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Chapter 4

No evidence for allelopathic effects of arbuscular mycorrhizal fungi on the non-host plant *Stellaria media*

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Summary

Background and Aims

Increasing evidence suggests that several plants, particularly non-mycorrhizal species, are negatively affected by the presence of arbuscular mycorrhizal fungi (AMF). Mechanisms explaining suppressive effects of AMF are, however, still poorly understood. Here we test whether growth suppression of the non-host weed *Stellaria media* in the presence of AMF can be explained by mycorrhizal alellopathy.

Methods

We grew *S. media* in microcosms where an active AM mycelium was supported by neighboring wheat (*Triticum aestivum*) plants. To test for allelopathy, we added activated carbon (AC) to the soil substrate. In addition, we performed two complementary experiments where extracts from roots extensively colonized by AMF (AM exudates) were directly applied to *S. media* seeds and seedlings.

Results

Stellaria media plants grown in microcosms with AM mycelium showed an 8-fold biomass reduction compared to microcosms where AMF were absent. The addition of AC, which is thought to reduce allelopathic effects by binding organic compounds, did not greatly mitigate the negative effect of AM mycelium on *S. media* growth. Moreover, AM exudates did not significantly reduce *S. media* germination and growth.

Conclusions

Results from this study confirm that non-hosts like *S. media* can be highly suppressed in the presence of AMF. However, we found no evidence that mycorrhizal allelopathy was a major mechanism responsible for growth suppression of *S. media* in the presence of AMF. Other mechanisms might therefore be more significant in explaining suppressive effects of AMF on non-host plant species.

Introduction

There is an increasing appreciation of the impact of soil biota on plant growth, abundance and distribution (e.g. Grime et al. 1987, Bever et al. 1997, van der Heijden et al. 1998b, Klironomos 2002, 2003, van der Heijden et al. 2008). Negative effects of soil biota on plants are particularly interesting for unwanted species, such as agricultural weeds. Understanding such effects of soil biota on agricultural weeds can not only provide new insights on weed biology and ecology, but also lead to the development of new tools to manage these plants to tolerable levels in sustainable agroecosystems (Boyetchko 1996).

The arbuscular mycorrhizal (AM) fungi are important components of the soil biota. These obligate root endophytes are present in most terrestrial environments and associate with the majority of land plants (Smith & Read 2008). AM fungi (AMF) colonize plant roots and develop an extensive extraradical mycelium in the soil that assists their hosts with nutrient uptake, mainly phosphorus (P), in return for plant carbohydrates (Leake et al. 2004, Smith & Read 2008). Even if this relationship is by definition mutualistic, the effects of AMF colonization on plant growth vary from positive to negative (Johnson et al. 1997). Interestingly, in ruderal plants, including several important agricultural weeds, there seems to be a predominance of negative growth responses to inoculation with AMF (Francis & Read 1995, Rinaudo et al. 2010, Veiga et al. 2011).

Some plant species, members of families like Amaranthaceae, Brassicaceae, Caryophyllaceae, Cyperaceae and Polygonaceae, do not establish a functional symbiosis with AMF and are hence characterized as “non-mycorrhizal” or “non-hosts” (Wang & Qiu 2006). Notably, many non-host species have a ruderal lifestyle and are problematic weeds of agricultural fields (Francis & Read 1994, Jordan et al. 2000). Despite not establishing AM symbiosis, non-mycorrhizal plants can also be affected by AMF and most studies report a negative effect of AMF presence on the growth of non-mycorrhizal species (Allen et al. 1989, Francis & Read 1994, Sanders & Koide 1994, Francis & Read 1995, Johnson 1998, Rinaudo et al. 2010) and, similarly, on some mycorrhiza-defective mutants (Neumann & George 2005, Facelli et al. 2010). Therefore, there is accumulating evidence to suggest that ruderal plants, in particular non-mycorrhizal species, can be suppressed in the presence of AMF. Several mechanisms explaining deleterious effects of AMF on non-hosts have been proposed by

the authors of the previously mentioned studies but most remain fairly unclear. Francis & Read (1994) reported that aqueous extracts of soil containing AM mycelium inhibited non-host root development. However, the hypothesis that AMF exude allelopathic compounds that interfere with the growth of non-mycorrhizal plants has not been further tested. Recently, extracts from AM roots have been shown to reduce the germination of parasitic weeds of the genera *Striga* (Lendzemo et al. 2007, 2009), *Orobanche* and *Phelipanche* (Fernandez-Aparicio et al. 2010). This effect was attributed to a down-regulation of exudation of signalling molecules by AM roots (to prevent further colonization; e.g. strigolactones) which are known to induce both hyphal branching in AMF and germination of parasitic weeds (Akiyama et al. 2005, Yoneyama et al. 2008), but not to allelopathy.

The aim of this study was to investigate whether suppressive effects of AMF on the growth of the non-host plant *Stellaria media* result from allelopathy. Allelopathy refers hence to the suppression of plant growth through toxic compounds (Fitter 2003). Both plants (e.g. Putnam 1988, Bais et al. 2003) and microorganisms, including fungi (e.g. Schulz et al. 2002, Macias-Rubalcava et al. 2010), have been shown to produce such compounds. *Stellaria media* (common chickweed; family Caryophyllaceae) is a ruderal plant species (Sobey 1981, Briggs et al. 1991) and a common and highly competitive weed in cereals and broad-leaved arable crops (Lutman et al. 2000, van Delden et al. 2002).

To test for the effects of AMF on *S. media,* we grew *S. media* in microcosms where neighboring wheat (*Triticum aestivum*; mycorrhizal host species) plants inoculated with the AM fungus *Glomus intraradices* had been previously planted, ensuring the presence of an active AM mycelium (Fig. 1). Microcosms without AMF served as a control. Simultaneously, to test for allelopathy, we added activated carbon to the soil substrate of half of the microcosms (with and without AMF). Activated carbon (AC) is often used in allelopathy studies due to its capacity of adsorbing allelochemicals and hence minimizing their effects on target species (e.g. Mahall & Callaway 1992, Callaway & Aschehoug 2000, Ridenour & Callaway 2001, Inderjit & Callaway 2003, Kulmatiski & Beard 2006). However, it has been shown that AC can alter nutrient availability and plant growth, as well as affecting plant symbiotic associations with AMF, which might lead to biased interpretations of the effects of allelopathic substances (Lau et al. 2008, Weisshuhn & Prati 2009, Wurst et al. 2010). For this reason, we included two

complementary experiments where aqueous extracts from roots colonized with the AM fungus *G. intraradices* were directly applied to *S. media* seeds and seedlings.

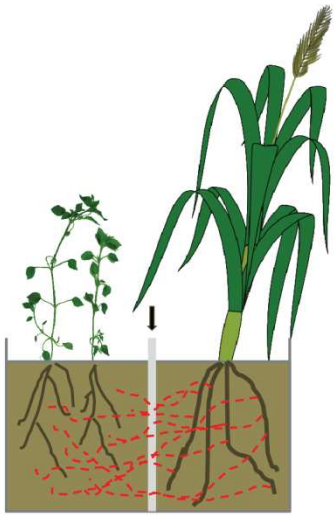

Figure 1. Schematic representation of the experimental system used in experiment 1. *Stellaria media* plants (left) were grown in the presence (or absence) of AM mycelium (dashed lines) supported by a neighboring host species (wheat; right). The root systems of the two plant species were separated by a 30 µm nylon mesh (permeable to AM hyphae but not to roots; arrow) to minimize the effects of direct root competition. The soil substrate in both halves of the microcosms was supplemented (or not) with activated carbon to test for allelopathic effects of the AM mycelium on *S. media* growth.

Materials & Methods

Plant material, fungal inoculum and soil mixture

In this study we used seeds of *Triticum aestivum* L. cv. Runal obtained from Agroscope Reckenholz-Tänikon Research Station ART, Switzerland, and of *Stellaria media* (L.) Vill. obtained from Appels Wilde Samen GmbH, Germany (www.appelswilde.de). Seeds were surface sterilized in 1.25% sodium hypochlorite for 10 min and subsequently rinsed with dH_2O.

We used soil inoculum of the AM fungus *Glomus intraradices* Schenck & Smith (isolate BEG 21, described in van der Heijden et al. 2006). Inoculum was propagated as a pure culture on *Plantago lanceolata* L. for 8 months, in pots filled with a sterilized (99 min at 121°C) mixture of quartz sand with 20 % (v:v) field soil. Roots and soil from similar but uninoculated *P. lanceolata* pot cultures served as non-mycorrhizal (NM) control inoculum. *G. intraradices* colonized 88% of the root length of *P. lanceolata* as assessed microscopically after staining with pen-ink vinegar (see details of the method below). No colonization of *P. lanceolata* roots was observed in the control inoculum.

Field soil was collected at a certified organic farm in Reckenholz (Zurich, Switzerland), sieved through 1 cm mesh to remove large stones and root fragments and mixed with quartz sand at a 1:1 ratio in volume. The soil-sand mixture was autoclaved for 99 min at 121°C and constituted the soil substrate used.

Experiment 1: effect of arbuscular mycorrhizal mycelium on the growth of *S. media* in the presence or absence of activated carbon

In this experiment we tested the effect of AM mycelium on the growth of *S. media* with and without active carbon (AC) added to the soil substrate. AC was used to adsorb potential allelochemicals exuded by AM mycelium, following earlier work (Callaway & Aschehoug 2000, Inderjit & Callaway 2003, Kulmatiski & Beard 2006, Weisshuhn & Prati 2009, Wurst et al. 2010). The experiment was set up as a randomized block design with two factors. One factor, "carbon presence", contained two levels: soil substrate with (AC treatment) and without AC (no-carbon – NC - treatment). The other factor, "AM mycelium presence", also contained two levels: with *G. intraradices* inoculum (AM treatment) and with non-mycorrhizal control inoculum (NM treatment). This makes a total of four treatment combinations. Each treatment was replicated eight times and assigned to a block, making a total of eight blocks and 32 microcosms (experimental units).

Each microcosm was divided in two equal parts by a 30 μm nylon mesh to separate roots (and hence reduce direct root competition) but still allowing the passage of AM hyphae (Fig. 1). Each half received, according to the treatment, 700 g of autoclaved soil substrate or the same amount of autoclaved soil substrate mixed with AC (Sigma) at a concentration of 20 mL/L (Weisshuhn & Prati 2009), with 7% (w:w) *G. intraradices* soil inoculum or with the same amount of NM inoculum. All the microcosms received 10 mL (5 mL each half) of inoculum washing (60 g of a mixture of equal parts in weight of *G. intraradices* soil inoculum and NM soil inoculum, suspended in 1 L dH_2O and filtered through Whatman filter paper) to correct for possible differences in microbial communities.

Four seeds of wheat were sown in one half of the microcosms and, upon germination, wheat seedlings were thinned down to two. To allow sufficient time for AMF colonization and the development of a hyphal network, wheat seedlings grew for 4 weeks before ten *S. media* seeds were sown in the other half of each microcosm. Upon germination, *S. media* seedlings where thinned down to four.

Plants were watered three times a week with the same volume of dH_2O. During the growing season, plants were twice supplied with 10 mL (5 mL 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 [6 mM KNO_3, 4

mM $CaCl_2$, 1 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$]. Plants were maintained in the glasshouse with constant lighting provided by 400 W high-pressure sodium lights to a daylength of 12 h. Temperatures in the glasshouse varied from 18ºC to 25ºC. Wheat and *S. media* plants were harvested 10 and 6 weeks after sowing, respectively.

At the time of harvest, shoots were cut at the soil surface, oven-dried at 80°C and the dry biomass recorded. Roots were carefully washed to remove soil particles, cut into 1 cm segments and mixed. A root subsample of known weight was taken for determining root colonization by AMF. The remaining roots were weighed, oven-dried at 80°C and the dry biomass was recorded. The dry biomass of the root subsample was inferred by multiplying its fresh weight with the dry to fresh weight ratio of the oven dried roots. Sum of shoot and root dry biomass gave the total biomass of each plant species per microcosm.

Roots taken for the assessment of AMF colonization were cleared in 10% KOH and stained with an ink-vinegar solution containing 5% Parker Quink Black in household vinegar (adaped from Vierheilig et al. 1998). The modified line intersection method (McGonigle et al. 1990) was used to calculate the percentage of root length colonized by *G. intraradices*. At least 100 intersections were examined, for each root sample.

Oven-dried roots and shoots were ground together and phosphorus (P) and nitrogen (N) concentrations of *S. media* and wheat were determined. Firstly, P concentration was determined photometrically after calcination and extraction with hydrochloric acid (Siegel 1976)**.** When there was enough ground plant material left from the latter analysis, N was determined according to the Dumas combustion procedure (Houba et al. 1989). Due to lack of enough ground material, only *S. media* plants grown in the absence of AM mycelium were analyzed and only for P concentration. The total P content of *S. media* and the total P and N content of wheat per microcosm was determined by multiplying the respective P or N concentration in a microcosm by the total biomass of that species in that microcosm. The mean of the 8 replicates per treatment was subsequently calculated and is presented.

Experiment 2: effect of arbuscular mycorrhizal exudates on the germination of *S. media*

In this experiment we tested the effect of AM fungal exudates on the germination of *S. media*. Exudates were collected from mycorrhizal and non-mycorrhizal *P. lanceolata*

plants. *P. lanceolata* plants were grown for 6 months in pots filled with an autoclaved (99 min at 121°C) mixture of quartz sand with 20 % (v:v) field soil and inoculated with *G. intraradices* BEG 21 (AM plants) or left un-inoculated (NM plants). Plants were gently removed from the microcosms and roots were carefully washed, not to break all extraradical AM mycelium attached to the roots. Entire plants were placed in 2-L flasks and dH_2O was added to completely immerse the root systems. The flasks were wrapped in aluminum foil and left for 48 hours in the glasshouse. Plants were then removed from the flasks, the root systems cut off, briefly dried in absorbent paper and weighed. Exudate solutions in each flask were adjusted with dH_2O to achieve a root density of 0.03 g of *P. lanceolata* root fresh weight per mL of root solution, following the approach by Fernández-Aparicio et al. (2010). Finally, exudate solutions were filtered through Whatman filter paper. Roots were further cut in 1 cm segments, mixed and a subsample of AM or NM roots taken for assessing root colonization (as previously described). *G. intraradices* colonized 93% of the root length of *P. lanceolata* while no colonization was observed in NM plants.

Thirty *S. media* seeds were placed on Whatman filter paper in 9 cm diameter Petri dishes. Petri dishes received 2 mL of either: AM exudate solution, NM exudate solution or dH_2O as an additional negative control. Each treatment was replicated 10 times making a total of 30 experimental units (Petri dishes). Individual Petri dishes were sealed with Parafilm and kept in the dark at RT for 2 weeks. Germination was determined by counting the number of germinated seeds in each Petri dish. A seed was considered germinated if the radicle was visible.

Experiment 3: effect of arbuscular mycorrhizal exudates on the growth of *S. media*

In this experiment we tested the effect of AM exudates on the growth of *S. media*. The exudate solutions were prepared as previously described for experiment 2 and stored at 4ºC in between applications. Five *S. media* seeds were sown in plastic pots containing 215 g of soil substrate and covered in plastic film to maintain moisture content. Upon germination, seedlings were thinned down to one seedling of similar size per pot. Germinated seedlings received 5 mL of either: AM exudate solution, NM exudate solution or dH_2O, 3 times per week, until the end of the experiment. Each treatment was replicated 10 times making a total of 30 experimental units (pots).

Pots were randomly distributed in the glasshouse and their positions randomized every week. Pots were watered with dH_2O by weight as required to maintain soil mixture in the range of 12-15%. Plants were allowed to grow for 5 weeks in the glasshouse where 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 14 h. During the growing season the temperatures in the glasshouse ranged from 15ºC to 27ºC.

At the time of harvest, shoots were cut at the soil surface, oven dried at 80°C and the biomass recorded. Roots were carefully washed to remove soil particles, briefly dried in absorbent paper, oven-dried at 80°C and the dry biomass recorded. Sum of shoot and root dry biomass gave the total biomass of *S. media* per pot (plant).

Statistical analyses

For the analyses of data from experiment 1 we used mixed-effects models (Pinheiro and Bates 2000) since our design included fixed and a random (block) effects. We used the lme function from the nlme library for R 2.9.0 (R Development Core Team 2009). For the analysis of AMF colonization in *S. media* and wheat, "carbon presence" was treated as fixed effect. For the analysis of *S. media* and wheat total biomass and for the analysis of P and N content in wheat, "AM mycelium presence" and "carbon presence" were treated as fixed effects. P content of *S. media* plants grown without AM mycelium (NM treatment) was analyzed with "carbon presence" treated as fixed effect. Whenever there was heterogeneity in the variance structure between treatments (*S. media* biomass between "AM mycelium presence" treatments and *S. media* P content between "carbon presence" treatments) we used the varIdent() function to allow each treatment to have a different variance. For the relationship between *S. media* and wheat AMF colonization, "carbon presence" was treated as fixed effect.

Experiments 2 and 3 did not include any random effect and were analyzed using generalized linear least squares with the gls function from the nlme library. We analyzed the effect of the solution applied (AM exudate solution, NM exudate solution or dH_2O) on the number of germinated *S. media* seeds (experiment 2) and on *S. media* total biomass (experiment 3) with ANOVA. Since there was heterogeneity in the variance structure of *S. media* germination and biomass between the three exudate solution treatments, we used the varIdent() function to allow each treatment to have a

different variance. In experiment 3, two *S. media* plants treated with dH_2O died and these replicates were therefore removed from the analysis.

In the text we present estimates of the means with their standard errors (s.e.m) and regression slopes with their 95% confidence interval (CI).

Results

Experiment 1: effect of arbuscular mycorrhizal mycelium on the growth of *S. media* in the presence or absence of activated carbon

The presence of AM mycelium significantly reduced the biomass of *S. media* both in the absence (NC treatment; $t = -17.9$, $P < 0.0001$) or presence (AC treatment; $t = -7.0$, $P < 0.0001$) of activated carbon (AC), compared to the respective NM controls (Fig. 2). In treatments without AC, the presence of AM mycelium caused an 8-fold biomass reduction of *S. media* while a ~5-fold biomass reduction was observed in treatments with AC. The biomass reduction by the AM mycelium was weaker in the AC treatment because the biomass of NM *S. media* plants was lower ($t = -6.0$, $P < 0.0001$), while the biomass of *S. media* plants grown with AM mycelium was similar between AC and NC treatments. As a consequence, a significant "AM mycelium presence" × "carbon presence" interaction term was observed ($F_{1,21} = 18.8$, $P < 0.001$), demonstrating that mycorrhizal effects on *S. media* biomass were not the same in treatments with or without AC. Similarly, the total P content of NM *S. media* plants was significantly lower in microcosms with AC (2.29 ± 0.25 mg) than without (4.18 ± 0.34 mg; $F_{1,5} = 20.4$, $P < 0.01$).

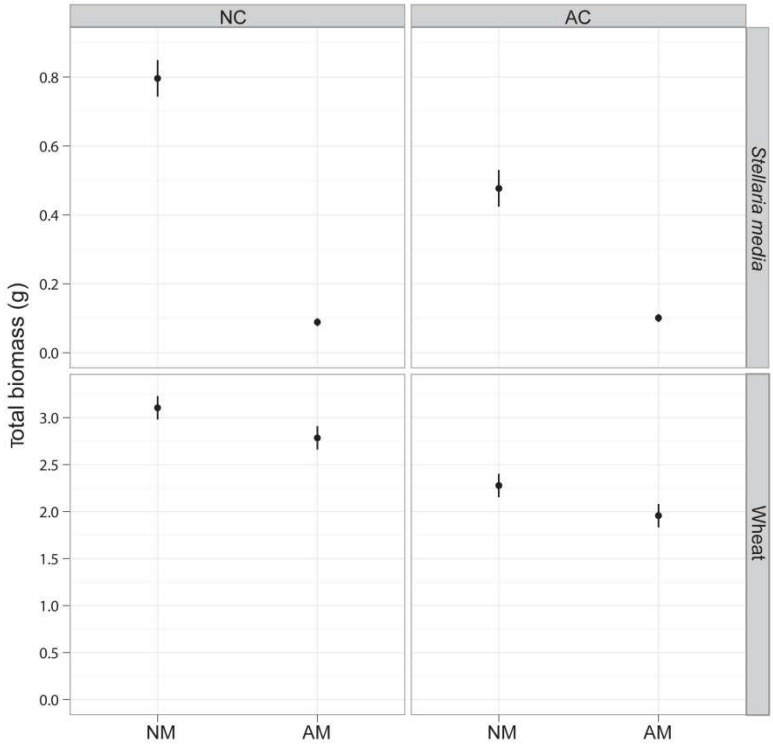

Figure 2. Total biomass (g) of *S. media* and neighboring wheat in experiment 1. Plants were grown with or without addition of activated carbon to the soil substrate (AC and NC, respectively) and in the presence or absence of *G. intraradices* (AM and NM, respectively). Points are means of 8 replicates ± s.e.m.

The total biomass of wheat was influenced by "AM mycelium presence" ($F_{1,22}$ = 9.6, P < 0.01) and "carbon presence" ($F_{1,22}$ = 63.8, P < 0.0001) but not by their interaction. The presence of *G. intraradices* (AM treatment) significantly reduced the biomass of wheat, equally in microcosms with or without AC (t = -3.1, P < 0.01) (Fig. 2). On the other hand, NM or AM wheat plants grown with AC had a significantly lower biomass than the respective NM or AM wheat plants grown without AC (t = -8.0, P < 0.0001). In contrast to the biomass, P and N content of wheat plants were strongly influenced by the interactive effect of "AM mycelium presence" and "carbon presence" ($F_{1,21}$ = 13.3, P < 0.01 for P; $F_{1,21}$ = 9.4, P < 0.01 for N). In microcosms without AC (NC treatment), AM wheat plants had significantly more P and N than NM plants (t = 10.84, P < 0.0001 for P; t = 5.9, P < 0.0001 for N) (Fig. 3). In the presence of AC, AM wheat plants still had higher P and N content than NM plants but the difference was smaller and only statistically significant for P (t = 5.67, P < 0.0001). Generally, wheat plants grown in microcosms with AC, either AM or NM, had significantly lower (P < 0.05) P and N content than the respective AM or NM plants grown without AC.

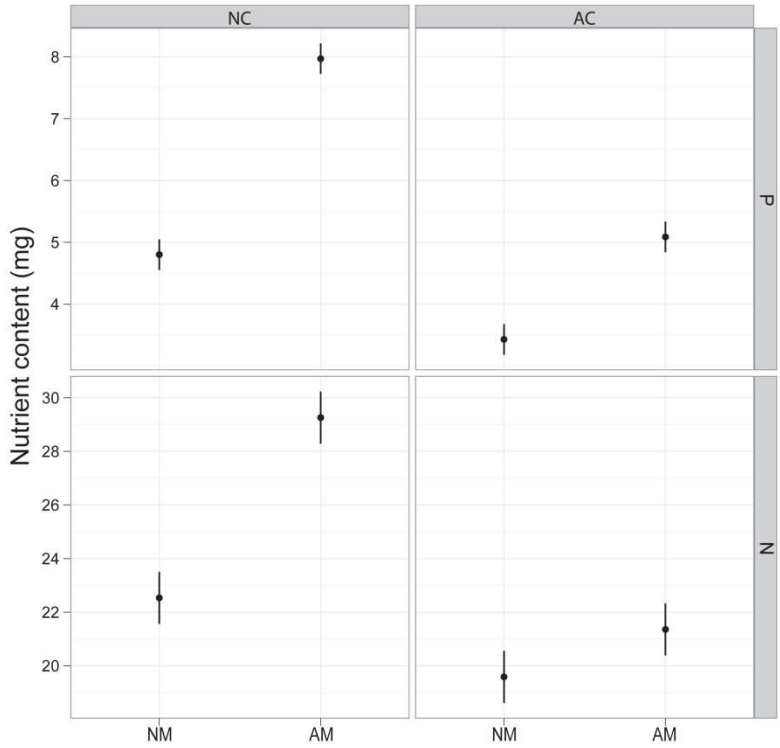

Figure 3. Total P and N content (mg) of wheat plants in experiment 1. Plants were grown with or without addition of activated carbon to the soil substrate (AC and NC, respectively) and in the presence or absence of *G. intraradices* (AM and NM, respectively). Points are means of 8 replicates ± s.e.m.

No colonization was observed in NM control plants for *S. media* or wheat. Wheat plants inoculated with *G. intraradices* showed extremely high levels of AMF colonization and all the typical fungal structures – hyphae, vesicles and arbuscules – were observed. The percentage of root length colonized by *G. intraradices* in wheat was significantly different between the carbon treatments ($F_{1,7}$ = 7.6, P = 0.028), being reduced from 95 ± 1.5% in the NC treatment to 91 ± 1.5% in the AC treatment. *S. media* plants grown in the presence of AM mycelium also showed some AMF colonization but only hyphae and vesicles were observed, and never arbuscules. Contrary to what we observed for wheat, the percentage of root length colonized by *G. intraradices* in *S. media* did not differ among carbon treatments: 15.5 ± 3.8% in both NC and AC treatments. There was a significant positive linear relationship between AMF colonization of *S. media* and AMF colonization of wheat in the other half of the microcosms ($F_{1,7}$ = 12.6, P = 0.012), similar for both carbon treatments (slope with 95% CI = 1.8 (0.6 – 3.1)) (see Supporting Information Fig. S1).

Experiment 2: effect of arbuscular mycorrhizal exudates on the germination of *S. media*

The number of germinated *S. media* seeds depended on the solution applied ($F_{2,27}$ = 92.3, $P < 0.0001$). Germination was significantly higher when seeds were treated with dH_2O compared to exudate solutions ($P < 0.05$; Fig. 4a). More importantly, the germination of *S. media* did not differ between seeds treated with NM and AM exudates (t = -0.73, P = 0.47).

Experiment 3: effect of arbuscular mycorrhizal exudates on the growth of *S. media*

The total biomass of *S. media* depended on the solution applied ($F_{2,25}$ = 43.1, $P <$ 0.0001). Plants treated with dH_2O had a significantly lower biomass compared to those treated with exudate solutions ($P < 0.05$; Fig. 4b). The biomass of *S. media* treated with AM exudates was slightly lower than the biomass of plants treated with NM exudates but this difference was not statistically significant (t = -1.53, P = 0.14).

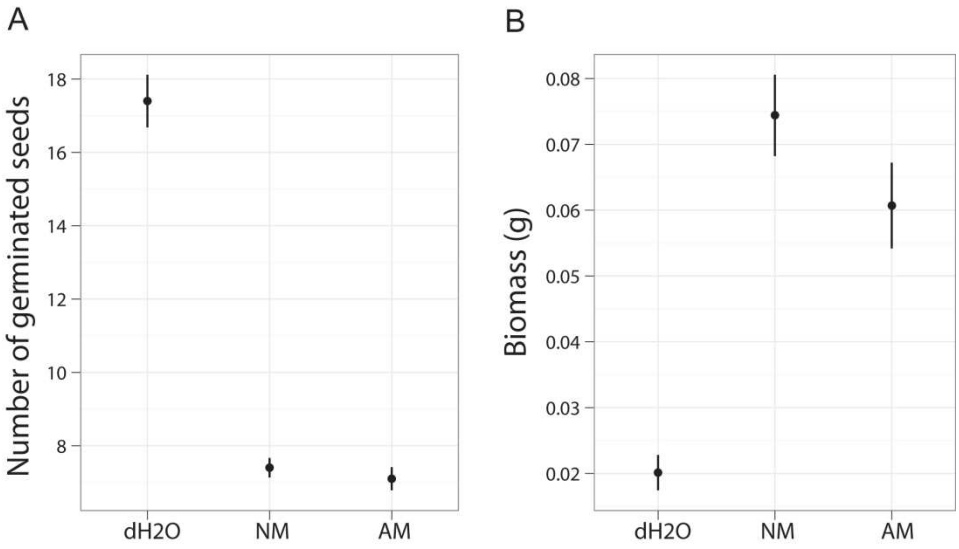

Figure 4. Effect of exudate solutions on the germination (A, experiment 2) and biomass (B, experiment 3) of *S. media*. Exudate solutions were obtained from *P. lanceolata* roots colonized with *G. intraradices* (AM) or non-mycorrhizal (NM) roots. dH_2O was used as an additional control. Points are means of 10 replicates ± s.e.m. except for the biomass of *S. media* plants treated with dH_2O (in B) which is a mean of 8 replicates ± s.e.m.

Discussion

Increasing evidence suggests that deleterious effects of AMF are common on ruderal plant species like many agricultural weeds (Francis and Read 1995, Rinaudo et al. 2010, Veiga et al. 2011) and, in particular, on non-mycorrhizal hosts (Allen et al. 1989, Francis & Read 1994, Sanders & Koide 1994, Johnson 1998). In agreement, this study shows that the presence of an active AM mycelium supported by a mycorrhizal host plant reduces the growth of the non-mycorrhizal weed *S. media*. However, we found no evidence that allelopathy is a major mechanism by which AMF can suppress *S. media* growth.

In the first experiment, *S. media* suffered a significant 8-fold biomass reduction in the presence of the AM fungus *G. intraradices* (see Fig. 5 for a strong visible effect).

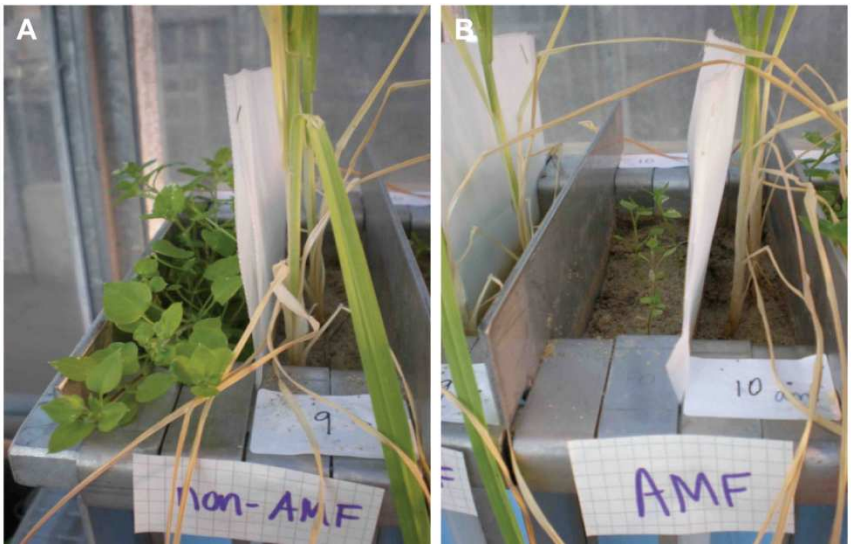

Figure 5. Suppressive effect of *G. intraradices* mycelium on 6-week-old *S. media* plants (panel B), compared to when the AM fungus was absent (panel A). The microcosms represented did not contain activated carbon.

In theory, if this effect resulted from toxic compounds produced by AMF, the deleterious effect on *S. media* growth should be mitigated by AC addition as illustrated in Fig. 6. We observed that, when AC was mixed into the soil substrate, the biomass of *S. media* was still significantly reduced in the presence of *G. intraradices* but the difference was smaller (*c*. 5-fold instead of 8-fold reduction). The interpretation of such result is complicated by a negative effect of AC on *S. media* biomass, as demonstrated by the lower biomass of NM *S. media* control plants. If we hypothesize that the addition of AC should cause a similar biomass reduction of *S. media* in both the absence or

presence of AM mycelium, like it was observed for wheat, then the smaller difference found in the AC treatment could be due to the adsorption of allelochemicals. Still, the effect of allelopathy on growth suppression of the non-host *S. media* would have been minimal.

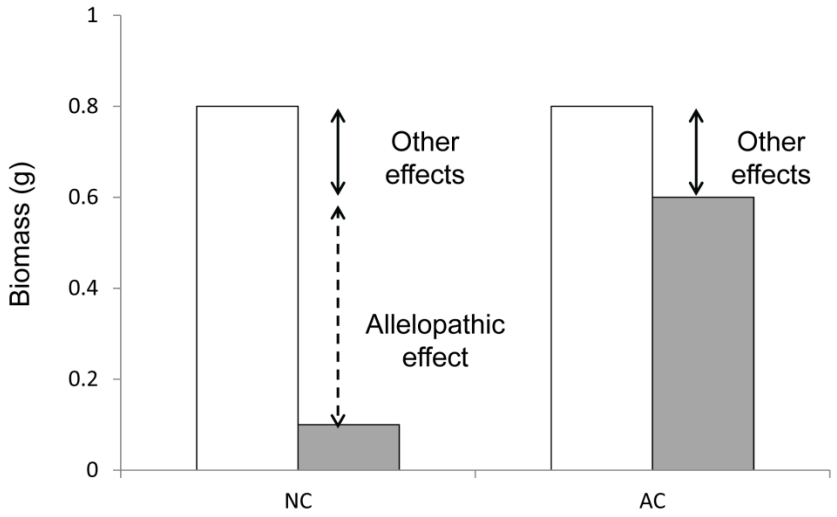

Figure 6. Expected biomass of *S. media* if allelopathy was the main mechanism responsible for biomass reduction in the presence of AM mycelium. Open bars represent the biomass of *S. media* plants grown in the absence of AM mycelium and closed bars represent the biomass of *S. media* plants growth in the presence of AM mycelium. When activated carbon (AC) is added to the soil substrate, only other effects (non-allelopathic) would occur because AC is supposed to bind most organic compounds. Adapted from Lau et al. (2008).

The use of AC has been highly criticized in studies of allelopathy due to unwanted effects on plant growth; it has been shown to alter nutrient availability directly or indirectly by affecting microbial communities (Wardle et al. 1998, Pietikainen et al. 2000, Callaway & Ridenour 2004, Lau et al. 2008, Weisshuhn & Prati 2009). The effects of AC are not consistent, though: in most cases AC has been shown to increase plant biomass/nutrient availability (Lau et al. 2008, Weisshuhn & Prati 2009) but, in some others, like in our study, addition of AC resulted in biomass decrease (Lau et al. 2008, Wurst et al. 2010). This discrepancy might be due to factors such as the source of AC, the plant species, the type of substrate and the experimental conditions (Lau et al. 2008). In this study, both *S. media* and wheat biomass were reduced by the addition of AC in microcosms without *G. intraradices*. In addition, total P content of *S. media* and total P and N content of wheat were also generally lower in plants grown in the presence of AC. Together, these results indicate that AC had a negative effect on

nutrient availability. It is possible that AC reduced nutrient availability by adsorbing organic N and P, consequentially decreasing their mineralization rates (Kulmatiski & Beard 2006). Another side effect of AC is the possible disruption of mycorrhizal associations with their hosts by adsorbing signaling molecules that control for mycorrhization (Weisshuhn & Prati 2009, Wurst et al. 2010). In this study we also observed a significant decrease of AMF colonization of wheat roots in the presence of AC, although average colonization remained quite high, above 90%.

We observed slight AMF colonization in 6-week-old *S. media* roots that was positively related to root colonization levels of the neighbor wheat plants. However, we did not observe any arbuscules which are the main organs where nutrient exchanges between symbiotic partners occur (Parniske 2008). Therefore, there is no indication that, at least at the time plants were harvested, a functional symbiosis between *S. media* and AMF was occurring. Root infection of non-hosts is not completely unexpected; it has been previously shown, using other plant species, that non-mycorrhizal plants can be colonized by AMF, often in low amounts, usually lacking arbuscules and, in most cases, when non-host plants are grown together with a mycorrhizal host that provides the carbohydrates necessary for the survival and development of AMF (Hirrel et al. 1978, Ocampo et al. 1980, Francis & Read 1995, Daisog et al. 2012). Occasionally, arbuscules have also been found in the roots of non-host species (e.g. Allen et al. 1989, Regvar et al. 2003, Rinaudo et al. 2010), as well as root infection in the absence of a host plant (e.g. Glenn et al. 1985, Allen et al. 1989).

The experiments employing extracts from AM roots further indicate that if there is an allelopathic effect of AMF, this effect is likely minimal; there was a slight, but not significant, biomass reduction of *S. media* plants treated with AM extracts compared to those treated with NM extracts, and AM extracts did not affect *S. media* germination. We cannot however exclude the possibility that this experimental approach precluded the expression of a putative allelopathic effect. For example, it is clear from the difference in *S. media* germination and biomass between the dH_2O treatment and the treatments with unsterile root extracts, that other factors (e.g. presence of nutrients and microbial communities in the extracts) influenced *S. media* behavior. One can hence suggest that the effect of these factors on plant germination/growth might have hindered a putative effect of allelochemicals produced by AMF. Still, an even more complex environment is expected in the field. Hence, if putative allelopathic effects of AMF are

counteracted or hidden by other factors that influence plant growth and development, such effects are anyway unlikely to be ecologically relevant. On the other hand, we collected AM extracts by detaching roots from the soil (thereby breaking up AM mycelium) and incubating the roots for 48 hours in dH_2O, thus diluting the extracts. This raises questions about whether putative AM allelopathic compounds were produced in sufficient amounts and/or were active and strong enough to cause an effect on *S. media* growth and germination. Ideally, exudates should be harvested from AM mycelium isolated from the substrate and concentrated before application. Nevertheless, in studies using similar extraction approaches, a differential effect of exudates from roots colonized with AMF (compared to exudates from non-mycorrhizal roots) was observed on the germination of parasitic weeds (Lendzemo et al. 2007, 2009, Fernandez-Aparicio et al. 2010). Finally, it is possible that putative allelopathic compounds are only expressed when the fungal hyphae are in contact with the roots of incompatible plant species. However, in the study by Francis and Read (1994) which reported inhibition of non-host root development by aqueous extracts of soil containing AMF mycelium, it is not mentioned whether there were non-host plants growing in contact with the fungal mycelium. Overall, despite the limitations of both experimental approaches used in this study (addition of AC or addition of AM root exudates), results from the three experiments put together, do not provide evidence that AM allelopathy is a main mechanism responsible for growth reduction of *S. media* in the presence of AMF.

Despite studies suggesting that allelopathy might be ecologically significant in the case of exotic invasions (Callaway & Aschehoug 2000, Bais et al. 2003, Callaway & Ridenour 2004, Vivanco et al. 2004), the general relevance of this mechanism in field conditions is controversial (Harper 1977, Fitter 2003, Inderjit & Callaway 2003). For example, it has been argued that, for normally coexisting organisms, both the producers of the toxin as the recipient species would evolve resistance to it (Fitter 2003, Callaway et al. 2005). Since AMF are present in most terrestrial ecosystems, though not so abundant in ruderal habitats, it is possible that, even if AMF produce allelochemicals, these might have little or no effect on resistant non-mycorrhizal plant species.

Mechanisms other than allelopathy might have hence been responsible for the observed growth reduction of *S. media* in the presence of AM mycelium supported by a host species. It is known that AMF can influence the competitive relationships between

coexisting plant species (Hartnett et al. 1993, Hetrick et al. 1994, Hart et al. 2003, Scheublin et al. 2007, Wagg et al. 2011b). Even if a recent study has shown that AMF favored a non-host plant species competing with a host (Daisog et al. 2012), the majority of research suggests that, in the presence of AMF, non-host species have a competitive disadvantage in the presence of plants hosting AMF (Sanders & Koide 1994, Francis & Read 1995, van der Heijden et al. 1998b). Similarly, competition studies between tomato (a mycorrhizal species) and a surrogate non-mycorrhizal mutant have shown that some AMF species changed competitive interactions to the detriment of the non-host mutant and this was mainly attributed to depletion of P by AM mycelium and delivery to the neighboring host plant (Cavagnaro et al. 2004, Facelli et al. 2010). Although the host species in our study (wheat) did not benefit from AMF colonization in terms of biomass increase, AM wheat plants still had higher P and N content than the respective NM controls, indicating that these nutrients might have been acquired at the expenses of the neighbor non-host. In addition, the deleterious effect of *G. intarardices* on *S. media* growth could be related to the presence of the fungus inside the roots. A relationship between root infection and negative effects on a non-mycorrhizal plant inoculated with AMF has been reported by Allen et al. (1989). These authors observed that AMF colonization of the non-mycorrhizal plant *Salsola kali* caused root browning and cell death, resembling a hypersensitive response (García-Garrido & Ocampo 2002). Investment in plant defense mechanisms following AMF root penetration of incompatible hosts could limit the allocation of recourses available for plant growth. Furthermore, Allen et al. (1989) also observed arbuscules in germinating *S. kali* seedlings and, in a later study, *S. kali* C4 signature was found on AMF spores in the field (Allen & Allen 1990). Such observations raise the interesting possibility that early-formed arbuscules (that may be no longer present at a later stage of plant development, as in our case, in 6-week-old *S. media*) enables AMF to take up C from non-host species, which could contribute to growth depressions. Finally, it has been suggested that AMF colonization or contact with the roots might inactivate the plant's direct root P-uptake pathway and reduce the total P uptake and growth (Neumann & George 2005, Smith et al. 2009, Facelli et al. 2010). Future studies should now focus on these alternative mechanisms.

The antagonistic effect of AM mycelium on non-mycorrhizal weeds such as *S. media* in our study is likely to be one of the factors responsible for the restriction of such species

to disturbed habitats where AMF are absent or low in abundance (Francis & Read 1995). Similarly, it can partially explain the success of these species as agricultural weeds, since several agroecosystems are highly disturbed environments where, accordingly, abundance and diversity of AMF has been shown to be reduced (Johnson 1993, Helgason et al. 1998, Jansa et al. 2002, Oehl et al. 2003). Several beneficial ecosystem services of AMF have been pointed out (e.g. van der Heijden & Horton 2009) and discussed in the context of more sustainable farming systems (Gianinazzi et al. 2010). The possibility of reducing the growth of some weed species and maintaining weed populations at tolerable levels further emphasizes the importance of preserving this component of the soil biota.

Acknowledgements

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Supporting Information

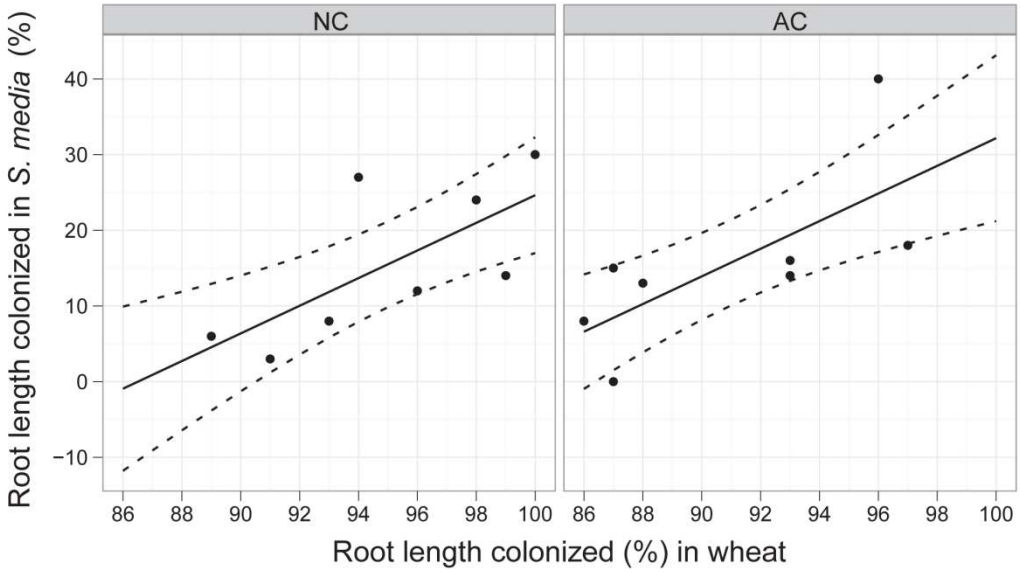

Figure S1 Relationship between percentage of root length colonized by *G. intraradices* in wheat and in neighbor *S. media*. Plants were grown with or without addition of activated carbon to the soil substrate (AC and NC, respectively). Results are shown as linear regression slopes and 95% CI.