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

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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 & Friesse 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.

Fig. 1: schematic representation of a dual-compartment microcosm containing a host plant (left), used to pre-establish the arbuscular mycorrhizal (AM) network (dashed lines), and Arabidopsis thaliana (right). The two root systems were separated by a 30µm nylon mesh (permeable to hyphae) to reduce the effects of direct root competition.

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 (99min 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 5mg 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 in 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 400 W 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 1cm 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 5min in 0.5% NaClO in phosphate buffer, pH6.8, washed three times for 10min in the same buffer and incubated for 2h with wheat germ agglutinin-fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Milan, Italy) at a final concentration of $10\mu\text{g}\cdot\text{mL}^{-1}$ to stain the chitin of fungal cell walls. Fluorescence was excited with the 488nm band of an argon laser and imaged using a 500–540nm emission window for FITC and a 600–690nm 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 0.1mM cacodylate buffer (pH7.2) for 2h 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 24h 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 (70nm) 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* ($F_{1,15}=16.05$, $P=0.001$). 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 \pm 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 ($0.5\pm0.2\%$) when grown without *T. pratense* but achieved a level of AM colonization of $12\pm2\%$ when grown with *T. pratense*. However, arbuscules were not observed. Hyphae of *R. irregularis* supported by *T. pratense* reached a density of $2.39\pm0.21\text{m g}^{-1}$ 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 ($0.04\pm0.01\text{m g}^{-1}$) 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 ($F_{1,5}=17.01$, $P=0.009$) (Fig. 2b). When inoculated with *R. irregularis*, $53\pm3\%$ 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* ($F_{1,47}=31.76$, $P<0.001$). 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 ($F_{1,45}=0.11$, $P=0.90$) (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 ($F_{2,18}=0.58$, $P=0.57$): $42\pm4\%$ in genotype Col-0, $38\pm4\%$ in myb72-1 and $43\pm4\%$ in jin 1-2. Hyphal density of *R. irregularis* in *A. thaliana* Col-0 side of the microcosms was $3.07\pm0.28\text{m g}^{-1}$ while almost no hyphae were observed in the corresponding NM microcosms ($0.03\pm0.01\text{m g}^{-1}$). 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 \pm least significant difference (LSD, $n=10$). Treatments with non-overlapping intervals are significantly different at $P=0.05$.

On average, $64\pm1\%$ 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 ($F_{2,47}=0.31$, $P=0.73$) (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* ($F_{1,47}=0.61$, $P=0.44$). 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 ($F_{1,9}=37.55$, $P<0.001$) and the shoots ($F_{1,9}=51.10$, $P<0.001$) (Table 1). Similarly, N concentration in the shoots of *L. multiflorum* was significantly higher when inoculated with *R. irregularis* ($F_{1,8}=11.93$, $P=0.008$). 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

semi-thin section from resin-embedded roots. Bars correspond to 100µm in (a), 50µm in (b), and 20µm in (c) and (d).

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 10µm 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.

Figure 6. Confocal microscopy imaging of wheat germ agglutinin (WGA)-stained *Rhizophagus irregularis* infecting *Arabidopsis thaliana* roots. Hyphae adhering to the root epidermis are shown in (a)–(c). The yellow lines in (a) indicate the positions of the confocal reconstructed transverse sections shown in (b) and (c), and schematized in the associated drawings, which confirm the localization of hyphae (H) on the root (R) surface. A hyphal tip growing inside an epidermal cell is shown in (d). Its location inside the epidermal cell lumen is clearly visible in the reconstructed cross section (yellow line) presented in (e), where the green fluorescence of the hypha (arrowhead, H) is surrounded by the weaker signal outlining the cell walls (arrows, CW). Figure (f) shows a more heavily colonized root where both vesicles (V) and hyphae (arrowheads, H) can be observed in the cross sections presented in (g) and (h) [positioned along the corresponding yellow lines in (f)]. Spore production also occurred from root-colonizing hyphae, as shown in (i), where several WGA-labelled spores (S) protrude outside the root surface. Bars correspond to 75 µm in (a), (f) and (i); 10µm in (d).

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 homogeneously 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.

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