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Arbuscular mycorrhizal fungi are necessary for the induced response to herbivores by *Cucumis sativus*

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Abstract

Aims

Although ecological interactions are often conceptualized and studied in a pairwise framework, ecologists recognize that the outcomes of these interactions are influenced by other members of the community. Interactions (i) between plants and insect herbivores and (ii) between plants and mycorrhizal fungi are ubiquitous in terrestrial ecosystems and may be linked via common host plants. Previous studies suggest that colonization by arbuscular mycorrhizal fungi (AMF) can modify plants' induced responses to herbivore attack, but these indirect effects of fungal symbionts are poorly understood. I investigated the role of AMF in induced plant response to a generalist herbivore.

Methods

I manipulated AMF status and herbivory in *Cucumis sativus* L. (cucumber, Cucurbitaceae) in a greenhouse to investigate induced responses in the presence and absence of the mycorrhizal fungus *Glomus intraradices* (Glomeraceae). *Spodoptera exigua* Hübner (Noctuidae) were used to manipulate prior damage and later as assay caterpillars. I also measured *G. intraradices* and herbivory effects on plant N and effects on plant growth.

Important Findings

AMF status affected the induced response of *C. sativus*, underscoring the importance of incorporating the roles of plant symbionts into plant defense theory. Assay caterpillars ate significantly more leaf tissue only on mycorrhizal plants that had experienced prior damage. Despite more consumption, biomass change in these caterpillars did not differ from those feeding on plants with other treatment combinations. Leaf N content was reduced by *G. intraradices* but unaffected by herbivory treatments, suggesting that the observed differences in assay caterpillar feeding were due to changes in defensive chemistry that depended on AMF.

Keywords: aboveground-belowground • cucurbitacin • induced defense • mutualist • resistance • trait-mediated indirect effect

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INTRODUCTION

Ecologists recognize that pairwise ecological interactions between species are embedded in complex interaction webs, and so the outcomes of pairwise interactions frequently depend on community context (Strauss 1997; Stanton 2003; Ohgushi 2005; Bascompte 2009). Two of the most widespread terrestrial ecological interactions are herbivorous insects feeding on plants (Strong et al. 1984) and the symbiotic association between plants and mycorrhizal fungi, in which plants exchange carbon compounds for mineral nutrients (Smith and Read 2008). These interactions are so abundant that (Gange 2007) proposed that every plant in the world that forms mycorrhizae is also attacked by herbivorous insects. Recent studies have focused on how these two groups of organisms may indirectly interact through a shared host plant. Herbivore attack can influence mycorrhizal colonization, and herbivore preference and performance may differ depending on the mycorrhizal status of host plants (Gehring and Whitham 2002; Bennett et al. 2006; Koricheva et al. 2009). The impact of mycorrhizae on herbivores may be due to modification of plant characteristics such as chemical defenses (Gange 2007; Koricheva et al. 2009), a trait-mediated indirect effect (Abrams 1995). This underscores the need to incorporate plant–mycorrhizal interactions into our understanding of plant defense theory. Insect herbivores frequently induce plant defense responses (Karban and Baldwin 1997), but the effects of mycorrhizae on induced responses of plants are poorly understood.

Kempel et al. (2010) predicted that arbuscular mycorrhizal fungi (AMF) should enhance plant-induced resistance because the formation of mycorrhizas 'primes' the defense response of plants, allowing them to respond more quickly to herbivore attack (Pozo and Azcón-Aguilar 2007). Indeed, they found evidence of this in several monocots and dicots where prior herbivore feeding reduced performance of a second herbivore in mycorrhizal, but not nonmycorrhizal, plants. These results contrast with another study in which AMF colonization tended to eliminate the induced chemical defense response of *Plantago lanceolata* L. (Bennett et al. 2009), although the magnitude of the effect varied with AMF species. Additionally, a role for AMF in induced plant defenses may be expected because the jasmonate response of plants, which plays an important role in resistance following herbivory (Thaler et al. 1996; Karban and Baldwin 1997), also interacts with and may regulate AMF colonization (Kiers et al. 2010).

Cucumis sativus L. (cucumber) represents a useful system for investigating the role of AMF in induced plant responses to herbivory. The primary defensive chemicals in *C. sativus* is cucurbitacin C; cucurbitacins are oxygenated tetracylic triterpenes produced by the Cucurbitaceae. Agrawal et al. (1999) showed that cucurbitacin C content increased following attack by generalist spider mites, resulting in increased resistance. Damage to *C. sativus* by generalist chewing herbivores can also increase concentration of N (Barrett and Agrawal 2004), which is often a limiting nutrient for insect herbivores (Mattson 1980). While AMF are often associated with P uptake in plants, they also supply N (Govindarajulu et al. 2005; Jin et al. 2005; Leigh et al. 2009).

Here, I investigated the role of AMF in induced responses of plants to herbivore attack by manipulating the mycorrhizal status of, and herbivore damage to, *C. sativus*. While previous studies of AMF effects on induced resistance have focused on actual or potential herbivore performance (Bennett et al. 2009; Kempel et al. 2010), I also measured nutrient content and herbivore tissue consumption to estimate impacts on plants.

MATERIALS AND METHODS

The experiment was a 2 × 2 factorial design manipulating AMF (presence and absence) and herbivory (prior herbivory or none). Plants were grouped in blocks of four plants with one treatment combination per block (n = 15 blocks, 60 plants total). I used *C. sativus* 'Marketmore 76' (Johnny's Selected Seeds, Winslow, ME), a 'bitter' variety that produces cucurbitacin C. Seeds were sterilized by soaking in 5% bleach solution for 15 min, rinsed thoroughly and germinated in a greenhouse in plug flats containing an autoclaved soil mix consisting of equal volumes field soil and sand. Seedlings were fertilized once using diluted low-P (NPK 24-8-16) fertilizer and transplanted to 2-l pots. All pots were cleaned with 10% bleach solution, rinsed, dried and filled with the same autoclaved soil mix to within 3 cm of the rim of the pot. Pots were inoculated with AMF (n = 30, 'Myke' *Glomus intraradices* Schenk and Smith, Premier Tech Biotechnologies, Rivière-du-Loup, Quebec) on a perlite carrier by mixing 50 ml into the top 3 cm of soil. Control plants (n = 30) were mixed with 50 ml autoclaved perlite without *G. intraradices*. Autoclaving should eliminate any soil pathogens, although other microbes may have been present in the 'Myke' inoculation mixture. Seedlings were transplanted at the one-leaf stage and allowed to grow in a greenhouse with daily watering and 6 h supplemental light daily. Blocks were rotated among benches every 7 days throughout the experiment.

Twelve days after transplanting, plants assigned to the herbivory treatment received a *Spodoptera exigua* Hübner caterpillar (approximately third instar) and were enclosed in a lightweight mesh bag. Control plants were enclosed in a mesh bag without a caterpillar. Caterpillars had been fed *C. sativus* leaves and cotyledons prior to being placed on plants. Caterpillars were checked daily, and missing or dead caterpillars were replaced. After 6 days, caterpillars were removed and damage was verified; four plants with no apparent damage (due to repeated dead or missing caterpillars) were re-categorized as control plants.

Herbivory to the most recently fully expanded leaf ('focal leaf') was measured using a clear grid. A single assay caterpillar was weighed and placed on the focal leaf of each plant, and the leaf was enclosed in a mesh bag. After 47 h, assay caterpillars were removed and reweighed. Caterpillar growth was calculated as the proportional change following the assay (= (postweight – preweight)/preweight). The damage on each focal leaf was estimated using the clear grid, and any previous damage from the herbivory treatment was subtracted to determine the leaf area consumed by the assay caterpillar. All plants were clipped at the soil surface, and aboveground tissues were dried at 60°C. Dried aboveground plant tissues were used to determine C and N content by microcombustion (Perkin-Elmer 2400 elemental analyzer). A sample of roots was taken from each of the plants in the first three blocks (n = 6 plants per AMF treatment) and stained with trypan blue to verify *G. intraradices* colonization, which was quantified using the gridline intersect method (McGonigle et al. 1990).

To understand the direct impacts of *G. intraradices* on *C. sativus* growth, I grew a second set of plants with and without the AMF inoculant, as above but with seeds sown directly into pots rather than plug flats. Six weeks after sowing, plants were harvested, above- and belowground tissues were separated and soil was carefully rinsed from roots. Tissues were dried at 60°C for 120 h and weighed to determine above- and belowground biomass.

Analysis

In the herbivory experiment, I tested for an effect of block by fitting linear mixed models with block as a random effect using lme() in the nlme package of R (Pinheiro et al. 2010) and fixed models using gls(). Both types of models were fit by

restricted maximum likelihood (Zuur et al. 2009). Likelihood ratio tests showed that block did not improve models for focal leaf area consumed or assay caterpillar growth (likelihood ratios < 0.12, all $P > 0.7$), so block was discarded from these models, which were fit treating AMF, herbivory and their interaction as fixed effects. Block was retained as a random factor in C and N content models (C, likelihood ratio = 3.39, $P = 0.066$; N, likelihood ratio = 14.13, $P < 0.001$). All models were fit using generalized linear models with Gaussian error distribution and identity link function. Focal leaf area consumed was square-root transformed to normalize residuals, but other variables did not require transformation. I analyzed differences in colonization between control plants and plants inoculated with *G. intraradices* with a *t*-test. In the separate plant growth experiment, I tested the effect of AMF on above- and belowground growth using a *t*-test.

RESULTS

In the focal leaf area consumed analysis, one plant was a highly influential outlier (Cook's $D_i = 0.378$) and was excluded. The interaction between *G. intraradices* and herbivory treatments was marginally significant due to greater leaf consumption by assay caterpillars on previously damaged plants only in the presence of *G. intraradices* (Table 1, Fig. 1A). Assay caterpillar growth was unaffected by treatments or their interaction (Fig. 1B, Table 1). Treatments and their interaction had no effect on C content (all $P > 0.2$), but N content was significantly lower in mycorrhizal plants and unaffected by herbivory or the AMF × herbivory interaction (Table 1, Fig. 1C). Colonization by *G. intraradices* was fairly low in the AMF treatment, but significantly greater than in control (mean ± SE: AMF, 3.3 ± 2.3%; control, 0.3 ± 0.5%; $t_{10} = 3.18$, $P < 0.01$).

AMF inoculation significantly reduced aboveground plant biomass ($t_{18} = 2.124$, $P = 0.048$) but had no effect on belowground biomass ($t_{18} = 1.468$, $P = 0.159$) in the separate plant growth experiment.

DISCUSSION

The outcome of an interaction between two species will often depend on the influences of other community members. By affecting nutrient content, carbon dynamics and hormones of plants, AMF can alter plant traits that are important in mediating plant–insect herbivore interactions. If the mycorrhizal symbiosis enhances plant resistance or tolerance to herbivory, it will further increase the net benefit the plant receives from the association. On the other hand, if AMF reduce plant defenses or make plants more susceptible to insects, the mycorrhizal association is less beneficial to plant fitness. These scenarios assume that the direct effect of AMF on plant fitness is positive, but AMF strains vary greatly in their carbon–nutrient exchange rates, and in some cases may act as 'cheaters,' taking in carbon while supplying few nutrients in return (Johnson 1993; Kiers et al. 2002).

In this experiment, I found an interactive relationship between *G. intraradices* colonization and herbivory on *C. cucumis* by the generalist herbivore *S. exigua*. On mycorrhizal *C. cucumis*, caterpillars feeding on previously damaged plants consumed almost four times the leaf area as those feeding on plants that had not received prior damage. Yet in the absence of *G. intraradices*, *S. exigua* feeding did not differ between plants with and without previous damage. Despite this effect on herbivory, *S. exigua* performance was unaffected. That is, although caterpillars on induced mycorrhizal plants ate four times as much leaf tissue as those in other treatments combinations, their mass change was no different. This suggests that the increased feeding by assay caterpillars on previously damaged plants colonized by *G. intraradices* was compensatory. Compensatory feeding is a widespread response by insect herbivores to low-quality host plants (Simpson and Simpson 1990) in which the amount of food consumed is increased to gain the same nutritional benefit as eating a smaller quantity of a high-quality plant.

This compensatory feeding cannot be attributed to leaf N content. Plants colonized by *G. intraradices* had significantly lower leaf N, which can increase herbivore feeding (Taylor 1989; Kingsolver and Woods 1998). However, assay caterpillars did not feed more on mycorrhizal plants without previous damage, which were also low in N content. In a similar induction experiment, *C. sativus* responded to *S. exigua* damage by increasing N in leaves (Barrett and Agrawal 2004), which the authors propose could be related to increased photosynthesis in leaves following damage and may allow herbivores to overcome the negative effects of plant defenses like cucurbitacins. Because no such N increase occurred following herbivory treatments in this experiment, it is unlikely that the observed differences in leaf consumption by assay caterpillars are due to leaf N. Phosphorus content of host plants can

Table 1: analysis of deviance results for generalized linear models testing AMF and herbivory treatments and interaction effects on leaf N content, focal leaf area consumed and assay caterpillar growth

	Leaf N content			Leaf area consumed			Caterpillar growth		
Source	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
AMF	1,41	20.99	<0.001	1,54	4.32	0.043	1,49	0.10	0.758
Herbivory	1,41	1.51	0.227	1,53	10.48	0.002	1,48	0.40	0.531
AMF × herbivory	1,41	1.21	0.278	1,52	3.99	0.051	1,47	0.02	0.881

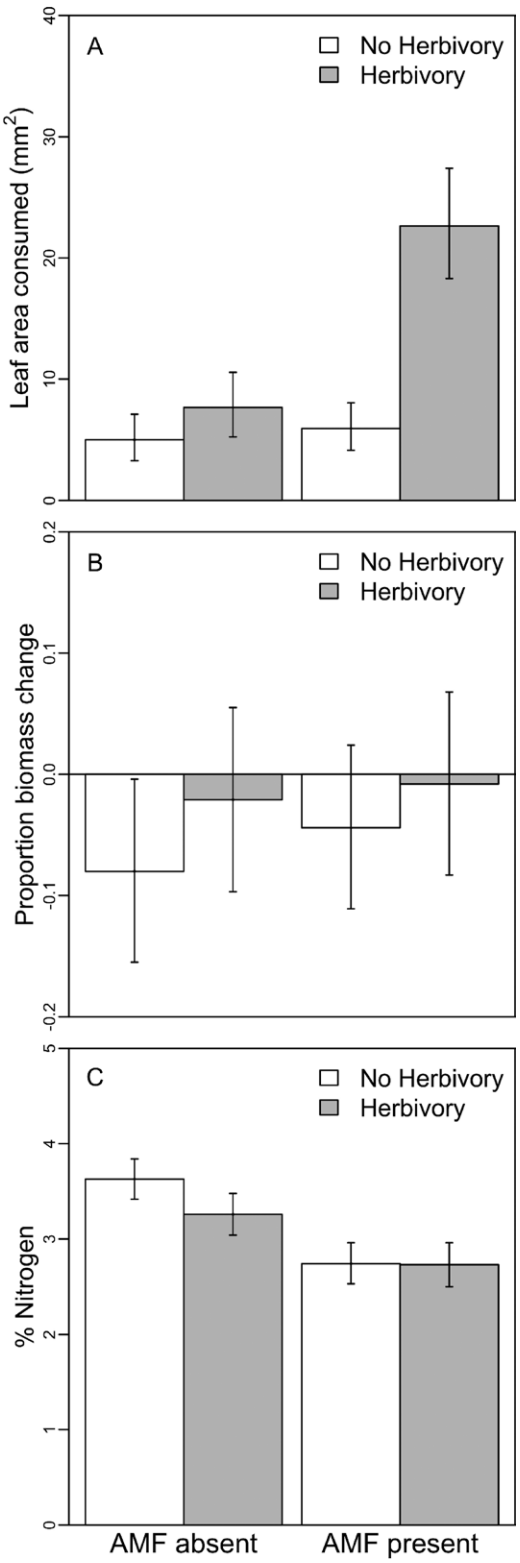

Figure 1: effects of AMF and prior herbivory treatments on (A) Leaf area consumed by assay caterpillars, (B) proportion biomass change in assay caterpillars after feeding and (C) N content of plants. Values are means ± 1 SE based on coefficient estimates of generalized linear models. Leaf area consumed was square-root transformed for analysis; back-transformed values are illustrated here.

influence insect herbivore growth (Janssen 1994; Perkins et al. 2004), although these effects may not be as strong as those of N (Huberty and Denno 2006) and likely depend on relative concentrations of other nutrients (Clancy and King 1993; Busch and Phelan 1999). Nonetheless future investigation of AMF-provided P may help clarify the indirect effects of the mutualism on herbivores, given the important role of AMF in plant P uptake (Smith and Read 2008).

The increased feeding and poor performance by assay caterpillars on damaged mycorrhizal plants suggests that an induced defense response to herbivory in *C. sativus* may have depended on *G. intraradices*. The establishment of mycorrhizas by AMF may 'prime' plant defenses, resulting in quicker or stronger induced responses to herbivores (Pozo and Azcón-Aguilar 2007). Cucurbitacin C is thought to be the primary defensive compound of *C. sativus,* and its production has been shown to increase following herbivore attack (Agrawal et al. 1999). However, direct measurement of cucurbitacin C was beyond the scope of this study, so I cannot attribute herbivore responses to cucurbitacin levels. An important next step in understanding the role of AMF in the induced responses of *C. sativus* and other members of Cucurbitaceae will be quantification of cucurbitacin induction in the presence and absence of AMF or when colonized by different AMF species. This is particularly important because *C. sativus* and other commercially important cucurbits are frequently attacked by diabroticite beetles (Chrysomelidae), for which cucurbitacins are phagostimulants (Metcalf et al. 1980). Additionally, AMF colonization can interact with the biosynthesis pathways of terpenes, which include precursors of cucurbitacins (Walter et al. 2000). Other potential defense traits, such as physical characteristics of leaves that interfere with feeding like trichomes (Copetta et al. 2006), should be investigated as well because these may be affected by mycorrhizal colonization.

Although *G. intraradices* presence may have increased plant defenses following induction, the overall effects on plants were negative. First, assay caterpillars consumed more leaf area on induced plants with *G. intraradices*. Leaf herbivory has strong effects on *C. sativus* fitness, reducing growth and production of flowers, fruits and seeds (Barber et al. 2012). Second, the growth experiment demonstrated that *G. intraradices* colonization significantly reduced aboveground biomass compared to non-AMF plants. Based on the results of this study, the *G. intraradices* strain used in this experiment is a poor mutualist for *C. sativus*. If, as discussed above, damage by a generalist such as *S. exigua* increases cucurbitacin content in leaves, it would likely make *C. sativus* more susceptible to attack from cucurbit-specialized diabroticite beetles. However, resistance is not the only plant strategy to minimize herbivore effects. Plants may also exhibit high tolerance, in which plants experience damage without reduced fitness relative to undamaged plants (Strauss and Agrawal 1999). It is possible that *G. intraradices* confers benefits to *C. sativus* that result in high tolerance (Bennett and Bever 2007), but determining this will require longer experiments and more direct measures of

fitness, such as seed production. The combined results of the induction experiment and the growth experiment highlight the importance of understanding both direct AMF effects on plant growth and indirect effects on other plant traits. That is, growth response alone may not be sufficient to assess the benefits or costs of a mycorrhizal mutualism.

Although these results provide strong support for an impact of *G. intraradices* on *C. sativus*-induced response to herbivory, it is unknown if other AMF species would have similar effects. Bennett et al. (2009) demonstrated that three AMF species differed in the magnitude of their effects on induced plant resistance and suppressed this plant response when mixed together. Importantly, these effects were nonadditive, indicating that the impacts of multiple AMF simultaneously colonizing a plant cannot easily be predicted from the effect of each species in isolation. This underscores the importance of conducting both single-species inoculation experiments and whole-community inoculations to fully understand how AMF affect plant traits that mediate herbivory (Gehring and Bennett 2009).

The dependency of *C. sativus*-induced response demonstrates that there is still much to understand about the role of AMF in plant defenses. There is strong evidence that the plant–AMF symbiosis interacts with plant jasmonate responses that also regulate defense responses to herbivores (Kiers et al. 2010). Scaling up from these organismal responses, the consequences of variation in AMF identity and diversity for plant–herbivore interactions in natural settings are poorly known and represent an important future avenue to understanding multispecies interactions in complex terrestrial ecosystems.

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