susceptible interactions involved a low level of JA accumulation 1 day after infection (where initial root contact was made), which is then suppressed by the time the parasite invades the host root and establish xylem continuity by 3 and 7 days, while a sharp increase in root SA content can be observed 7 days after infection. An increase in SA would likely have an antagonistic effect on AM colonisation, since successful AM colonisation appears to involve the suppression of SA accumulation (Herrera-Medina et al., 2007, Mohr and Cahill, 2007a). Therefore, in this chapter, it could be hypothesised that the strong localised suppression of *Striga* on mycorrhizal colonisation could have due to an induction of SA accumulation in the host roots, as shown by (Mutuku et al., 2015).

The mechanistic basis for the systemic suppression of AM colonisation seen in my results is unlikely to be due to systemic effects of *Striga* on host defences. For example, the study by Hiraoka and Sugimoto (2008) mentioned above and a study by Hiraoka et al., (2009) found little evidence for systemic effects of *S. hermonthica* on host gene expression. However, the priority effect of early arrival of *S. hermonthica* in acquiring host resources may be an important factor. As mentioned above, obligate hemiparasites parasites such as *Striga* spp. receive about 30% of their carbon from their host plant (Irving and Cameron, 2009), AM fungi can obtain as much as 20% of host photosynthate (Jakobsen and Rosendahl, 1990). The systemic suppression of mycorrhizal colonisation seen in this chapter probably occurs because the host plant cannot support a further demand for resources.

4.4.3 Conclusions

This chapter has shown that priority effects can determine the success of AM fungi and *Striga* as they compete for host infection. Overall, a combination of the antagonistic effects of AM colonisation and *Striga* infection, together with their high demand for host resources, is probably what led to their suppression of each other when plants were simultaneously colonised/infected by both symbionts. It is unclear exactly how *R. irregularis* suppressed *S. hermonthica* infection and *vice versa* in this chapter because direct measurements of host defence hormones and nutrient transfers were not made. However, the individual spatiotemporal regulation of host defences during each symbionts interaction with the host can provide us with some hypotheses for the mechanisms involved. The use of rhizotrons to allow controlled timing of symbiont arrival in combination with gene expression, metabolomic analyses and analysis of nutrient transfers could be a useful tool for deciphering the mechanistic basis of tripartite interactions. Critically though, it must always be remembered that the suitability of highly controlled experimental results for considering what happens in the field must be evaluated. For example, in the field, rapid colonisation by AM fungi may occur because hyphal networks and spores already exist in plant roots and the soil left behind by previous crops. Whether or not *Striga* will infect in advance of AM colonisation, as was enabled in this experiment, is something that should be considered when relating these results to the field.

Chapter 5

General discussion

5.1 Beyond the SL mechanism for understanding the interaction between AM fungi and *Striga*

In this thesis, I have significantly developed our understanding of the interaction between AM fungi and *Striga* by exploring beyond the SL mechanism, and by taking into account the importance of the order of arrival on symbiont success. In chapter 2, I showed for the first time that the presence of AM fungi can increase *Striga* infection. In chapter 3, a more competitive isolate of the same fungal species resulted in no alteration to *Striga* infection under two different nutrient conditions, thus showing the importance of context on the result of the interaction. In chapter 4, I developed a novel system to explore the effect of the order of arrival on symbionts success, and showed that early arrival by one symbiont suppresses the invader. Indeed, the only time that AM fungi suppressed *Striga* infection in this thesis is when their arrival was manipulated to occur simultaneously or before the parasite.

Striga spp. are the most widespread parasitic weeds in sub-Saharan Africa, and severely reduce yields of economically important cereal crops such as rice (Rodenburg et al., 2010). In contrast, AM fungi are often described as a key component to the future of sustainable agriculture, where their exploitation will benefit crops by enhancing plant nutrient uptake and plant defence against disease (Johansson et al., 2004, Fitter et al., 2011, Vejan et al., 2016). In agreement with this view, AM fungi have been shown to decrease *Striga* infection (Lendzemo et al., 2005, Lendzemo et al., 2007, Othira et al., 2012). The mechanistic basis for this is that the enhancement of host nutrient status by the fungus down-regulates the release of parasite seed germinating strigolactones (SLs) from host roots (Lendzemo et al., 2009, Lopez-Raez et al., 2011). The adoption of the SL signal, which induces hyphal branching in AM fungi, as a germination cue by *Striga*, is illustrative of the similarities between the two symbioses. Individually, both symbionts need to regulate host defences, and both impose a C demand on the host. So far, the interaction is mainly understood in terms of the signalling role of SLs. In such a complex interaction, further understanding is required, and this can be garnered when considering the individual effect of both symbionts on host physiology.

The SL mechanism makes critical assumptions about the progression of the interaction. It requires AM fungi to be recruited by the SL signal, and for them to colonise the host, enhance host nutrient status, and down-regulate host SL release, all before *Striga* detects the same signal and germinates. This seems unlikely considering the fact that *Striga* has evolved an incredibly high sensitivity to the presence of host SLs, with the ability to detect SLs down to concentrations of just 10^{-12}M (Kim et al., 2010, Conn et al., 2015, Tsuchiya et al., 2015, Toh et al., 2015), and that germination to infection can take as little as 3 days (Gurney et al., 2006). Furthermore, caution must be exercised when making assumptions about mycorrhizal interactions, because the AM symbiosis is known to lead to commensal and even parasitic effects on host plants (Johnson et al., 1997, Klironomos, 2003), and that farming practices such as tillage, fertilisation, and monoculture can select for AM species which favour nutrient storage strategies over nutrient trade to host plants (Nijjer et al., 2010, Verbruggen and Kiers, 2010). Therefore, despite the fact that AM fungi and the parasitic plant *Striga* appear to have opposing symbiotic strategies, it is important that the interaction is fully understood.

5.2 The order of colonisation/infection is critical for determining the influence that nutrients or defences will have on symbiont and host success

Fig. 5.1 shows the progression of AM colonisation and the possible impacts on *Striga* infection and host growth reported in this thesis, and the SL mechanism reported previously. The different results of the interaction can ultimately be attributed to symbiont and host identity and abiotic conditions (e.g. nutrient availability). These factors influence the timing of colonisation/infection, mycorrhizal nutrient exchange, and the ability of the host to support symbiont demand for resources. The progression of the interaction can be considered as a timeline of events with the outcome dependent on the timing of symbiont arrival. Plants produce and release more SLs under N and P stress (Lopez-Raez et al., 2008, Yoneyama et al., 2012). SLs represent the initial signal to which AM fungi and *Striga* respond by initiating hyphal branching and germination respectively (Akiyama et al., 2005, Cook et al., 1972, Matusova et al., 2005).

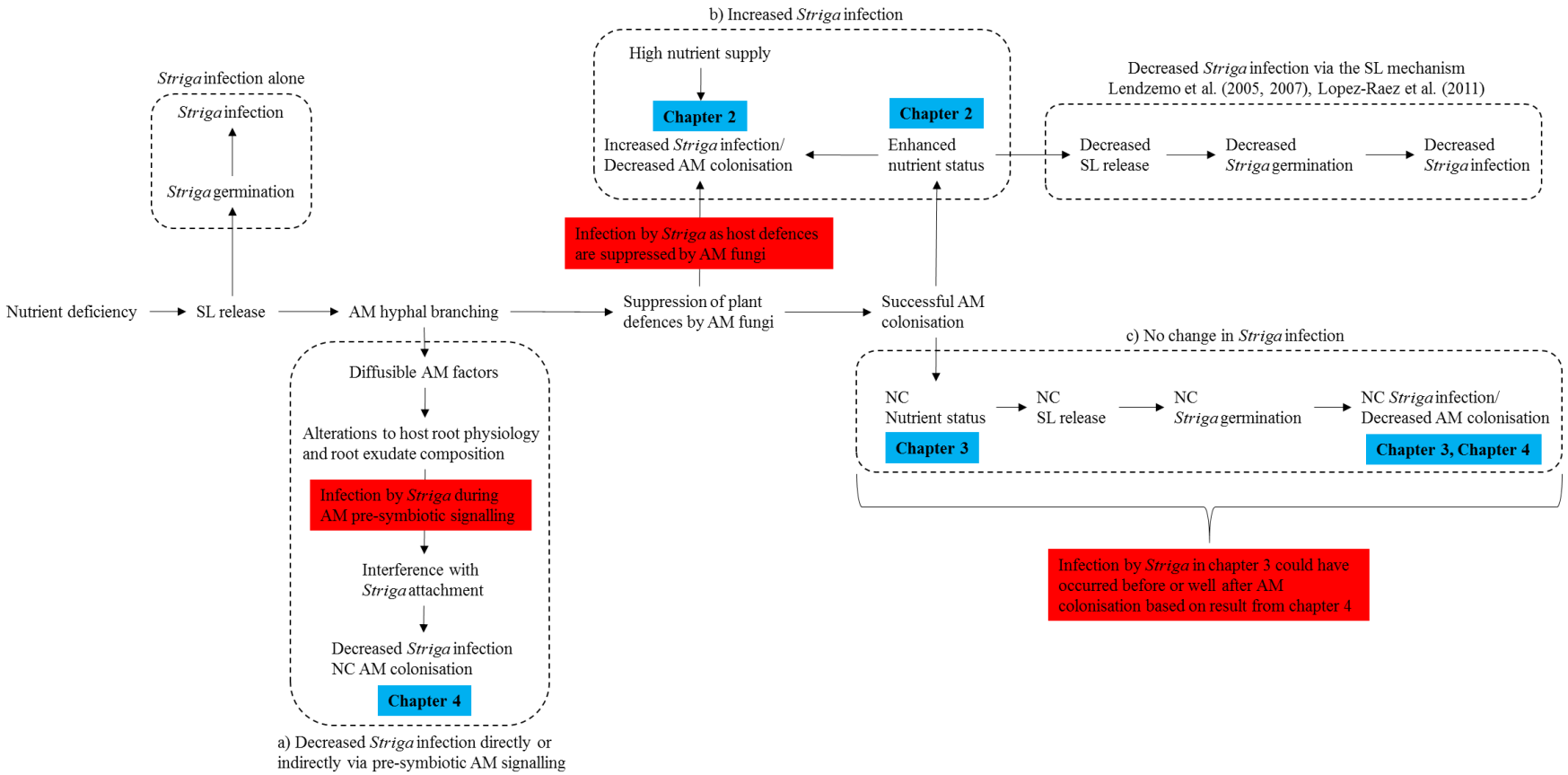


Fig. 5.1 Summary of the possible outcomes of the interaction between AM fungi and *Striga* for symbiont success. Symbiont success depends largely on the timing of colonisation/infection. (a) Arrival of *Striga* during the pre-symbiotic stages of AM colonisation may favour AM colonisation and suppress *Striga* infection as seen in chapter 4. NC = no change (b) Arrival of *Striga* during the suppression of host defences may benefit *Striga* infection and suppress AM colonisation as seen in chapter 2, particularly if the host has enough resources to support increased parasite biomass. (c) Arrival of *Striga* after successful colonisation by AM fungi may not alter *Striga* infection but could still suppress AM colonisation, potentially due to an increased demand on host resources by the invading parasite.

The SL signal allows plants to enhance recruitment of AM fungi so as to exploit their hyphal network to scavenge for nutrients, while the same signal has been hijacked by *Striga* for germination near to a host root (Akiyama et al., 2005). If the *Striga* seeds are appropriately conditioned, then infection may occur rapidly before AM colonisation. It can take days for *Striga* seeds to condition in the laboratory (Matusova et al., 2004). However, the warm, rain fed, nutrient-poor arable soils of SSA are where *Striga* thrives (Ejeta, 2007, Rodenburg et al., 2010), so it is likely that there is always at least a subset of seeds which are ready to germinate in the field. Nutrient-poor soils can also favour AM colonisation (Mader et al., 2000). However, colonisation may be perturbed in agricultural soils by tillage, which disrupt hyphal networks (Jansa et al., 2003, Verbruggen and Kiers, 2010). On the other hand, in the field, rapid colonisation by AM fungi may occur because hyphal networks, both in the soil but critically already existing in plant roots left behind by previous crops, may allow rapid contact with the growing roots of new crops. Indeed, the mycorrhizal state in roots is considered a normality for mycorrhizal plants. An often quoted statement by American plant pathologist Stephen Wilhelm says that, '...in agricultural field conditions, plants do not, strictly speaking, have roots, they have mycorrhizas'. The speed at which both AM fungi and *Striga* can colonise/infect is therefore variable, and as such is difficult to predict.

Fig. 5.1a shows a possible outcome of the interaction where *Striga* infects during the pre-symbiotic signalling stages of AM colonisation. In chapter 4, it was observed that the application of AM inoculum onto rice roots just five days before the application or pre-germinated *Striga* seed resulted in the suppression of *Striga* infection. In the rhizotron system used, intraradical AM colonisation was not established by 5 days, so it was hypothesised that pre-symbiotic AM signalling, which is known to occur even before hyphal contact with the host root (Kosuta et al., 2003, Czaja et al., 2012, Genre et al., 2013), could be responsible for the suppression of *Striga* observed. Despite not forming intraradical structures in the 5 days before *Striga* invasion, early arrival resulted in a clear priority effect, and fungal colonisation was not altered by *Striga* invasion.

Fig. 5.1b shows how parasite infection may increase if *Striga* infects as AM fungi suppress host defences. In chapter 2, it was observed for the first time that the

presence of AM fungi can increase *Striga* infection. This occurred despite the fact that the fungus was clearly mutualistic, enhancing host nutrient status alone and in combination with the parasite. This suggested that the fungus could not have improved host nutrient status in time to decrease SL release and *Striga* seed germination. I hypothesised that the transient, localised down-regulation of host defences known to occur at the early stages of AM colonisation (Herrera Medina et al., 2003, Herrera-Medina et al., 2007, Mohr and Cahill, 2007, Kloppholz et al., 2011) were responsible for enhancing the ability of *Striga* to infect the host plant. In addition to this, the supply of nutrients to the plants in this experiment, possibly in combination with the fungal supply, allowed the host to support this increase in resource demand by the parasite.

In contrast to the result seen in chapter 2, previous studies had shown that AM fungi can suppress *Striga* infection as a result of host nutrient status enhancement and a reuction in SL release (Lendzemo et al., 2005, Lendzemo et al., 2007, Lopez-Raez et al., 2011). Assuming a mutualistic interaction, the enhancement of host nutrient status is one of the end results of AM colonisation, so must therefore occur significantly before *Striga* germination.

The SL mechanism assumes a mutualistic interaction, where the fungus enhances host nutrient status quickly enough to sufficiently down-regulate SL release and *Striga* germination. However, Fig. 5.1c shows the possible outcome of the interaction of *Striga* with a more competitive isolate of AM fungi than was used in chapter 2. In chapter 3, the same pot experiment design was used as in chapter 2, but one of the major differences was the isolate of AM fungus used. The isolate of AM fungus used in chapter 3 colonised the host roots extensively, and had a commensal effect on host nutrient status and growth. The more competitive isolate did not alter host nutrient status or growth. Furthermore, the vigorous nature of this isolate suggests that relative timing of colonisation/infection was different in chapter 3 in comparison to chapter 2. Overall, the interaction in chapter 3 did not result in an increase in *Striga* infection as seen in chapter 2. Interestingly, results from chapter 4 showed no change in *Striga* infection when *Striga* arrived 5 days before the fungus, but also when the parasite arrived 21 days after the fungus. One of these two timing scenarios may have also occurred in the pot experiment in chapter 3. Additionally, the high fungal

demand showed some signs of competition with *Striga* for host resources, with some reduction in parasite biomass observed at the first harvest in chapter 3.

This thesis has shown that the impact of AM fungi on the *Striga* problem is context-dependent and may not necessarily be beneficial. However, it is noted that experimental contexts are likely to significantly alter the result of the interaction. For example, a particularly crucial factor in the field may be the diversity of AM fungi and the presence of other plant growth-promoting rhizobacteria in the soil compared to highly controlled experiments. A number of studies have shown that plants grown with multiple AM species benefit from increased biomass (van der Heijden et al., 1998). This suggests that partner choice may benefit host plants, although some studies have shown that single isolates can be as beneficial as mixtures (Lekberg et al., 2007). Regardless, in the field at least, partner selection may completely alter the interaction with *Striga* due to variable nutrient exchange dynamics and effects of AM fungi on host defences. Some studies suggest that the addition of AM inoculum can enhance crop yields (Ceballos et al., 2013) although results of inoculum addition do vary (Tarbell and Koske, 2007). Furthermore, the study by Lendzemo et al. (2005) suggested that inoculum addition reduced *Striga* infection in the field. Ultimately, our present uncertainty about the interaction between AM fungi and other plant symbionts, combined with the desire to exploit natural over chemical means to enhance crop yields means that further understanding of AM fungal interactions is required. Using approaches like those used in this thesis in combination with field trials will allow us to assess the value of AM fungi to sustainable agriculture, and provide insights into the mechanistic basis underlying complex interactions such as the interaction with *Striga*.

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Appendix A

ANOVA tables for statistics carried out in chapter 2

Table A1 Results of the ANOVA testing for the effects of *R. irregularis* and *S. hermonthica* on above ground and root dry weight of IAC165.

Adjusted R^2	93.13%			44.29%		
None	IAC165 Above ground dry weight (g)			IAC165 Root biomass (g)		
Source of variation	df	*F*	*P*	df	*F*	*P*
AM	1	73.31	<0.000	1	1.37	0.252
Striga	1	294.55	<0.000	1	25.43	<0.001
AM x *Striga*	1	55.11	<0.000	1	0.85	0.365
Error	28			28		

Table A2 Results of the ANOVA testing for the effects of *R. irregularis* and *S. hermonthica* on leaf and root nitrogen concentration of IAC165.

Adjusted R^2	15.75%			46.34%		
None	IAC165 Leaf N (mg/g)			IAC165 Root N (mg/g)		
Source of variation	df	*F*	*P*	df	*F*	*P*
AM	1	2.94	0.97	1	4.59	0.041
Striga	1	3.55	0.7	1	22.1	<0.001
AM x *Striga*	1	2.3	0.141	1	3.08	0.09
Error	28			28		

Table A3 Results of the ANOVA testing for the effects of *R. irregularis* and *S. hermonthica* on leaf and root phosphorus concentration of IAC165.

Adjusted R^2	71.64%			81.02%		
	IAC165 Leaf P (mg/g)			IAC165 Root P (mg/g)		
Source of variation	df	*F*	*P*	df	*F*	*P*
AM	1	55.75	<0.001	1	95.23	<0.001
Striga	1	21.04	<0.001	1	35.18	<0.001
AM x *Striga*	1	4.51	0.043	1	4.96	0.034
Error	28					

Table A4 Results of the ANOVA testing for the effects of *R. irregularis* and *S. hermonthica* on main stem diameter and height of IAC165.

Adjusted R^2	87.73%			83.16%		
	IAC165 Diameter of main stem (cm)			IAC165 Height of main stem (cm)		
Source of variation	df	*F*	*P*	df	*F*	*P*
AM	1	0.09	0.767	1	0.99	0.329
Striga	1	223.54	<0.001	1	154.11	<0.001
AM x *Striga*	1	0.97	0.333	1	0.99	0.329
Error	28			28		

Table A5 Results of the ANOVA testing for the effects of *R. irregularis* and *S. hermonthica* on total number of leaves and tillers of IAC165.

Adjusted R^2	47.45%			68.86%		
	IAC165 Total number of leaves			IAC165 Total number of tillers		
Source of variation	df	*F*	*P*	df	*F*	*P*
AM	1	30.4	<0.001	1	31.11	<0.001
Striga	1	0	0.945	1	25.2	<0.001
AM x *Striga*	1	0.59	0.449	1	15.24	0.001
Error	28			28		

Table A6 Results of the ANOVA testing for the effects of *R. irregularis* and *S. hermonthica* on above ground and root dry weight of Shiokari.

Adjusted R^2	90.93%			0%		
	Shiokari Above ground dry weight (g)			Shiokari Root dry weight (g)		
Source of variation	df	*F*	*P*	df	*F*	*P*
AM	1	40.65	<0.001	1	0.07	0.799
Striga	1	261.23	<0.001	1	0.68	0.415
AM x *Striga*	1	11.75	0.002	1	1.06	0.312
Error	28			28		

Table A7 Results of the ANOVA testing for the effects of *R. irregularis* and *S. hermonthica* on leaf and root nitrogen concentration of Shiokari.

Adjusted R^2	44.67%			12.13%		
	Shiokari Leaf N (mg/g)			Shiokari Root N (mg/g)		
Source of variation	df	*F*	*P*	df	*F*	*P*
AM	1	18.05	<0.001	1	2.56	0.121
Striga	1	5.11	0.032	1	3.88	0.059
AM x *Striga*	1	4.86	0.036	1	0.84	0.366
Error	28			28		

Table A8 Results of the ANOVA testing for the effects of *R. irregularis* and *S. hermonthica* on leaf and root phosphorus concentration of Shiokari.

Adjusted R^2	54.67%			74.12%	
	Shiokari Leaf P (mg/g)			Shiokari Root P (mg/g)	
Source of variation	df	*F*	*P*	df	*F*	*P*
AM	1	34.8	<0.001	1	53.6	<0.001
Striga	1	5.37	0.028	1	29.76	<0.001
AM x *Striga*	1	0.22	0.64	1	8.44	0.007
Error	28			28		

Table A9 Results of the ANOVA testing for the effects of *R. irregularis* and *S. hermonthica* on main stem diameter and height of Shiokari.

Adjusted R^2	69.31%			89.82%	
	Shiokari Diameter of main stem (cm)			Shiokari Height of main stem (cm)	
Source of variation	df	*F*	*P*	df	*F*	*P*
AM	1	3.32	0.079	1	3.06	0.092
Striga	1	66.55	<0.001	1	222.59	<0.001
AM x *Striga*	1	3.15	0.087	1	36.52	<0.001
Error	28			28		

Table A10 Results of the ANOVA testing for the effects of *R. irregularis* and *S. hermonthica* on total number of leaves and tillers of Shiokari.

Adjusted R^2	46.83%			18.42%		
	IAC165 Total number of leaves			IAC165 Total number of tillers		
Source of variation	df	*F*	*P*	df	*F*	*P*
AM	1	23.08	<0.001	1	8	0.009
Striga	1	3.13	0.088	1	2	0.168
AM x *Striga*	1	4.09	0.053	1	0	1
Error	28			28		

Appendix B

ANOVA tables for statistics carried out in chapter 3

Table B1 Results of the ANOVA testing for the effects of *R. irregularis*, *S. hermonthica* and nutrients (high and low N/P) on the above ground and root dry weight of rice cultivar IAC165 at 5 weeks after sowing (WAS).

Adjusted R^2	97.51%			84.36%		
	Above ground dry weight (g)			Root dry weight (g)		
Source of variation	df	*F*	*P*	df	*F*	*P*
Nutrients	1	2054.33	<0.001	1	251.74	<0.001
AM	1	16.97	<0.001	1	0.03	0.862
Striga	1	380.31	<0.001	1	68.84	<0.001
Nutrients x AM	1	0.86	0.357	1	0.02	0.878
Nutrients x *Striga*	1	11.84	0.001	1	25.15	<0.001
AM x *Striga*	1	2.97	0.09	1	0.14	0.706
Nutrient x AM x *Striga*	1	8.63	0.005	1	0.91	0.344
Error	56			56		

Table B2 Results of the ANOVA testing for the effects of *R. irregularis*, *S. hermonthica* and nutrients (high and low N/P) on leaf and root nitrogen concentration of rice cultivar IAC165 at 5 WAS.

Adjusted R^2	86.85%			63.87%		
	Leaf N (mg/g)			Root N (mg/g)		
Source of variation	df	*F*	*P*	df	*F*	*P*
Nutrients	1	412.59	<0.001	1	94.69	<0.001
AM	1	7.17	0.01	1	4.14	0.047
Striga	1	0.06	0.813	1	16.08	>0.001
Nutrients x AM	1	0.16	0.693	1	1.98	0.165
Nutrients x *Striga*	1	2	0.163	1	0.32	0.575
AM x *Striga*	1	0.39	0.537	1	0.31	0.581
Nutrient x AM x *Striga*	1	0.75	0.389	1	0.84	0.363
Error	56			56		

Table B3 Results of the ANOVA testing for the effects of *R. irregularis*, *S. hermonthica* and nutrients (high and low N/P) on leaf and root phosphorus concentration of rice cultivar IAC165 at 5 WAS

Adjusted R^2	63.90%			21.56%		
		Leaf P (mg/g)			Root P (mg/g)	
Source of variation	df	*F*	*P*	df	*F*	*P*
Nutrients	1	52.18	<0.001	1	4.12	0.047
AM	1	0.13	0.723	1	0.09	0.761
Striga	1	44.3	<0.001	1	16.01	<0.001
Nutrients x AM	1	0.44	0.509	1	0.37	0.546
Nutrients x *Striga*	1	20.23	<0.001	1	0.88	0.351
AM x *Striga*	1	0.01	0.923	1	1.02	0.317
Nutrient x AM x *Striga*	1	1.25	0.268	1	1.82	0.182
Error	56			56		

Table B4 Results of the ANOVA testing for the effects of *R. irregularis*, *S. hermonthica* and nutrients (high and low N/P) on the above ground and root dry weight of rice cultivar IAC165 at 10 WAS.

Adjusted R^2	96.10%			82.14%		
	Above ground dry weight (g)			Root dry weight (g)		
Source of variation	df	*F*	*P*	df	*F*	*P*
Nutrients	1	469.74	<0.001	1	81.71	<0.001
AM	1	12.37	0.001	1	0.11	0.745
Striga	1	716.9	<0.001	1	210.41	<0.001
Nutrients x AM	1	0.96	0.332	1	0.14	0.706
Nutrients x *Striga*	1	358.4	<0.001	1	0.19	0.668
AM x *Striga*	1	0.72	0.399	1	0.15	0.696
Nutrient x AM x *Striga*	1	0.13	0.724	1	4.11	0.047
Error	56			56		

Table B5 Results of the ANOVA testing for the effects of *R. irregularis*, *S. hermonthica* and nutrients (high and low N/P) on leaf and root nitrogen concentration of rice cultivar IAC165 at 10 WAS.

Adjusted R^2	33.05%			66.97%	
	Leaf N (mg/g)			Root N (mg/g)	
Source of variation	df	*F*	*P*	df	*F*	*P*
Nutrients	1	18.48	<0.001	1	24.87	<0.001
AM	1	2.7	0.106	1	1.13	0.293
Striga	1	9.70	0.003	1	94.1	<0.001
Nutrients x AM	1	0.39	0.537	1	1.12	0.293
Nutrients x *Striga*	1	5.29	0.025	1	1.11	0.297
AM x *Striga*	1	1.44	0.236	1	5.79	0.019
Nutrient x AM x *Striga*	1	0.11	0.743	1	6.63	0.013
Error	56			56		

Table B6 Results of the ANOVA testing for the effects of *R. irregularis*, *S. hermonthica* and nutrients (high and low N/P) on leaf and root phosphorus concentration of rice cultivar IAC165 at 10 WAS.

Adjusted R^2	58.12%			70.74%		
	Leaf P (mg/g)			Root P (mg/g)		
Source of variation	df	*F*	*P*	df	*F*	*P*
Nutrients	1	9.73	0.003	1	0.07	0.793
AM	1	0.87	0.354	1	1.64	0.205
Striga	1	81.2	<0.001	1	125.65	<0.001
Nutrients x AM	1	0.76	0.388	1	1.84	0.18
Nutrients x *Striga*	1	0.01	0.918	1	24.21	<0.001
AM x *Striga*	1	1.49	0.227	1	4.09	0.048
Nutrient x AM x *Striga*	1	0.35	0.556	1	1.82	0.183
Error	56			56		

Table B7 Results of the ANOVA testing for the effects of *R. irregularis*, *S. hermonthica* and nutrients (high and low N/P) on main stem diameter and height of rice cultivar IAC165 at 10 WAS.

Adjusted R^2	87.68%			84.82%		
	Diameter of main stem (mm)			Height of main stem (mm)		
Source of variation	df	*F*	*P*	df	*F*	*P*
Nutrients	1	55.91	<0.001	1	69.64	<0.001
AM	1	5.31	0.025	1	5.46	0.023
Striga	1	390.07	<0.001	1	281.54	<0.001
Nutrients x AM	1	1.12	0.295	1	0.07	0.792
Nutrients x *Striga*	1	1.99	0.164	1	1.21	0.277
AM x *Striga*	1	0.87	0.355	1	1.05	0.311
Nutrient x AM x *Striga*	1	0.17	0.685	1	0.01	0.93
Error	56			56		

Table B8 Results of the ANOVA testing for the effects of *R. irregularis*, *S. hermonthica* and nutrients (high and low N/P) on total number of leaves of rice cultivar IAC165 at 10 WAS.

Adjusted R^2	41.68%

	Total number of leaves		
Source of variation	df	*F*	*P*
Nutrients	1	41.89	<0.001
AM	1	0.03	0.874
Striga	1	3.48	0.068
Nutrients x AM	1	1.84	0.18
Nutrients x *Striga*	1	3.18	0.08
AM x *Striga*	1	0.18	0.672
Nutrient x AM x *Striga*	1	1.44	0.236
Error	56		

Table B9 Results of the ANOVA testing for the effects of *R. irregularis*, *S. hermonthica* on total number of tillers of rice cultivar IAC165 supplied with high N/P at 10 WAS. Rice supplied with low did not develop any tillers at any stage of the experiment.

Adjusted R^2	47.84%

	Total number of tillers		
Source of variation	df	*F*	*P*
AM	1	1.14	0.294
Striga	1	23.14	<0.001
AM x *Striga*	1	7.14	0.012
Error	28		

Table B10 Results of the ANOVA testing for the effects of *S. hermonthica* and nutrients (high and low N/P) on *R. irregularis* colonisation of rice cultivar IAC165 at 5 WAS.

Adjusted R^2	90.18%			81.62%			75.27%		
	Intraradical root colonisation			Vesicles colonisation			Arbuscule colonisation		
Source of variation	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Nutrients	1	256.68	<0.001	1	118.6	<0.001	1	94.93	<0.001
AM/*Striga*	1	17.83	<0.001	1	12.47	0.001	1	0.54	0.47
Nutrients x AM/*Striga*	1	13.15	0.001	1	9.55	0.004	1	1.89	0.18
Error	28			28			28		

Table B11 Results of the ANOVA testing for the effects of *S. hermonthica* and nutrients (high and low N/P) on *R. irregularis* colonisation of rice cultivar IAC165 at 10 WAS.

Adjusted R^2	35.97%			49.81%			24.90%		
	Intraradical root colonisation			Vesicles colonisation			Arbuscule colonisation		
Source of variation	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Nutrients	1	0.59	0.45	1	1.07	0.31	1	0.19	0.665
AM/*Striga*	1	19.59	<0.001	1	32.52	<0.001	1	12.9	0.001
Nutrients x AM/*Striga*	1	0.24	0.631	1	0.18	0.671	1	0.19	0.665
Error	28			28			28		

Table B12 Results of the ANOVA testing for the effects of *R. irregularis* and nutrients (high and low N/P) on *S. hermonthica* infection of rice cultivar IAC165 at 5 WAS.

Adjusted R^2	50.68%			30.86%		
	Striga number			*Striga* number per unit root biomass		
Source of variation	df	*F*	*P*	df	*F*	*P*
Nutrients	1	43.06	<0.001	1	16.48	<0.001
AM/*Striga*	1	0.49	0.491	1	0.04	0.842
Nutrients x AM/*Striga*	1	0.3	0.586	1	0.31	0.58
Error	28			28		
Adjusted R^2	40.68%			12.25%		
	Striga biomass (mg)			Average *Striga* biomass		
Source of variation	df	*F*	*P*	df	*F*	*P*
Nutrients	1	16.66	<0.001	1	0.59	0.449
AM/*Striga*	1	6.36	0.018	1	5.39	0.028
Nutrients x AM/*Striga*	1	1.24	0.275	1	1.35	0.255
Error	28			28		

Table B13 Results of the ANOVA testing for the effects of *R. irregularis* and nutrients (high and low N/P) on *S. hermonthica* infection of rice cultivar IAC165 at 10 WAS.

Adjusted R^2	16.84%			13.57%		
	Striga number			*Striga* number per unit root biomass		
Source of variation	df	*F*	*P*	df	*F*	*P*
Nutrients	1	7.51	0.011	1	6.71	0.015
AM/*Striga*	1	1.49	0.232	1	0.08	0.784
Nutrients x AM/*Striga*	1	0.28	0.601	1	1.09	0.306
Error	28			28		
Adjusted R^2	48.31%			24.64%		
	Striga biomass (mg)			Average *Striga* biomass		
Source of variation	df	*F*	*P*	df	*F*	*P*
Nutrients	1	29.28	<0.001	1	12.26	0.002
AM/*Striga*	1	0.99	0.328	1	0.17	0.685
Nutrients x AM/*Striga*	1	0.06	0.814	1	0.03	0.868
Error	28			28		