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This is a pre print version of the following article:

Original Citation:

Availability:
This version is available http://hdl.handle.net/2318/129330 since

Published version:
DOI:10.1111/pce.12102

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Arbuscular mycorrhizal fungi reduce growth and infect roots of the non-host plant Arabidopsis thaliana

Authors
RITA S. L. VEIGA, ANTONELLA FACCIO, ANDREA GENRE, CORNÉ M. J. PIETERSE, PAOLA BONFANTE, MARCEL G. A. van der HEIJDEN

Correspondence: M. G. A. van der Heijden. e-mail: marcel.vanderheijden@art.admin.ch

Abstract

The arbuscular mycorrhizal (AM) symbiosis is widespread throughout the plant kingdom and important for plant nutrition and ecosystem functioning. Nonetheless, most terrestrial ecosystems also contain a considerable number of non-mycorrhizal plants. The interaction of such non-host plants with AM fungi (AMF) is still poorly understood. Here, in three complementary experiments, we investigated whether the non-mycorrhizal plant Arabidopsis thaliana, the model organism for plant molecular biology and genetics, interacts with AMF. We grew A.thaliana alone or together with a mycorrhizal host species (either Trifolium pratense or Lolium multiflorum) in the presence or absence of the AMF Rhizophagus irregularis. Plants were grown in a dual-compartment system with a hyphal mesh separating roots of A.thaliana from roots of the host species, avoiding direct root competition. The host plants in the system ensured the presence of an active AM fungal network. AM fungal networks caused growth depressions in A.thaliana of more than 50% which were not observed in the absence of host plants. Microscopy analyses revealed that R.irregularis supported by a host plant was capable of infecting A.thaliana root tissues (up to 43% of root length colonized), but no arbuscules were observed. The results reveal high susceptibility of A.thaliana to R.irregularis, suggesting that A.thaliana is a suitable model plant to study non-host/AMF interactions and the biological basis of AM incompatibility.

Introduction

The majority of land plants form a symbiosis with arbuscular mycorrhizal fungi (AMF), widespread soil fungi belonging to the phylum Glomeromycota (Schüßler, Schwarzott & Walker 2001; Smith & Read 2008). AMF acquire nutrients from the soil and deliver these to host plants in return for photosynthates (Smith & Read 2008). Nutrient exchanges between the fungus and its host occur in symbiotic structures inside plant root cells known as arbuscules (Parniske 2008). In addition to having an effect on plant nutrition, AMF can provide drought tolerance, disease protection (Newsham, Fitter & Watkinson 1995; Zamioudis & Pieterse 2012), and influence a number of important ecosystem functions such as plant productivity, plant diversity, soil structure and nutrient cycling (Grime et al. 1987; van der Heijden et al. 1998; van der Heijden 2010).

An estimated 18% of all vascular species do not associate with AMF (Brundrett 2009). These plants, denominated ‘non-host’ or ‘non-mycorrhizal’ (NM) plants, can be broadly divided in two groups: those with highly specialized nutrition such as carnivores, parasites and species with cluster roots (e.g. in Cyperaceae and Proteaceae
families) that often grow in severely phosphorus (P)-impoverished soils, and more
generalistic species without specialized strategies for nutrient acquisition that grow
mainly in wet, arid, saline, very cold and disturbed habitats (Lambers et al. 2008,
2010; Brundrett 2009). NM species from the latter group are especially abundant in
families such as Brassicaceae, Polygonaceae, Amaranthaceae and Caryophyllaceae
(Wang & Qiu 2006), and many are considered important agricultural weeds (Jordan,
Zhang & Huerd 2000). Overall, although NM species are clearly less abundant than
those that establish an arbuscular mycorrhizal (AM) symbiosis, they are present (and
sometimes dominant) in a wide range of environments.

Until now, only few studies investigated the interactions of AMF with non-hosts.
Most of these studies reported a negative effect of AMF on non-host growth and
survival (Allen, Allen & Friese 1989; Francis & Read 1994, 1995; Sanders & Koide
1994; Veiga, Howard & van der Heijden 2012). Proposed mechanisms include (1)
competitive disadvantage compared with mycorrhizal plants (Sanders & Koide 1994);
(2) release of allelopathic compounds by the AM mycelium which inhibit the growth
of non-host plants (Francis & Read 1994, 1995; Veiga et al. 2012); and (3) activation
of strong plant defence responses that result in a loss of plant fitness (Allen et al.
1989; Francis & Read 1995).

Despite these observations, the precise mechanism(s) responsible for negative effects
of AMF on non-hosts is still poorly understood due to the absence of a suitable model
system. For this reason, we focused on Arabidopsis thaliana (L.) Heynh., the most
studied model organism in plant biology, biochemistry and genetics. A. thaliana
belongs to the Brassicaceae family, does not have any specialized root adaptations for
nutrient acquisition and is generally considered a non-host plant that cannot establish
AM symbiosis (Wang & Qiu 2006). It occurs naturally in open or disturbed habitats
(Koornneef, Alonso-Blanco & Vreugdenhil 2004). Based on the hypothesis that,
similar to other plants from the same group of NM species, A. thaliana growth is
affected by AMF, we set out to investigate the interaction between A. thaliana and the
widespread AM fungus Rhizophagus irregularis in terms of growth responses and root
infection. Our aim was to evaluate the suitability of A. thaliana as a model plant to
further investigate the molecular basis of AMF/non-host interactions. Note that, for
the sake of simplicity, when we mention NM species throughout this paper we are
referring exclusively to the group of non-hosts similar to A. thaliana, that is, without
specialized nutrition strategies.

We combined two approaches to study the interaction between AMF and A. thaliana.
We grew A. thaliana plants with and without AM fungal inoculum, an approach used
by most physiologists studying plant–AMF interactions. In addition, we chose a more
‘ecological approach’ and grew A. thaliana in microcosms where an active AM
mycelium had been pre-established by a host plant that was sown 4–5 weeks earlier in
a neighbouring soil compartment (Fig. 1). This approach has similarities to many
natural ecosystems where seedlings establish and grow in the presence of AM
mycelium already developed by the surrounding vegetation (Leake et al. 2004; van
der Heijden & Horton 2009). The latter approach is especially interesting because a
vital and active AM mycelium continuously interacts with the roots, thus amplifying
potential negative interactions with non-host plants.
In order to gain more insight on the nature of A. thaliana growth responses to AMF, we tested, in one of the experiments, three A. thaliana genotypes: the ‘wild-type’ accession Columbia-0 (Col-0) and the mutants myb72-1 and jin1-2 that are impaired in their response to colonization of the roots by beneficial plant growth-promoting rhizobacteria and/or fungi (Pozo et al. 2008; Van der Ent et al. 2008; Segarra et al. 2009). We hypothesized that if A. thaliana plants would recognize R. irregularis as a beneficial fungus, this would be reflected in differential growth responses among the mutants and the wild-type. We also performed bright field, confocal and transmission electronic microscopy studies to visualize whether AMF colonize A. thaliana roots and to better describe the infection process. We show that the AM fungus R. irregularis infects roots of A. thaliana more extensively than what was foreseen and that plant growth is highly inhibited by this fungus.

**Materials and Methods**

In this paper, three experiments are presented. The first two experiments were conducted to assess A. thaliana growth responses to R. irregularis while the third was aimed to describe the root infection process. In the first experiment, interactions between A. thaliana and R. irregularis were investigated in microcosms where A. thaliana was grown either alone or together with the host species Trifolium pratense L. (red clover), sown 4 weeks earlier in a neighbouring soil compartment to pre-establish an active AM mycelium (Fig. 1). To understand whether the effects of R. irregularis on A. thaliana depend on the identity of the neighbour host species and its AMF dependency/response, in the second experiment A. thaliana was grown in the presence of a pre-established AM mycelium, but this time supported by the host Lolium multiflorum Lam. (Italian ryegrass). In addition, three different A. thaliana genotypes were used. In the third experiment, A. thaliana was grown in pots with R. irregularis mycelium supported by T. pratense and roots of A. thaliana were collected for extensive microscopic analyses.

**Plant material, fungal inoculum and soil mixture**

In this study, we used seeds of wild-type A. thaliana Col-0 and the mutants myb72-1 and jin1-2 that are impaired in their response to colonization of the roots by beneficial plant growth-promoting rhizobacteria and/or fungi (see Lorenzo et al. 2004; van der Ent et al. 2008; Pozo et al. 2008; Segarra et al. 2009 for a description). Seeds of T. pratense and L. multiflorum were surface sterilized in 1.25% sodium hypochlorite for 10min and rinsed with H2O.

Inoculum of R. irregularis, previously named Glomus intraradices (BEG 21, described in van der Heijden et al. 2006; Stockinger, Walker & Schüßler 2009; Krüger et al. 2012), was propagated on Plantago lanceolata L. for 5 months in pots filled with a sterilized mixture (99min at 121°C) of quartz sand with 20% field soil.
The soil substrate used for all the experiments consisted of an autoclaved (99 min at 121°C) mixture of 10% field soil collected at a certified organic farm in Reckenholz (Zurich, Switzerland) with quartz sand. The autoclaved soil mixture had pH (H2O) 7.5 and plant available P, extracted by CO2 saturated water, of 5 mg kg⁻¹.

Experiment 1: Effects of AMF on A. thaliana grown alone or in combination with T. pratense

This experiment was set up as a randomized block design with two factors. One factor, plant mixture, contained two levels: A. thaliana Col-0 grown alone and A. thaliana Col-0 grown in combination with T. pratense in the other half of the microcosm (monocultures and mixtures, respectively). The other factor, AMF presence, also contained two levels: with R. irregularis (AMF) and with NM inoculum. This makes a total of four treatment combinations. Each treatment was replicated six times and assigned to a block, making a total of 6 blocks and 24 microcosms.

Each microcosm was divided into two equal parts by a 30μm nylon mesh to separate roots but still allowing the passage of AMF hyphae (see Fig. 1). Each half received 0.5L of autoclaved soil mixture with 5% R. irregularis soil inoculum or the same amount of sterilized (2×99 min at 121°C) inoculum for the NM control treatment. All the microcosms received 10mL (5mL each half) of inoculum washing (100g of the soil inoculum suspended in 600mL water and filtered through filter paper) to correct for possible differences in microbial communities.

According to the treatment (mixtures or monocultures), six seeds of T. pratense were sown in one-half of the microcosms, or these were left unsown. Upon germination, smaller seedlings were removed, leaving three seedlings. T. pratense seedlings grew for 4 weeks before A. thaliana seeds (2.5mg) were sown in the other half of each microcosm. At the same time, A. thaliana seeds (2.5mg) were added to microcosms without T. pratense. Upon germination, smaller seedlings were removed, leaving 12 A. thaliana seedlings of similar size.

Plants were watered three times a week with the same volume of H2O and were supplied weekly with 10mL (5mL each half) of a nutrient solution based on Hoagland solution (Hoagland & Arnon 1950) but with half of the normal N and P concentrations and containing only macronutrients (6mm KNO3, 4mm CaCl2, 1mm NH4H2PO4, 1mm MgSO4). Plants were maintained in the glasshouse and additional lighting was provided by 400W high-pressure sodium lamps, when natural light levels were below 250 W m⁻², to a daylength of 14h. During the growing season, the temperatures in the glasshouse ranged from 14 to 23°C. T. pratense and A. thaliana plants were harvested 10 and 6 weeks after sowing, respectively.

Experiment 2: Effects of AMF on three A. thaliana genotypes grown in combination with L. multiflorum

This experiment was set up as a randomized block design with two factors. One factor, A. thaliana genotype, contained three levels: Col-0, myb72-1 and jin1-2. The other factor, AMF presence, contained two levels: with R. irregularis (AMF) and with NM inoculum. This makes a total of six treatment combinations. Each treatment was replicated 10 times and each replicate was assigned to a block, making a total of 10 blocks and 60 microcosms.
Microcosms were divided with 30μm nylon mesh, filled with soil mixture and inoculum exactly as described in experiment 1. Similarly, all microcosms received 10mL (5mL each half) of inoculum washing (170g of the soil inoculum suspended in 1L water and filtered through filter paper).

Six L. multiflorum seeds were sown in one-half of the microcosms. Upon germination, smaller seedlings were removed, leaving three seedlings. L. multiflorum seedlings grew for 5 weeks before A. thaliana seeds (2.5mg) were sown in the other half of each microcosm. Upon germination, smaller seedlings were removed, leaving eight A. thaliana seedlings of similar size.

Plants were watered and received nutrient solution like in experiment 1. Plants were maintained in the glasshouse with constant temperature (25°C) and constant lighting provided by 400 W high-pressure sodium lights to a daylength of 14h. L. multiflorum and A. thaliana plants were harvested 11 and 6 weeks after sowing, respectively.

Experiment 3: AM colonization of A. thaliana roots

T. pratense and A. thaliana plants were grown in 0.75L pots filled with the same soil/sand mixture and R. irregularis inoculum as in the previously described experiments. Three T. pratense seeds were sown in the centre of the pot. Upon germination, two of the seedlings were removed, leaving only one plant. This plant grew for 4 weeks before A. thaliana (Col-0) was sown. A. thaliana seeds were sown in a circle around the T. pratense plant in each pot. Plants received microbial wash, nutrient solution, and were watered as previously described. After 6 weeks (maintained in the same glasshouse conditions as in experiment 2), A. thaliana plants were harvested and roots were carefully washed. The complete root system was excised under the stereomicroscope. In a similar set-up, A. thaliana plants were grown for 6 weeks and maintained in the same conditions, but in the absence of fungal inoculum (non-infected, control roots).

Harvest and analyses of samples from experiments 1 and 2

At harvest, shoots of A. thaliana, T. pratense and L. multiflorum were cut at the soil surface, oven dried (80°C) and weighed to determine the aboveground biomass. Roots were carefully removed from the soil substrate. Soil from A. thaliana Col-0 half of microcosms containing T. pratense or L. multiflorum in the other half (experiment 1 or experiment 2, respectively) was collected, separated in two subsamples and weighed. One subsample was oven dried (80°C) and weighed. The other soil subsample was used to determine the length of the fungal hyphae by an aqueous extraction and membrane filter technique (Jakobsen, Abbott & Robson 1992). R. irregularis total hyphal length per gram was estimated (on dry weight basis) using the modified Newman formula (Tennant 1975).

Roots were carefully washed, cut into 1cm segments and mixed, and the fresh weight was recorded. A subsample of T. pratense or L. multiflorum roots of known weight was taken for measurement of AM colonization. In the case of A. thaliana, there was not sufficient root material for assessing belowground biomass and AM colonization. For this reason, all roots retrieved were taken for measurement of colonization. The remaining roots of T. pratense and L. multiflorum were oven dried (80°C) for 5d and
weighed. The dry weight of the subsample taken for measurement of AM colonization was inferred by multiplying its fresh weight with the dry-to-fresh weight ratio of the oven-dried roots. The inferred dry weight of the subsample was added to the dry weight of the remaining roots to calculate the belowground biomass. The sum of belowground and aboveground biomass gave the total biomass of T. pratense and L. multiflorum per microcosm.

Root samples for measurement of AM colonization were cleared with 10% KOH and stained with trypan blue (Phillips & Hayman 1970). The percentage of root length colonized by AMF and frequency of hyphae, vesicles and arbuscules was estimated according to McGonigle et al. (1990) using at least 100 intersections per root sample.

Oven-dried roots and shoots of L. multiflorum (experiment 2) grown with A. thaliana Col-0 were ground and analysed separately for P and N concentrations. Firstly, P was determined spectrophotometrically after calcination and extraction with hydrochloric acid (Siegel 1976). Nitrogen was determined on the remaining plant material according to the Dumas combustion procedure (Houba et al. 1989).

Statistical analyses

Plant biomass, AM colonization, R. irregularis hyphal length and nutrient (P and N) concentration in plant material were analysed separately with mixed-effects models (Pinheiro & Bates 2000) using the lme function from the nlme library for R 2.9.0 (R Development Core Team 2009).

In the experiment with T. pratense (experiment 1), for the analyses of A. thaliana biomass and AM colonization, AMF presence and plant mixture were treated as fixed effects as was the AMF presence in the analysis of T. pratense biomass and in the analysis of the hyphal length. Because there was heterogeneity in the variance structure of T. pratense biomass, R. irregularis hyphal length, and AM colonization between the AMF treatment and NM controls, we used the varIdent() function to allow each treatment to have a different variance.

In the experiment with L. multiflorum (experiment 2), for the analyses of A. thaliana and L. multiflorum biomass, AMF presence and genotype were treated as fixed effects as was the AMF presence in the analysis of hyphal length and genotype in the analysis of AMF colonization. We used the varIdent() function to take into account the heterogeneity in the variance structure of R. irregularis hyphal length and A. thaliana biomass between the AMF treatment and NM controls. For the P and N concentrations in L. multiflorum plants, AMF presence was treated as a fixed effect.

Block was treated as a random effect. In the text, we present estimates of the means from the mixed-effects models with their standard errors (SEs) and regression slopes with their 95% confidence interval (CI).

Microscopic analyses of samples from experiment 3

Bright field microscopy
Complete root systems excised from four A. thaliana plants growing in the presence of R. irregularis mycelium were cut into segments of about 1 cm long. Root segments were stained overnight at room temperature in 0.1% cotton blue in lactic acid, and washed several times in lactic acid. Stained root segments were observed under a Nikon Eclipse E400 optical microscope (Nikon Instruments, Firenze, Italy). Some segments were not stained and were left for confocal and electron microscope analyses.

**Confocal microscopy**

Root segments were treated for 5 min in 0.5% NaClO in phosphate buffer, pH 6.8, washed three times for 10 min in the same buffer and incubated for 2 h with wheat germ agglutinin-fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Milan, Italy) at a final concentration of 10 μg·mL⁻¹ to stain the chitin of fungal cell walls. Fluorescence was excited with the 488 nm band of an argon laser and imaged using a 500–540 nm emission window for FITC and a 600–690 nm window for root background autofluorescence. All images were acquired and processed using a Leica TCS SP2 confocal microscope and software (Leica Microsystems GmbH, Wetzlar, Germany).

**Electron microscopy**

Selected root segments were fixed in 2.5% glutaraldehyde in 0.1 mM cacodylate buffer (pH 7.2) for 2 h at room temperature and then overnight at 4°C. After rinsing three times with the same buffer, they were dehydrated in an ascending series of ethanol to 100%, incubated in two changes of absolute acetone, infiltrated in Epon-Araldite resin (Hoch 1986) and flat embedded in a thin resin layer between Teflon-coated glass slides (Howard & O'Donnell 1987). The resin was polymerized for 24 h at 60°C.

Samples in resin were selected under an optical microscope, excised using a razor blade and mounted on resin stubs prior to ultramicrotomy. Semi-thin sections of 0.5 μm were stained with 1% toluidine blue and ultra-thin (70 nm) sections were counter-stained with uranyl acetate and lead citrate (Reynolds 1963), and used for electron microscopy analyses under a Philips CM10 transmission electron microscope (FEI Europe, Eindhoven, Netherlands).

**Results**

**Experiment 1: Effects of AMF on A. thaliana grown alone or in combination with T. pratense**

The effect of R. irregularis on A. thaliana growth (measured as aboveground biomass) depended on the presence or absence of the host species T. pratense (F1,15=16.05, P=0.001). In the absence of T. pratense, R. irregularis had no effect on the growth of A. thaliana compared with the respective controls, that is, A. thaliana plants inoculated with NM inoculum (Fig. 2a mono). However, when grown with T. pratense, R. irregularis significantly reduced the growth of A. thaliana by more than 50% (t=−5.05, P<0.001) (Fig. 2a mix).
Figure 2. Aboveground biomass (dry weight) of Arabidopsis thaliana grown in microcosms with (mix) and without (mono) Trifolium pratense (a) and total biomass (dry weight) of T. pratense (b). Plants were grown in microcosms inoculated with Rhizophagus irregularis [arbuscular mycorrhizal fungi (AMF)] or with non-mycorrhizal (NM) inoculum. Points are means±least significant differences (LSD, n=6). Treatments with non-overlapping intervals are significantly different at P=0.05.

Similarly, AM root colonization of A. thaliana depended on the presence of T. pratense. Roots of A. thaliana were barely colonized (0.5±0.2%) when grown without T. pratense but achieved a level of AM colonization of 12±2% when grown with T. pratense. However, arbuscules were not observed. Hyphae of R. irregularis supported by T. pratense reached a density of 2.39±0.21 m g⁻¹ in A. thaliana side of the microcosms. When NM inoculum was used, no colonization of A. thaliana roots was observed and the hyphal length density found in the A. thaliana side of the microcosms (0.04±0.01 m g⁻¹) is most likely due to non-AMF or dead R. irregularis hyphae present in the soil at the beginning of the experiment.

Contrary to A. thaliana, T. pratense plants inoculated with R. irregularis had significantly higher biomass than the NM control plants (F1,5=17.01, P=0.009) (Fig. 2b). When inoculated with R. irregularis, 53±3% of the root length of T. pratense was colonized with the formation of vesicles and arbuscules. No AM colonization was observed in NM T. pratense. Root nodules indicating symbiosis with rhizobia were also observed in T. pratense roots regardless of the presence or absence of AM colonization.

The reduction of A. thaliana biomass in the presence of R. irregularis could be (partially) due to increased above- and belowground competition with the neighbour T. pratense. In fact, in microcosms inoculated with R. irregularis we observed a trend, albeit statistically non-significant, of decreasing A. thaliana biomass with increasing T. pratense biomass (Supporting Information Fig. S1).

Experiment 2: Effects of AMF on three A. thaliana genotypes grown in combination with L. multiflorum

Growth of A. thaliana was significantly affected by R. irregularis supported by the host L. multiflorum (F1,47=31.76, P<0.001). As in the experiment with T. pratense (experiment 1), A. thaliana aboveground biomass was significantly reduced (always more than 50%) by the presence of R. irregularis, regardless of its genotype (F1,45=0.11, P=0.90) (Fig. 3a). In addition, similar to experiment 1, A. thaliana plants grown in the presence of R. irregularis mycelium showed root colonization by hyphae and vesicles but no arbuscules. Percentage of root length colonized by R. irregularis was comparable among the three genotypes (F2,18=0.58, P=0.57): 42±4% in genotype Col-0, 38±4% in myb72-1 and 43±4% in jin 1–2. Hyphal density of R. irregularis in A. thaliana Col-0 side of the microcosms was 3.07±0.28 m g⁻¹ while almost no hyphae were observed in the corresponding NM microcosms (0.03±0.01 m g⁻¹). When NM inoculum was used, no AM colonization was observed in A. thaliana plants.

Figure 3. Aboveground biomass (dry weight) of Arabidopsis thaliana genotypes Col-0, myb72-1 and jin1-2 grown in microcosms with Lolium multiflorum (a) and total
biomass (dry weight) of L. multiflorum according to A. thaliana genotype in the same microcosm (b). Plants were grown in microcosms inoculated with Rhizophagus irregularis [arbuscular mycorrhizal fungi (AMF)] or with non-mycorrhizal (NM) inoculum. Points are means±least significant difference (LSD, n=10). Treatments with non-overlapping intervals are significantly different at P=0.05.

On average, 64±1% of the root length of the host species L. multiflorum inoculated with R. irregularis was colonized, with the formation of vesicles and arbuscules. No AM colonization was observed in NM L. multiflorum plants. Growth of L. multiflorum was not affected by the genotype of the coexisting A. thaliana plants (F2,47=0.31, P=0.73) (Fig.3b) and, opposite to what we observed in the experiment with T. pratense (experiment 1), also not affected by the presence of R. irregularis (F1,47=0.61, P=0.44). Not surprisingly therefore, there was no relationship between the aboveground biomass of A. thaliana and the aboveground biomass of neighbour L. multiflorum, independent of the presence of R. irregularis (Supporting Information Fig. S2).

Despite the lack of a growth response, mycorrhizal L. multiflorum plants grown with A. thaliana Col-0 showed a significantly higher P concentration compared with NM controls in both the roots (F1,9=37.55, P<0.001) and the shoots (F1,9=51.10, P<0.001) (Table 1). Similarly, N concentration in the shoots of L. multiflorum was significantly higher when inoculated with R. irregularis (F1,8=11.93, P=0.008). However, there was also no relationship between P and N concentrations in the shoots of L. multiflorum and the aboveground biomass of neighbour A. thaliana (Supporting Information Fig. S3).

Table 1. Phosphorus (P) and nitrogen (N) concentrations in roots and shoots of Lolium multiflorum grown in combination with Arabidopsis thaliana Col-0 in experiment 2

To better describe the infection process and to understand the nature of the interaction between A. thaliana and R. irregularis, 6-week-old roots of A. thaliana were investigated with a combination of bright field, confocal and electron microscopy.

Bright field microscopy observations of cotton blue-stained roots highlighted the presence of a network of hyphae which mostly penetrated larger and thinner roots via hairs (Fig. 4, arrows). As illustrated in the details of Fig. 4b, the hypha, which is penetrating a root hair, is continuous with a small vesicle and with intraradical hyphae that showed limited branching. These observations were confirmed by sections from resin-embedded roots to be used for electron microscopy (Fig. 4c) and provided a first indication that the fungus was infecting A. thaliana roots and not simply growing on their surface. A rarer penetration way was directly through the epidermal cells (Fig. 4d). Many roots were strongly colonized by hyphae that reached the vascular cylinder and moved from primary roots to the secondary ones producing a high number of intraradical vesicles (Fig. 5a–c). Arbuscules were never observed.

Figure 4. Rhizophagus irregularis hyphae (F) penetrate Arabidopsis thaliana roots through root hairs (H, arrow) (a, b and c) and, more rarely, directly through the epidermal cells (d). Figures (a), (b) and (d) are cotton blue-stained roots while (c) is a
Figure 5. Rhizophagus irregularis produces vesicles inside Arabidopsis thaliana roots. Large vesicles (V), strongly blue stained, are localized in the root cells while a fungal hypha (F) penetrates through a root hair (arrow) (a and b). Electron microscopy of the vesicles: huge lipid globules (L) and electron-dense granules (arrowheads) (c). Figure (a) is a cotton blue-stained root while (b) and (c) are respectively bright field and transmission electron microscopy images from the same resin-embedded root. Bars correspond to 50 μm in (a), 75 μm in (b) and 10 μm in (c).

Confocal microscopy analysis of wheat germ agglutinin-FITC-stained root samples further confirmed fungal presence inside the roots of A. thaliana, providing more details on the colonization of outer and inner tissues. Figure 6a–c shows a root with superficial colonization. The reconstructed transverse sections show that hyphae are only adhering to the root surface. An example of epidermal cell penetration is presented in Fig. 6d,e, where the reconstructed cross section clearly shows that the fluorescent hypha is located in the centre of the epidermal cell lumen. The root shown in Fig. 6f–h is more heavily colonized and hosts both vesicles and hyphae located in the inner zone of the root. Lastly, Fig. 6i shows the production of spores from root-colonizing hyphae. Such spores protrude outside the root surface, while vesicles are hosted inside the root tissues.

Different from the non-infected control roots (Supporting Information Fig. S4a,b), the infected root tissues seemed senescent, with extensive areas of dead and partially collapsed cell walls (Fig. 5b). This observation was clearly confirmed by transmission electron microscopy when comparing the images of non-infected, live cells from control roots (Supporting Information Fig. S4c,d), rich in cytoplasm and cellular organelles, with those from colonized roots (Fig. 7a), where only the cell walls are recognizable and the cellular content has disappeared almost completely. Moreover, rod-shaped bacteria (Fig. 7a) and signs of cell wall degradation (Fig. 7c arrow) were occasionally found in the colonized tissues. By contrast, the fungus was actively thriving inside plant cells (Fig. 7a) and all the fungal organelles were easily distinguishable (Fig. 7a,b). The fungal wall was thick and homogenously layered.
without changes between the extraradical and the intraradical hyphae (Fig. 7b,c respectively).

Figure 7. Electron microscopy of Rhizophagus irregularis infecting Arabidopsis thaliana roots. The morphology of A. thaliana roots was greatly affected when compared with the control roots (see Supporting Information Fig. S4): cell cytoplasm was highly degraded, no organelles were distinguishable and bacteria (B) colonized the dead/dying tissues (a). The fungus (F) was actively thriving inside such dead root cells and all the fungal organelles were easily distinguishable: a nucleus (arrow) with an electron transparent chromatin, lipid globules (L), electron-dense granules (arrowheads) (a). Figure (b) is a detail of an extraradical hypha: lipid globules (L), electron-dense granules (arrowheads), mitochondria (M) with long cristae. The fungal wall (FW) was thick and homogenously layered without changes between the extraradical and the intraradical hyphae (b and c, respectively). Occasionally, the plant cell wall appeared degraded at the point of contact with the fungal hyphae (arrow) (c). Bars correspond to 2 μm in (a), 0.8 μm in (b), 1 μm in (c).

Discussion

This study is the first to demonstrate that the growth of the model plant A. thaliana is reduced in the presence of a previously established and active AM mycelium. Moreover, we show that A. thaliana roots can be extensively colonized by AM even if a functional symbiosis is not occurring, as suggested by the lack of arbuscules.

Results from the experiment with T. pratense (experiment 1) emphasize the importance of adding a host plant to the study system in order to assess the impact of AMF on non-host plants. The biomass of A. thaliana grown alone (in monocultures) was not influenced by the presence of R. irregularis and its roots were also not colonized. However, when A. thaliana was grown together with a host plant, either with T. pratense or L. multiflorum (experiment 2), considerable root infection levels were observed and biomass of A. thaliana was greatly reduced in the presence of R. irregularis. This is in concordance with other reports showing that NM plants can be infected, although usually in low levels and lacking arbuscules, in the presence of a host (Ocampo, Martin & Hayman 1980; Francis & Read 1995; Veiga et al. 2012). Together, the lack of AM colonization of A. thaliana roots in the absence of a host species and the absence of arbuscules in colonized A. thaliana roots confirms that interactions of A. thaliana with AMF are different from those of known host plants. It has been shown that, contrary to host species, A. thaliana is unable to recognize bioactive molecules present in AM fungal exudates that are important for the establishment of a functional AM symbiosis (Navazio et al. 2007; Genre et al. 2013). Therefore, experimental data strongly support the notion that the first steps of the pre-symbiotic dialogue between non-host species and AMF are already impaired.

It is possible that the growth reduction observed in A. thaliana in the presence of R. irregularis is, to some extent, due to nutrient removal from the A. thaliana side of the microcosm by AM hyphae that allocate nutrients to the coexisting host plant. In addition, increased light and water competition caused by larger host plants could affect the growth of neighbour A. thaliana. Indeed, in experiment 1, the biomass of host T. pratense was enhanced in the presence of R. irregularis while the biomass of A. thaliana was reduced. In order to reduce effects of competition, we performed
experiment 2 with the grass L. multiflorum as host because in earlier work it has been observed that many grasses are not very responsive to AMF (van der Heijden 2002; Smith, Grace & Smith 2009). Although the biomass of L. multiflorum did not differ between mycorrhizal and NM plants, P and N concentrations were still significantly higher in L. multiflorum inoculated with R. irregularis. Therefore, it is likely that the host species benefited from R. irregularis partially at the expenses of the neighbour A. thaliana. However, we could not find a clear relationship between growth reduction of A. thaliana and benefit (in biomass, P or N concentration) to the coexisting host plant species.

Another possibility is that the continuous root contact with AMF mycelium and root penetration activates costly plant defence responses (Walters & Heil 2007) that might result in reduced plant growth. In a study by Allen et al. (1989), cell death resembling a hypersensitive response (García-Garrido & Ocampo 2002) was observed in roots of the non-host plant Salsola kali upon colonization by AMF. Similarly, we observed that colonized A. thaliana roots were senescent or dead. It is however still unclear from our observations whether root cell death occurred as a defence response to AMF infection or if roots were previously dead (but see below).

Although an extensive transcriptomic analysis of R. irregularis did not provide any evidence of cell-wall-degrading enzymes, unlike saprotrophic fungi (Tisserant et al. 2012), indications exist suggesting the possibility of monosaccharide uptake by the extraradical mycelium of AMF (Helber et al. 2011). It has anyway been shown that AMF can take up P and obtain substantial amounts of N from decomposing organic materials (Ritz & Newman 1985; Hodge & Fitter 2010). Therefore, R. irregularis may have been using dead or senescent A. thaliana roots to acquire the latter nutrients, also using monosaccharides originating from the degrading cell walls. Moreover, dead roots may be a good substrate for spore formation as it has been shown for non-fixing root nodules (Scheublin & van der Heijden 2006), organic debris and old AMF spores in soil (Koske 1984). This may be another reason why we observed spores in A. thaliana roots. If this is the case, though, the cause of root death would still remain unexplained. The A. thaliana plants were only starting to flower and even secondary roots were colonized. Hence, as confirmed by the non-colonized control roots, which appeared to be viable, it is unlikely that all colonized roots were roots that naturally died due to root turnover and senescence.

In recent years, A. thaliana served as a valuable tool in unravelling plant responses to beneficial microorganisms (Van Wees, Van der Ent & Pieterse 2008). Mutants myb72-1 and jin1-2 that are impaired in genes encoding the transcription factors MYB72 and MYC2, respectively, are incapable of responding to beneficial Pseudomonas rhizobacteria or Trichoderma fungi (Pozo et al. 2008; Van der Ent et al. 2008; Segarra et al. 2009). However, in our microcosm experiments, both mutants behaved similar to wild-type Col-0 plants, suggesting that these components of the A. thaliana immune response to beneficial microbes are not associated with the effects of AMF observed in our experiments.

Our aim was to establish a model system to study non-host/AMF interactions. In the next step, the mechanism(s) responsible for the observed growth suppression/root cell death should be investigated, particularly by exploring the available ‘Arabidopsis toolbox’. For example, the potential involvement of defence mechanisms should be
tested by analysing the expression of well-characterized defence-related A. thaliana genes and/or using A. thaliana mutants that are impaired in their defence responses.

Conclusions

Recent studies have increased our understanding of the molecular dialogue going on between AMF and host plants (Pozo & Azcon-Aguilar 2007; Parniske 2008; Bonfante & Genre 2010; Bonfante & Requena 2011). The mechanisms responsible for negative interactions between AMF and non-host plants are, however, still poorly understood. A mechanistic understanding of such negative interactions is not only interesting from a biological perspective, but it also has the potential to be applied in weed management in view of recent observations that several aggressive NM weeds respond negatively to AMF (Jordan et al. 2000; Rinaudo et al. 2010; Veiga et al. 2012). By developing a model system with hyphal networks and showing negative mycorrhizal effects on the model plant A. thaliana, we set up the basis for future physiological, molecular and genetic studies on the mechanisms responsible for negative responses of non-hosts to AMF and AM incompatibility. Moreover, methodological advances have now made it possible to demonstrate that A. thaliana interacts with a wide range of soil bacteria to form a so-called root microbiome (Bulgarelli et al. 2012; Lundberg et al. 2012). In this context, our work highlights how AMF may be an important component of A. thaliana microbiome, notwithstanding its nature of NM host.

Acknowledgments

We thank Jan Jansa for providing the experimental study system and Beat Boller for the Trifolium pratense and Lolium multiflorum seeds. We also wish to thank Yann Hautier for the statistical advice. This work was supported by the Swiss Federal Government (Agroscope Reckenholz-Tänikon) and a grant from the Swiss National Science Foundation (Grant No. 315230_130764/1).