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Do fungivores trigger the transfer of protective metabolites from host plants to arbuscular mycorrhizal hyphae?

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Abstract. A key objective in ecology is to understand how cooperative strategies evolve and are maintained in species networks. Here, we focus on the tri-trophic relationship between arbuscular mycorrhizal (AM) fungi, host plants, and fungivores to ask if host plants are able to protect their mutualistic mycorrhizal partners from being grazed. Specifically, we test whether secondary metabolites are transferred from hosts to fungal partners to increase their defense against fungivores. We grew *Plantago lanceolata* hosts with and without mycorrhizal inoculum, and in the presence or absence of fungivorous springtails. We then measured fungivore effects on host biomass and mycorrhizal abundance (using quantitative PCR) in roots and soil. We used high-performance liquid chromatography to measure host metabolites in roots, shoots, and hyphae, focusing on catalpol, aucubin, and verbascoside. Our most striking result was that the metabolite catalpol was consistently found in AM fungal hyphae in host plants exposed to fungivores. When fungivores were absent, catalpol was undetectable in hyphae. Our results highlight the potential for plant-mediated protection of the mycorrhizal hyphal network.

Key words: cooperation; defense; Folsomia candida; Glomus *sp.; mutualism; networks;* Plantago lanceolata; *species interactions; symbiosis.*

Introduction

All mutualistic interactions are embedded in larger ecological webs (Bascompte 2009). This means that external species, including predators, parasites, herbivores, and even other mutualists (e.g., Palmer et al. 2010) can influence the benefit : cost ratios of mutualisms, and alter their ecological and evolutionary outcomes (Afkhami and Rudgers 2009). Anthropogenic disturbances are increasingly linked to the disruption of species networks (Kiers et al. 2010), and this has prompted a call to focus on understanding how cooperative strategies evolve and are maintained in species networks (Bascompte 2009).

The 450-million-year-old arbuscular mycorrhizal (AM) symbiosis is likely the world's most prevalent mutualism (van der Heijden et al. 2008). It primarily involves the exchange of carbohydrates from plants for mineral nutrients from the fungal partner (Parniske 2008). Estimates suggest that up to 20% of total host carbon can be transferred to AM fungi (for review see Bago et al. 2000). In return, AM fungi improve the host plant's supply of phosphorus (Parniske 2008) and nitrogen (Fellbaum et al. 2012), and provide a diversity of other benefits to the host plant (van der Heiden et al. 2008). The symbiosis contributes to massive global nutrient transfer, global carbon sequestration, and soil stabilization (Rillig and Mummey 2006). These features make it paramount to health and ecosystem function.

Like all mutualisms, the mycorrhizal symbiosis exists in a rich web of interactions. A given host is colonized by multiple AM fungal species (e.g., Vandenkoornhuyse et al. 2002), and a single fungus can simultaneously colonize several plant individuals belonging to different plant species (e.g., Vandenkoornhuyse et al. 2007, Mikkelsen et al. 2008). This common mycelial network represents a dynamic underground environment: AM fungal hyphae can account for up to 30% of the total soil microbial biomass (for review see Leake et al. 2004).

The plant–AM fungal network coexists with populations of soil microarthropods (Hishi et al. 2008) that feed on rhizosphere fungi, including AM fungal hyphae (Jonas et al. 2007). Collembola, known collectively as springtails, are among the most abundant soil arthropods (Petersen and Luxton 1982), and most Collembola species feed on fungal hyphae (Fountain and Hopkin 2005). Depending on their densities, fungivores may either enhance or degrade the symbiosis (Gange 2000). At low densities, the presence of fungivores has been shown to increase AM fungal colonization and hyphal development by acting as a transporting agent for

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nutrients in the soil (Klironomos and Moutoglis 1999, Bakonyi et al. 2002). Conversely, when fungivore densities increase, grazing pressure can negatively affect the AM fungal hyphal development (Klironomos and Ursic 1998). This grazing effect can represent a significant cost to the AM fungi and their host plants (Harris and Boerner 1990, Klironomos and Ursic 1998, Johnson et al. 2005).

It is well known that plants employ a great variety of biologically active secondary metabolites as defensive compounds to deter herbivores (Bowers and Puttick 1988, Marak et al. 2002, Wurst et al. 2010), but it is unknown if soil fungi use a similar chemical-based defense strategy. Recent work suggests that soil-borne fungi have developed strategies to decrease their palatability (Böllmann et al. 2010, Kempken and Rohlfs 2010), such as the utilization of poisonous or repellent compounds to discourage hyphal consumption. So far only a few examples of fungal-synthesized repellents have been described (Rohlfs et al. 2007, Böllmann et al. 2010, Staaden et al. 2010). Relative to saprotrophic free-living fungi, mycorrhizal fungi (both AM and ectomycorrhizal types) appear to be well protected from grazing by fungivores. Several laboratory-based food choice studies have shown that fungivorous springtails preferentially consume saprophytic free-living fungi over mycorrhizal taxa (e.g., Klironomos and Kendrick 1996, Klironomos and Ursic 1998, Schreiner and Bethlenfalvay 2003). When AM fungal hyphae are the only available food source, a diminished growth performance and fecundity is found in many springtail species (Klironomos and Moutoglis 1999, Larsen et al. 2008), suggesting that the consumption of these hyphae may be disadvantageous (Gange 2000, Böllman et al. 2010, Kempken and Rohlfs 2010). There is also some evidence that plant colonization by AM fungi can induce protective secondary metabolites in roots and leaves (Gange and West 1994, De Deyn et al. 2009). The question arises, whether fungal partners benefit, either directly or indirectly, from secondary metabolite production of their plant host.

Here we test the idea that secondary metabolites, used by the host plant for its own protection against herbivory, can be transferred to the fungal partner to increase its defense against fungivores. We hypothesize that the presence of fungivores elicits the transfer of secondary metabolites to the fungal hyphae by the mycorrhizal plant. To test this hypothesis, we utilized microcosms to study the interaction between the host plant *Plantago lanceolata*, *Glomus* sp. fungal symbionts, and the fungivorous springtail *Folsomia candida*. We focused on the production of catalpol, aucubin, and verbascoside, the main defensive secondary metabolites known to occur in *P. lanceolata* (Bowers et al. 1992). Catalpol and aucubin are iridoid glycosides and act as direct defense compounds (Fontana et al. 2009), with generalist anti-feedant properties (Bowers and Puttick 1988, Biere et al. 2004), and antimicrobial activity (Marak et al. 2002). Verbascoside is a caffeoyl phenylethanoid glycoside known for its antimicrobial and cytotoxic activity (Pardo et al. 1993). We used high-performance liquid chromatography (HPLC) to measure secondary metabolite concentrations in roots, shoots, and fungal hyphae and quantitative PCR (qPCR) to determine the mycorrhizal abundance in roots and soil in the presence and absence of fungivores. Ultimately, our aim was to determine if plants protect their mycorrhizal hyphae in the presence of fungivores.

Methods

Plantago lanceolata was chosen as the host plant because it has become a model plant species in mycorrhizal research: it is readily colonized and highly responsive to a broad range of AM fungal taxa (Maherali and Klironomos 2007, Verbruggen et al. 2012), and is known to employ secondary metabolites for defense and protection (Marak et al. 2000, Biere et al. 2004, Wurst et al. 2008, 2010, De Deyn et al. 2009). *P. lanceolata* seeds (Cruydthoek, Assen, The Netherlands) were sterilized using diluted bleach (NaOCl 2.5% mass : mass), then planted in autoclaved quartz sand (15% humidity) and grown for 14 days under plastic foils.

For each pot, three randomly selected seedlings of *P. lanceolata* (three seedlings to ensure ample root growth and hyphal growth) were planted together in a single mesh bag (diameter 6.5 cm, height 16 cm). The mesh bags were prepared from 20-μm pore size nylon mesh, which allowed the hyphae, but not the roots, to cross over, and protected the roots from springtail exposure (Appendix A). After filling the quartz-dune sand mixture in the bags, they were placed in the center of pots (15 cm diameter) with autoclaved quartz sand (~15% humidity, 1.7 kg/pot) mixed with 25% (mass : mass) glass beads (4 mm in diameter), to create spaces for springtails.

Seedlings were inoculated with spore material produced in in vitro root organ cultures (provided by S.L. Biotechnología ecológica, Granada, Spain) in one of three treatments. The seedlings were inoculated either with: (1) a single fungal inoculum of *G. intraradices*, strain 09 (Schenck and Smith [1985], see Stockinger et al. [2009] for discussion of *G. intraradices* reclassification), (2) a single fungal inoculum of *G. custos*, strain 010 (Cano et al. 2009), or (3) a 50:50 mixture of *G. intraradices* and *G. custos*, (see Kiers et al. [2011] for further description of fungal species). In all cases, a total of ~1000 spores were added to the roots of the host plants in each mesh bag. Nonmycorrhizal controls were inoculated with heat-sterilized inoculum. Pots were randomized into treatments, with or without springtails, of 10 replicates each.

We used the springtail *Folsomia candida* (Berlin clonal line), a ubiquitous soil microarthropod with a global distribution (Fountain and Hopkin 2005) as our fungivore. This collembolan has been shown to consume AM fungal hyphae, although saprophytic fungi are the

preferred food source (Gange 2000, Larsen et al. 2008). Individuals of *F. candida* (size range 0.25–0.5 mm) were raised in a climate room at 15°C, fed with a diet of common baker's yeast, and starved for a week before being added to pots. One month after transplanting seedlings, we added 200 *F. candida* per pot, outside the mesh bag in a shallow trench, providing a final density of ~120 individuals/kg soil (i.e., 1.4×10^4 individuals/m^2). Our aim was to match Collembola density found in natural habitats, which vary in grasslands from 0.5 to 8 $\times 10^4$ individuals/m^2 (Petersen and Luxton 1982) to agricultural fields with densities from 0.5 to 2.5×10^4 (Moore et al. 1984). The plants were grown for 12 weeks in a greenhouse (temperature 20°–25°C, relative humidity 60–70%), and watered to maintain ~15% humidity. A Hoagland's nutrient solution with a reduced P content (50%) of 4 mL/kg of dry sand was added once every two weeks (Hoagland and Arnon 1950; see also Appendix A). Pots were randomized on benches once per week.

Harvest

At harvest, the aerial plant portions were removed, freeze-dried and weighed. The roots were removed from mesh bags, washed, freeze-dried, weighed, and a subsample was taken for DNA extraction. Both root and shoot masses were corrected for raw ash content. One soil core (diameter 2.7 cm) was collected outside the mesh bags and weighed for DNA extraction and qPCR. Glass beads were removed from the soil and cores were stored at −20°C until DNA extractions. To measure the hyphal mass, blocks of sand were removed from the pot, placed on a sieve with a 0.5-mm mesh, and subjected to wet sieving/washing (Appendix A). The ERM fraction was snap-frozen in liquid N_2, freeze-dried, weighed, and stored at −80°C for later HPLC analysis. Fungal biomass was determined as ash-free dry mass by the mass difference upon loss on ignition at 500°C (see *Chemical analysis*). A random subsample of roots was stained with Trypan Blue in lactoglycerol, using the modified method by Phillips and Hayman (1970) following treatment of the roots with KOH (10% KOH for 30 min at 90°C) and acidification with 1% HCl for 15 min. The roots were then aligned on a slide and 100 intersections were scored for presence/absence of hyphae, arbuscules, and vesicles using the method described by McGonigle et al. (1990).

Molecular analysis

We extracted fungal DNA from roots using DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) and soil using FastDNA SPIN kit for Soil (MP Biomedicals, Santa Ana, California, USA), following manufacturer's recommendations. One gram of crushed (with vortexer), thawed (but not dried) soil was used for the extraction. For root extractions, fresh roots were blotted dry, cut into small pieces, and mixed, with a random subsample of 100 mg fresh mass taken for further processing. Liquid nitrogen and micropestles were used to pulverize the roots, following supplier recommendations, with DNA eluted in 50 μL elution buffer. The abundance of the two AM fungal species in the different samples was quantified using taxon-specific markers, the mitochondrial large ribosomal subunit RNA genes (mtLSU), with hydrolysis probes (Kiers et al. 2011, Thonar et al. 2012; and see Appendix B). Our qPCR tests revealed that the inoculation with the AM fungal species *Glomus custos* was unsuccessful. This fungal species was undetectable in qPCR tests in most root samples that were inoculated with this strain (both single and mixed), and we found no hyphal biomass in the single *G. custos* treatment. A positive qPCR was recorded in only one out of four analyzed plants that were inoculated with *G. custos*, but the abundance was still two orders of magnitude lower than for *G. intraradices*. We therefore removed the treatments containing *G. custos* from our plant analyses. However, we did still test for the presence of secondary metabolites in the hyphae of the mixed (*G. intraradices* + *G. custos*) treatment. Although this treatment only contained *G. intraradices* (i.e., *G. custos* did not successfully colonize hosts), it was still a valid test for the presence/absence of secondary metabolites in AM fungal hyphae.

Chemical analysis

Freeze-dried roots and shoots were ground to powder using a metal lockable tube and a metal bullet for 50 s at the highest speed (30 strokes/s; Retsch MM200 [Retsch GMBH, Haan, Germany]). The hyphae were ground cryogenically at liquid N_2 temperature in an Eppendorf tube using a fitted pestle. The powdered roots, shoots, and hyphae were stored at −80°C until HPLC analyses. The secondary metabolites were extracted from 10 mg of leaf or root material in 2 mL of methanol, following a modified analytical protocol used by Sesterhenn et al. (2007) for iridoid glycoside determination. The extraction vials were sonicated for four minutes, heated for 30 minutes at 50°C, and shaken overnight at 150 rpm. Subsequently, samples were centrifuged at 3000 rpm for 10 minutes, and passed through a 4.0-μm filter. Because no suitable internal standard was available, care was taken at all steps to maintain the absolute (secondary metabolites) concentration of the methanol extracts. For HPLC analysis, 50 μL of the extract and 100 μL of mobile phase A were transferred to a vial (Appendix A). The preparation of the hyphal extracts followed the same protocol as the plants, except that 10 mg of crude hyphal material was extracted in 1.5 mL of methanol. The concentrations of hyphal extracts were then increased 10-fold by evaporation of the methanol under a stream of N_2. For hyphal measurements, there was a total of six replicates in the *G. intraradices* + springtail treatment, and seven replicates in the mixed AM fungi + springtail treatment, because some samples were pooled to achieve a sufficient amount of hyphae. All freeze-dried hyphal material was cryogenically ground, and a subsample was subsequently ashed at 500°C to measure

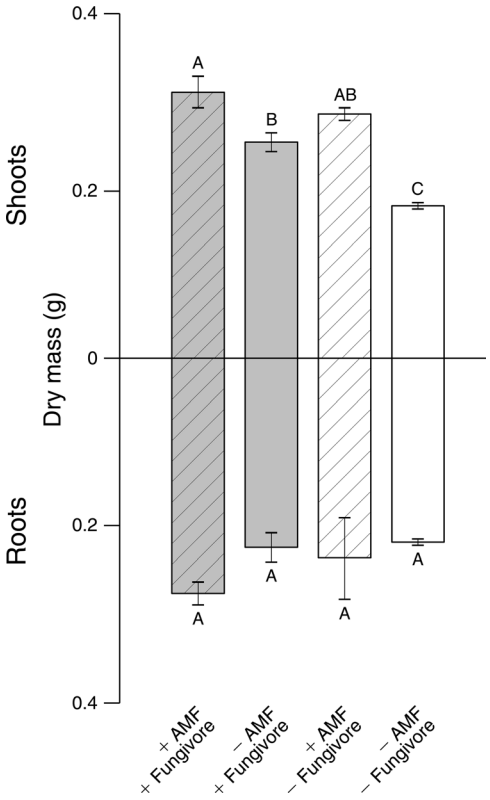

FIG. 1. Effects of AM fungal species *Glomus intraradices* (hatched bars) and the fungivore *Folsomia candida* (gray bars) on *Plantago lanceolata* biomass aboveground (shoots, above the 0 axis line) and belowground (roots, below the 0 axis line). Letters indicate significant differences between treatment means according to Tukey's HSD test ($P \leq 0.05$). Bars represent the means of 10 replicates ± SE.

fungal biomass (the ash-free dry mass) by the loss on ignition.

Hyphal isolation from in vitro cultures

To begin to assess if secondary metabolites originated from the fungus itself, we analyzed secondary metabolites in hyphae from in vitro root organ cultures (Doner and Bécard 1991). In vitro-grown hyphae from *G. intraradices* (provided by Mycovitro) were grown on a gellan gum medium on a split plate together with carrot roots (*Daucus carota*). To extract the hyphae from the medium, the fungal compartment of the medium was suspended in 25 mL of 10 mmol/L sodium citrate buffer (pH 6, 37°C; Appendix A). The hyphae were freeze dried, stored, and processed as previously described.

Choice tests in presence and absence of catalpol

To determine if catalpol was a feeding deterrent for *F. candida*, we constructed food choice arenas as described by Larsen et al. (2008). We divided Petri dishes with plaster of Paris bottoms into two equal sections using a transverse wall, while leaving an opening to allow migration of springtails to either section. One section of the arena received clean yeast, the other side yeast with the catalpol spiked at four different treatment concentrations: 0, 0.1, 1.0, or 2.0% mass : mass. We used yeast rather than fungal mycelium because: (1) a high amount was required for all the different choice treatments, (2) it is a more uniform test material than mycelium grown on a series of replicate plates, and (3) it is free of any possible secondary metabolite material. We placed 20 springtails in the middle opening of each arena with 10 replicates per treatment. We recorded the distribution of the springtails over the two sections, four times per day for three consecutive days. For each arena the collembolan distribution was averaged per day, and each treatment was tested for significant deviations from a random 50:50 distribution. The first day was not taken into account, since the springtails were still actively exploring both sections.

Statistical analysis

Plant data, hyphal biomass, and secondary metabolite concentrations were analyzed using a two-way ANOVA with R 2.13.0 (R Development Core Team 2010). If significant differences were found with ANOVA, a Tukey post hoc test was applied. All data were first tested for normality and homogeneity of variances (Kolmogorov-Smirnov test and Levene's test) and a logarithmic link function was used when required. To confirm ANOVA test results, a complementary statistical analysis was performed using parametric generalized linear model (GLM), using R (GLM approach, Appendix A). All molecular analyses and data on secondary metabolites in hyphae were analyzed by a one-sample *t* test. Differences were considered significant at $P < 0.05$. We ran a power analysis using R 2.13.0 on hyphal biomass and the molecular root colonization data to determine the number of replicates we would have needed to detect a significant difference with a given power of 90% possibility to detect a significant result with $P < 0.05$.

RESULTS

To investigate the potential transfer of protective secondary metabolites from the host plant to the fungal symbiont, we studied the effects of AM fungi on *P. lanceolata* in the presence or absence of springtails (overview table and statistics, Appendices C and D). We found a highly significant effect of AM fungal inoculation (ANOVA, df = 1, 38, $F = 50.2$, $P < 0.001$) and springtail treatment (ANOVA, df = 1, 38, $F = 17.8$, $P < 0.001$) on aboveground plant growth (Fig. 1). The inoculation with *G. intraradices* led to an increase in aboveground growth of 74% and of 60%, respectively, in the presence (Tukey, $P < 0.05$) and in the absence of springtails (Tukey, $P < 0.001$). We also found that the presence of springtails reduced the positive effect of AM fungi on plant biomass (23% vs. 60%; Fig. 1), as indicated by a significant interaction term (ANOVA, df = 1, 38, $F = 4.12$, $P = 0.0498$). There was no significant

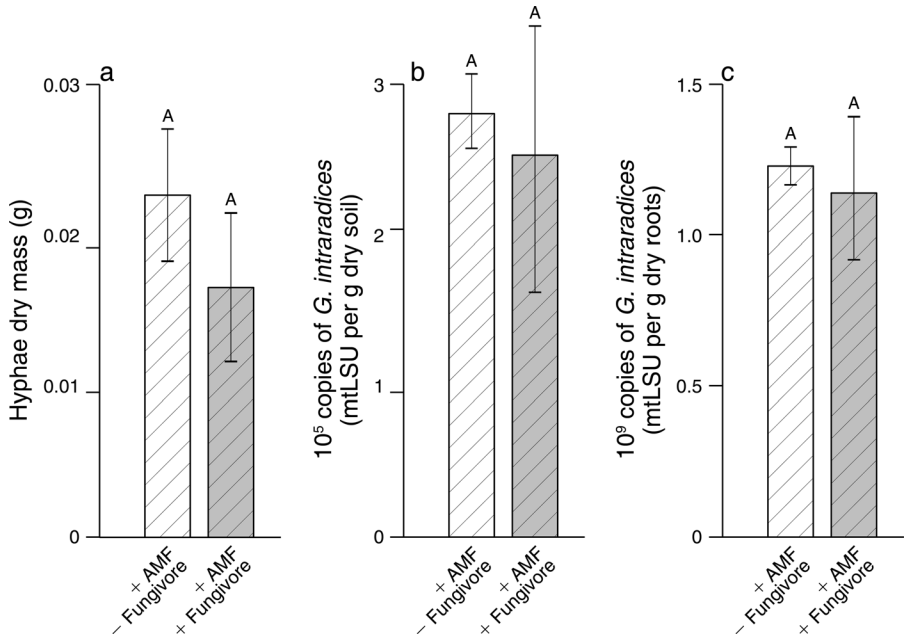

FIG. 2. The effect of *Folsomia candida* presence (gray) and absence (white) on (a) the dry mass of *Glomus intraradices* hyphae, $N = 10$; (b) mtLSU copies of *G. intraradices* per gram of soil, $N = 7$, and (c) mtLSU copies of *G. intraradices* per gram of root, $N = 7$. Values are shown as mean ± SE. Letters above the bars indicate significant differences between treatments according to Tukey's HSD test ($P \leq 0.05$).

effect of inoculation or springtail addition on root biomass (ANOVA, df = 3, 36, $F = 1.27$, $P = 0.299$; Fig. 1). As discussed previously, treatments containing *G. custos* were excluded from plant growth analyses because of a failure to successfully establish *G. custos* colonization.

We measured AM fungal colonization in both the soil outside the mesh bags (using hyphal biomass corrected for raw ash content as well as qPCR) and roots (using qPCR and visual counts) of the *G. intraradices* microcosms (Fig. 2). In the soil, hyphae of *G. intraradices* were found (biomass and by qPCR) in all but one pot. We found no significant reduction in the hyphal biomass in the soil in the presence of springtails (Student's *t* test, $t = 0.93$, df = 18, $P > 0.05$; Fig. 2a). Based on our microscopic counts, we found the plant roots had a total mean colonization of 57%, with no significant effect from the springtail treatment (ANOVA, $F = 0.6434$, df = 1, 17, $P > 0.05$), nor in the percentage of vesicles (mean = 12%; ANOVA, $F = 0.6933$, df = 1, 17, $P > 0.05$) or arbuscles (mean = 46%; ANOVA, $F = 0.538$, df = 1, 17, $P > 0.05$). Likewise, in the qPCR analysis, we found no statistical difference on soil fungal colonization due to springtails (Student's *t* test: $t = 0.3025$, df = 12, $P > 0.05$; Fig. 2b), nor on root colonization (Student's *t* test: $t = 0.36$, df = 12, $P > 0.05$; Fig. 2c). To determine if these nonsignificant differences were due to the low sample number, we ran a power analysis and found that 17, ~100, or ~1000 samples would be needed to detect differences in springtail addition on root biomass, hyphal biomass, and qPCR data, respectively.

P. lanceolata roots and shoots contained the secondary metabolites catalpol, aucubin, and verbascoside in varying concentrations depending on the treatment. Fungal inoculation led to a 62.5% decrease in the verbascoside root concentration in the absence of springtails (ANOVA, df = 3, 36, $F = 7.62$, $P < 0.001$; Fig. 3a). This trend was reversed in shoots where AM fungal inoculation increased the verbascoside concentration by 37%, but only when springtails were present (ANOVA, df = 3, 36, $F = 4.41$, $P = 0.001$; Fig. 3a). In contrast, AM fungal inoculation consistently reduced the concentrations of catalpol in the shoots by 48% and 53%, respectively, both when springtails were present and absent (ANOVA, df = 3, 36, $F = 8.43$, $P < 0.001$; Tukey: for both $P < 0.05$; Fig. 3c). Inoculation with AM fungi had no effect on the catalpol concentrations in the root (ANOVA, df = 3, 36, $F = 0.93$, $P = 0.44$). Of the three secondary metabolites found in roots, the catalpol concentrations were the lowest: in 13 of the 40 root samples, catalpol levels were under the detection limit. While aucubin was detectable in all leaf and root samples, none of the treatments had a significant effect on the aucubin concentration in plant shoots (ANOVA, df = 3, 36, $F = 2.45$, $P = 0.08$) or roots (ANOVA, df = 3, 36, $F = 1.99$, $P = 0.13$; Fig. 3b). The power analysis indicates that ~17 replicates would be required to detect a significant effect of the fungal treatment on the aucubin concentration of the roots.

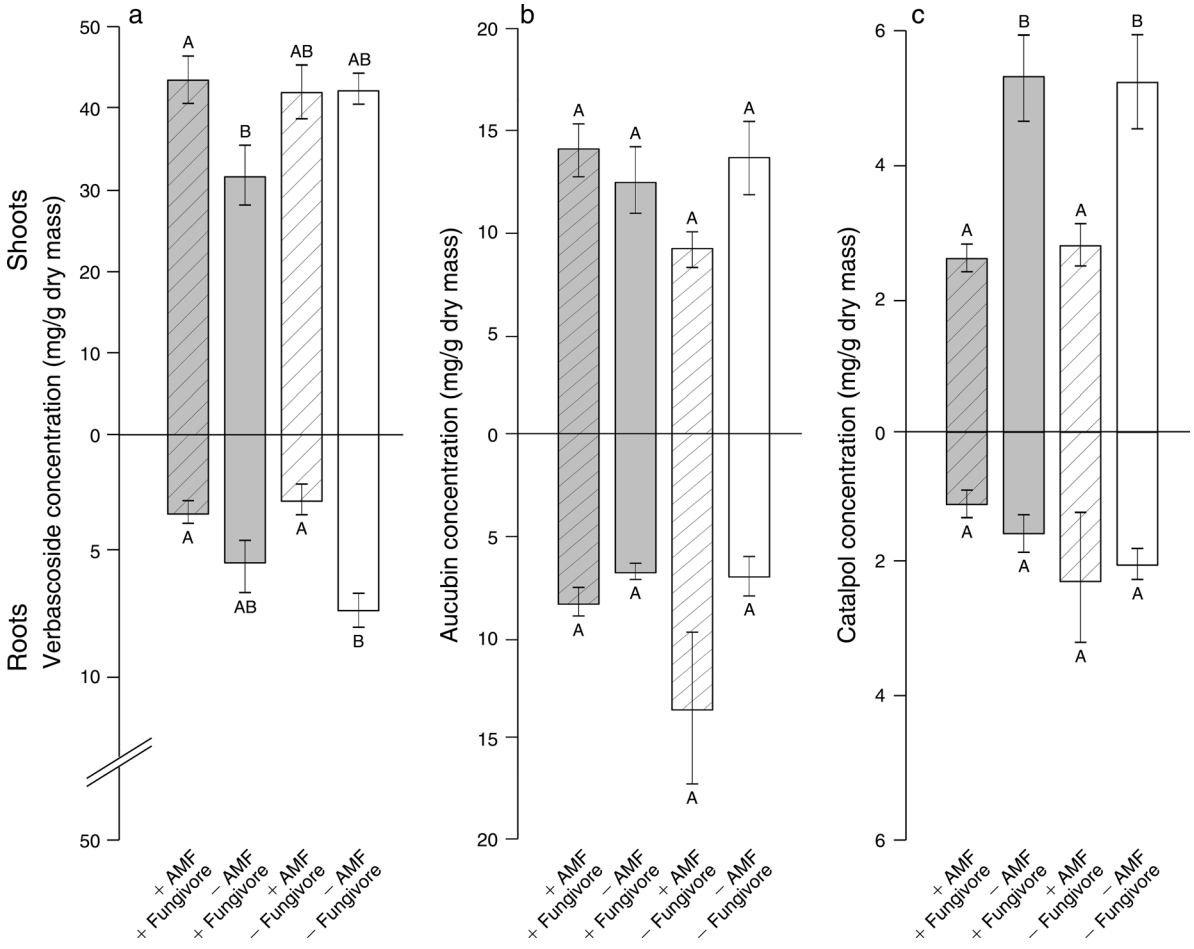

FIG. 3. Effects of AM fungal species *Glomus intraradices* (hatched bars) and the fungivore *Folsomia candida* (gray bars) on (a) verbascoside, (b) aucubin, and (c) catalpol concentration in *P. lanceolata* shoots and roots. Above the *x*-axis corresponds to aboveground concentrations (shoots); below the *x*-axis corresponds to belowground concentrations (roots); note the different scales above and below 0 in panel (a). Values are shown as mean ± SE of 10 replicates. Letters indicate significant differences between treatment means according to Tukey's HSD test ($P \leq 0.05$). Bars represent the means of 10 replicates ± SE.

We measured the presence of secondary metabolites in AM fungal hyphae in treatments with and without springtails. While aucubin and verbascoside were undetectable in all hyphal samples, we consistently identified catalpol (concentration of 0.35 ± 0.12 mg/g [mean ± SE] of dry hyphae; Fig. 4) in the fungal hyphae of each sample ($n = 6$ because of pooling) from the treatment with springtails. In contrast, catalpol was undetectable in the fungal hyphae when no springtails were added. To confirm this finding, we also tested hyphae from the mixed fungal treatment (Appendix E), which due to inoculation failure of *G. custos*, contained only *G. intraradices*. Again, when springtails were present, AM fungal hyphae contained catalpol (mean concentration of 0.26 ± 0.08 mg/g of dry hyphae, $n = 7$; Fig. 4), while catalpol was undetectable when springtails were absent. As an initial test of whether the catalpol was produced by the fungus (e.g., in the absence of a photosynthetically active host), we tested the catalpol concentration of hyphae from in vitro root organ cultures. In all in vitro replicates, the catalpol level was below the detection limit (i.e., <250 ng/mL).

Lastly, we tested whether catalpol was a feeding deterrent for *F. candida*, using food choice arenas. We found that at all levels tested (0.1%, 1%, and 2% mass/mass), catalpol acted as an efficient repellent for the springtails. When catalpol was present, 79–91% (depending on concentration) of the springtails chose to feed from material on the unspiked, control side (Appendix F).

DISCUSSION

Here we investigated the effects of fungivores on the concentration of secondary metabolites in shoots and roots of host plants, and hyphae of AM fungi. The most striking result of our study was that AM fungal hyphae contained catalpol (Fig. 4). This iridoid glycoside was consistently identified in all hyphal samples exposed to springtails, suggesting that its presence is triggered by the presence of fungivores.

Secondary metabolites have been well studied in plants but less is known about these compounds in fungi. Reported fungal secondary metabolites broadly fall into five diverse chemical categories: polyketides, polyketide–peptide hybrids, fatty acid-derived compounds, amino acid-derived compounds, and nonribosomal peptides (Roze et al. 2011). Previous work has identified secondary metabolites in Basidiomycota and Ascomycota phyla (Rohlfs and Churchill 2011), and it is known that endophytic fungi can synthesize various secondary metabolites, like ergovaline, peramine, loline, or indol derivatives (Yue et al. 2000, Fleetwood et al. 2007, Tanaka et al. 2012). These compounds have been shown to negatively affect microarthropods (Rohlfs and Churchill 2011), and exhibit antifungal and antimicrobial properties (Aly et al. 2010). However, the secondary metabolite class of iridoid glycosides seem to be exclusive to the plant kingdom (Dinda et al. 2007).

As this is the first evidence of secondary metabolites in AM fungal hyphae, it is not clear whether catalpol is synthesized by the plant or the fungus. As an initial test of this question, we collected hyphae from in vitro root organ cultures that lack a photosynthetic top. We did not find any evidence for secondary metabolites, suggesting that AM fungi do not synthesize catalpol de novo. However, these hyphae were not exposed to fungivores, and thus iridoid secondary metabolites synthesis may not have been induced. While we utilized a different *G. intraradices* isolate than the one currently being sequenced, a preliminary search through the available genome data of *G.* (*Rhizophagus*) *intraradice*s failed to provide any evidence for a functional biosynthetic pathway for iridoid glycosides in its genome. The fact that catalpol is one of the major secondary metabolites found in *P. lancelota*, and that there are no reports of catalpol being synthesized by other fungi in nature (Dinda et al. 2007), is supportive of our hypothesis that catalpol is transferred by the host to the fungi to protect against springtails.

How is catalpol transferred to the hyphae? Recent work suggests that mycorrhizal networks can facilitate a transfer of allelopathic compounds, compounds produced by one plant that limit the growth of surrounding plants (Barto et al. 2011), but it is unknown if these compounds simply move along hyphal surfaces or whether they move inside hyphae. A transfer of biologically active secondary metabolites has been shown to exist in some root-hemiparasitic plants and their hosts, enabling these root parasites to reduce their susceptibility to herbivory by an uptake (via the haustorium) and sequestration of host-produced deterrents (Schädler et al. 2005, Rasmussen et al. 2006). The selective uptake and subsequent transport and storage of plant-derived secondary metabolites has also been found in several herbivorous insects (Leptidoptera, Coleoptera) to support antipredator defense (Kuhn et al. 2004). These observations all indicate the existence of specific mechanisms that enable the uptake and handling of

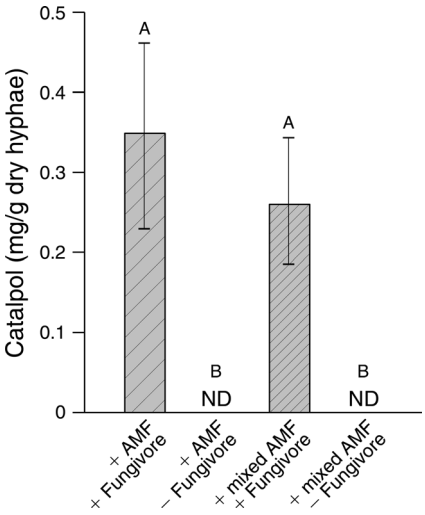

FIG. 4. Catalpol concentration in hyphae of *G. intraradices* (widely hatched bar) and in mixed treatment (tightly hatched bar) that contained *G. intraradices* (+ *G. custos*, which failed to establish). Fungal treatments were either exposed to the fungivore *F. candida* or experienced no fungivores. "ND" indicates no data. Letters indicate significant differences between treatment means according to Tukey's HSD test ($P \leq 0.05$). Bars represent the means of six replicates (*G. intraradices* alone) or seven replicates (mixed treatment) ± SE.

"foreign" biologically active compounds without adverse effects on the organisms own physiological processes (e.g., involving "vesicle trafficking" in transfer/transport [see Field et al. 2006]). However, more work is needed to explore the movement and transfer (active or passive) of chemicals across hyphal networks.

A second result was that the hyphal biomass of the AM fungus was not reduced in the presence of springtails (Fig. 2a). There was a trend toward reduced biomass in the presence of springtails, but this was never significant and the power analysis suggests that the lack of the significant difference was not the result of a small sample number. Biomass measurements were consistent with the qPCR analyses of roots and soil. Visually, we found the roots were well colonized (~50% root length colonized), and that this is the same or higher than root colonization rates reported for the field-grown *Plantago* plants (Šmilauerová and Šmilauer 2002). No significant differences were found for the springtail treatments for any colonization data. Measurements of the mtLSU were used as a proxy for active fungal biomass (Alkan et al. 2006), and again springtails did not lead to a significant reduction in fungal copy number (Fig. 2b, c).

There are two potential explanations for why we do not see a significant reduction in hyphal biomass. First, it is possible that the survival of the springtails was low due to the lack of appropriate food sources. We added ~120 individuals/kg soil, which is within the range for natural densities (Petersen and Luxton 1982, Moore et al. 1984). However, previous greenhouse experiments have shown that springtail numbers under ~200

individuals/kg of soil result in no negative reduction of fungal growth, and can even stimulate fungal colonization (Giller 1996, Bakonyi et al. 2002). There could be a compensatory effect to grazing by the springtails, with fungi allocating more to hyphal regeneration and increased mycelium turnover in presence of these fungivores. The second possibility is that we are seeing an interplay between two opposing factors: while AM fungal hyphae was the only food source for the springtails, it was also an undesirable food source (Klironomos and Ursic 1998). While the effects of plant-derived secondary metabolites vary depending on fungivore (Larsen et al. 2008), they are generally very strong feeding deterrents to herbivores (Biere et al. 2004). For example, Collembola prefer to graze fungi containing less secondary metabolites, even if they may contain less nutrients (Jørgensen et al. 2005, Staaden et al. 2010). Our food choice experiments demonstrate that catalpol is a strong repellent for *F. candida* when spiked in the springtail's regular laboratory food (baker's yeast) at concentrations $<0.1\%$ mass : mass (Appendix F). So while we would expect a decrease in the AM fungal biomass as sole food source, the reduction may be less pronounced due to the repellent qualities of the hyphae themselves.

As expected, we found a positive effect of AM fungal colonization on plant biomass in the treatments with *G. intraradices* (Fig. 1). However, we did not expect that the presence of springtails, in the absence of AM fungal colonization, would increase plant biomass (Fig. 1). While one possible explanation is that dead springtails provided extra nutrients or other growth promoter, our calculations indicate that the nitrogen content in 200 springtails ($\sim$130 μg of N per pot) is insignificant compared to what was added as nutrient solution ($\sim$4.5 mg of N per pot). All growth data from hosts inoculated with *G. custos* were removed from the analysis because of the inoculation failure with this fungus. While we have had success with this AM fungal species in the past (e.g., Verbruggen et al. 2012), the soil characteristics of our pot cultures (composition, pH, moisture) were potentially not favorable for its growth.

Consistent with the results of other authors (Gange and West 1994, De Deyn et al. 2009), we found that inoculation with AM fungi resulted in changes in the secondary metabolite contents of plant shoots and roots (Fig. 3). While secondary metabolite levels are known to vary depending on numerous factors like plant age, pathogen presence, AM fungal colonization, nutrient availability, and genetic factors (Marak et al. 2002, Fuchs and Bowers 2004, Barton 2007), our secondary metabolite levels were in a similar range to those found by others in greenhouse experiments (Fajer et al. 1992 [shoots only], Fontana et al. 2009 [shoots only], De Deyn et al. 2009 [roots and shoots]). In a manipulative experiment similar to ours, De Deyn et al. (2009) studied the effect of AM fungi on selected lines of *P. lanceolata*, containing high and low levels of iridoid glycosides. They found a catalpol range of 0.05–0.8% and aucubin range of 0.05–1.0% in the low and high lines, respectively. These levels are in the range of our experiment, with catalpol levels found at 0.1–0.52% and aucubin at 0.6–1.4%. Also in agreement with a trend identified by De Deyn et al. 2009, we demonstrated that inoculation with AM fungi decreased catalpol levels in shoots (Fig. 3c). We found that aucubin levels were unaffected by our experimental treatments (Fig. 3b), and that colonization by AM fungi resulted in a decrease in the verbascoside levels in plant roots (Fig. 3a). In the presence of springtails and absence of AM fungi, the verbascoside concentration was lower than in any other treatment. A possible explanation is that the reduced verbascoside production explains benefits to plant biomass. However, previous studies suggest that the costs of secondary metabolite products in *Plantago lanceolata* are minor (e.g., Darrow and Bowers 1997), especially when nutrients are in short supply, giving rise to a relative surplus of photosynthate available in the synthesis of the iridoids (Marak et al. 2003). Therefore explaining an 80-mg increase in plant biomass from a 2.5 mg savings in verbascoside content is probably unlikely. Previous work has shown increases in secondary metabolites in leaves after AM fungal colonization (Gange and West 1994), or no effect at all (Wurst et al. 2004, Fontana et al. 2009), highlighting the variability of secondary metabolite synthesis. Levels of secondary metabolites may also be higher in field-grown plants compared to greenhouse plants, potentially due to exposure to even more threats (e.g., Bower et al. 1992). Changes in plant secondary metabolite levels can also be very local (Stout et al. 1996, Darrow and Bowers 1999), which explains how we can see variations in catalpol in the aboveground portions and variations of verbascoside in roots only.

Conclusion

Given the substantial investment of plants and fungi to form a mycorrhizal network, both partners have a shared interest in protecting it. Fungivores present a constant threat. What strategies do plant and/or fungus employ to safeguard the hyphal network from grazing? Our results suggest that the plant may contribute to the chemical protection of the hyphal network. In the presence of fungivores, catalpol was found in the hyphae of AM fungi. When fungivores were absent, the catalpol concentrations in the hyphae were below the detection limit. This suggests that catalpol can be triggered by fungivore grazing pressure. As the synthesis of allelochemicals may involve costs, it is understandable why these compounds are only found in the hyphae when there is a strong threat, such as fungivores.

Several aspects of the origin and transfer of protective compounds in hyphal networks warrant further study. For instance, we need more research to deduce whether AM fungi are capable of a deterrent metabolite synthesis of their own, and to test for the presence of a wider array

of compounds such as mycorradicin and blumenin that may be transferred by mycorrhizal plant species as feeding deterrents (Maier et al. 1995; see Strack et al. 2003 for a review). We also need a better understanding of the origin and/or transfer mechanisms of protective compounds, and whether compounds travel along the hyphae extracellularly (e.g., Barto et al. 2011) or intracellularly, as we predict. Lastly, we utilized only one host, one AM fungal species, and one fungivore. More work is needed to broaden these conclusions and determine whether this is a common strategy across mycorrhizal host plants.

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Supplemental Material

Appendix A

Methodological details of plant propagation/setup, nutrient regime, and hyphal chemical analysis (*Ecological Archives* E094-184-A1).

Appendix B

The qPCR methodology and probe design (*Ecological Archives* E094-184-A2).

Appendix C

Summary table of results and statistics (*Ecological Archives* E094-184-A3).

Appendix D

Comparison of ANOVA and GLM analyses for biomass measurements and secondary metabolites in roots and shoots (*Ecological Archives* E094-184-A4).

Appendix E

Dry mass of fungal hyphae from "mixed" fungal treatment (*Ecological Archives* E094-184-A5).

Appendix F

Choice experiment data testing catalpol palatability on *Folsomia candida* (*Ecological Archives* E094-184-A6).