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PLANT ANIMAL INTERACTIONS

# Arbuscular mycorrhizal fungi reduce the construction of extrafloral nectaries in *Vicia faba*

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**Abstract** Arbuscular mycorrhizal fungi (AMF) can alter the physiology and morphology of their host plant, and therefore may have indirect effects on insect herbivores and pollinators. We conducted this study to test the hypothesis that AMF can also affect insects involved in protection-for-food mutualisms. We examined the constitutive and inducible production of food rewards [extrafloral (EF) nectaries] in *Vicia faba* plants by manipulating the presence/absence of AMF and by simulating various levels of herbivory. Plants inoculated with AMF produced significantly fewer EF nectaries than uninoculated plants, even after accounting for differences in plant growth. In contrast to earlier studies, EF nectaries were not inducible: damaged plants produced significantly fewer EF nectaries than undamaged plants. Moreover, the effects of mycorrhizal and damage status on EF nectary production were additive. The reduction in EF nectaries in mycorrhizal plants potentially represents a mechanism for indirect effects of AMF on the protective insects that exploit EF nectaries as a food source (e.g., ants). Reduced reward size should result in reduced protection by ants, and could therefore be a previously unappreciated cost of the mycorrhizal symbiosis to host plants. However, the overall effect of AMF will depend upon the extent to which the reduction of EF nectaries affects the number and activity of ants and the extent to which AMF alter other aspects of host plant physiology. Our results emphasize the complexity of multitrophic interactions, particularly those that span belowground and aboveground ecology.

**Keywords** Ant–plant interactions · Cost of symbiosis · Extrafloral nectar · Indirect defence · Trait-mediated indirect interactions (TMIIs)

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## Introduction

Mutualisms, mutually beneficial interspecific interactions, are an important component of ecological communities (Boucher 1985). Mutualisms are both ubiquitous and typically diffuse (i.e., they involve multiple species partners; Janzen 1985). Coupled with the high diversity of functional types of mutualisms (Boucher 1982, 1985; Connor 1995), these attributes emphasize the fact that many organisms engage in multiple mutualisms simultaneously. The diversity of mutualisms involving plants is impressive and includes belowground associations with mycorrhizal fungi and nitrogen-fixing bacteria, and aboveground associations with fungal endophytes and a wide array of animal seed dispersers, pollinators and "bodyguards." Considering the diversity of these taxa that serve as plant mutualists, it is apparent that plants are particularly likely to participate in multiple, simultaneous mutualisms. These can include situations in which the "multiple mutualists" are distantly related, e.g., mycorrhizal fungi and pollinating insects (Gange and Smith 2005; Wolfe et al. 2005), or situations in which they are comparatively closely related, e.g., several ant species that protect a host (Dejean et al. 2000; Hossaert-McKey et al. 2001; Labeyrie et al. 2001; Orivel and Dejean 2002). In either scenario, there is a strong possibility of indirect effects between multiple mutualists, as well as the

potential for each mutualist to modify the benefits and costs the other mutualists impose on their host plant. In this paper, we investigate the potential for plant-mediated indirect effects between arbuscular mycorrhizal fungi and ant bodyguards, and discuss the implications for plant protection.

Arbuscular mycorrhizal fungi (AMF, phylum Glomeromycota; Schüßler et al. 2001) form symbioses with the roots of most plant species (Bever et al. 2001). In these typically mutualistic relationships, soil nutrients collected by AMF are transferred to the host plant (Bonfante-Fasolo and Scannerini 1992). During this process, AMF procure a large proportion of their host's carbon budget for fungal biomass and respiration, and also promote increased plant root growth and respiration (Bryla and Eissenstat 2005). Therefore, in addition to their nutritional benefits to plants, AMF also exact a carbon cost from their host, and these costs may outweigh the benefits when soil nutrients are abundant (e.g., Buwalda and Goh 1982; Peng et al. 1993). The changes generated by AMF in their host plants can have important indirect effects on plant–animal interactions, with herbivory receiving the most attention (reviewed in Gehring and Whitham 2002). The presence of plant–AMF associations (and associations between plants and other types of mycorrhizal fungi) can result in a continuum of positive, neutral, and negative indirect effects on herbivores such as insects and nematodes (e.g., Rabin and Pacovsky 1985; Tylka et al. 1991; Gange and West 1994; Gange et al. 1994, 1999, 2002a, 2002b, 2005; Borowicz 1997; Gehring et al. 1997; Manninen et al. 1999; Goverde et al. 2000; Wamberg et al. 2003). The position of a particular AMF–plant–herbivore indirect interaction along this continuum depends on the specific changes that AMF elicit in their host plants (i.e., on the quantity, as well as the nutritional and defensive qualities of edible plant tissues), and the abilities of different species of herbivores to tolerate or exploit these changes (Gehring and Whitham 2002).

In accordance with the multiple mutualist phenomenon, AMF may also have indirect effects on plant–insect mutualisms. Many plants rely on insect mutualists to perform a variety of beneficial services, such as the transport of their gametes or offspring (i.e., pollination and seed dispersal), protection from herbivores, or the provision of nutrients through frass deposition. In most cases, host plants attract mutualistic insects with rewards of food [e.g., floral and extrafloral (EF) nectar, pollen, fruits, seeds, and food bodies] and/or shelter (e.g., hollow thorns, stems and leaves). The quantity and quality of these rewards—just like the quantity and quality of plant tissues consumed by herbivores—are potentially influenced by the host plant's mycorrhizal status. To date, two studies have investigated the indirect effects of AMF on insect mutualists via changes to plant rewards. In *Chamerion angustifolium*, AMF increased pollinator visitation and seed set, because mycorrhizal plants had larger, more conspicuous inflorescences than non-mycorrhizal plants (Wolfe et al. 2005). In *Centaurea cyanus*, *Tagetes erecta* and *T. patula*, AMF increased pollinator visitation due to increased flower numbers, inflorescence size, and floral nectar production and sugar concentration (Gange and Smith 2005).

In our study, we focus on the effects of AMF on plant-protection mutualisms instead of pollination mutualisms, examining the effects of AMF on the production of one type of reward that host plants offer to their insect mutualists: EF nectar. EF nectar is secreted by EF nectaries that occur on the leaves, stems, stipules, bracts and other parts of many plant species (Elias 1983; Koptur 1992). EF nectar is mainly composed of sugars, but also contains other organic compounds such as amino acids (Baker et al. 1978). The function of EF nectaries is to attract aggressive, mutualistic insect "bodyguards," such as ants, which, while foraging for nectar, protect their host plant from herbivorous insects (Bentley 1977; Rogers 1985; Koptur 1992; but see Arimura et al. 2005 for counterexamples). The importance of these bodyguards to their host plants is underscored by the potentially negative effects of herbivory on plant fitness. For example, in *Vicia hirsuta* and *V. sativa*, two plants closely related to the focal species in this study, herbivory led to reduced total leaf biomass and various measures of seed quality and quantity (Brown et al. 1987).

Variation in reward quality and quantity can have strong effects on the level of ant attendance, and therefore on plant protection and fitness. For instance, Ness (2003) demonstrated that ant attendance increased and herbivore abundance decreased on *Catalpa bignonioides* leaves whose EF nectary's sugar concentrations were increased after experimental damage. In another example, Rudgers (2004) experimentally reduced the number of EF nectaries expressed by *Gossypium thurberi*, and observed decreased ant attendance, greater plant damage, and reduced seed set. Similarly, in two species of *Vicia*, EF nectary removal lead to a reduction in ants, greater plant damage, and reduced fruit set (Koptur 1979). Finally, and most importantly, the experimental removal of EF nectaries in *Vicia faba* (the same species used in this study) led to a reduction in the number of attending ants and a reduction in those ants' efficiency of herbivore removal (Katayama and Suzuki 2004).

The flow of plant-produced carbohydrates from host plants to AMF is one of the key features of the mycorrhizal symbiosis (Bonfante-Fasolo and Scannerini 1992; Finlay and Söderström 1992). Indeed, from a carbon budget perspective, mycorrhizal associations can be very expensive for a host plant to maintain (e.g., 10% of carbon fixed daily in *V. faba* (Pang and Paul 1980; reviewed in Bryla and Eissenstat 2005). Moreover, this acquisition of

plant-produced carbohydrates by symbiotic AMF can reduce the availability of carbohydrates for aboveground plant functions (e.g., Buwalda and Goh 1982). Thus, we predict that AMF have the potential to alter both the constitutive and inducible production of carbohydrate-requiring EF nectaries. The direction of this effect will reflect the balance between AMF's probable positive effects on plant growth, and their simultaneous preemption of carbohydrates. If there is indeed a net effect of AMF on EF nectar production, this may have an indirect effect on the host plant's interaction with mutualistic ants, depending on whether the magnitude of the effect of AMF on EF nectar production is sufficient to elicit a response in ant foraging patterns. Moreover, any subsequent changes to the level of plant protection by ants will depend on the relationship between ant attendance and plant protection, which may be a saturating function (e.g., Ness et al. 2006). Of course, determining whether AMF are indirectly responsible for influencing EF nectary-mediated ant–plant mutualisms will ultimately require a study system in which all the participants in the interaction are present (i.e., fungi–plants–ants–herbivores). Nevertheless, given that the importance of EF nectaries themselves has already been established for *Vicia faba*–ant interactions (Katayama and Suzuki 2004), demonstrating whether AMF can alter the production of EF nectaries is clearly the next step in this line of research.

To this end, we report the results of a controlled growth chamber experiment using *V. faba* as a focal species. By simultaneously manipulating plants' mycorrhizal status (using selective inoculation) and damage status (to simulate herbivory), we address the question of whether the AMF–plant association influences the constitutive and inducible production of EF nectaries.

## Materials and methods

### Study species

*Vicia faba* L. (Fabaceae) cv. "Broad Windsor" is mycorrhizal, with up to 10% of its photosynthates appropriated by its AMF and their metabolic processes (Pang and Paul 1980; Paul and Kucey 1981; Kucey and Paul 1982). This mycorrhizal association can lead to enhanced nutrient uptake and increased plant growth and photosynthetic rate (El-Ghandour et al. 1996; Jia et al. 2004). *V. faba* also produces conspicuous, ant-attended EF nectaries (Bugg and Ellis 1990; Engel et al. 2001). In *V. faba*, EF nectaries occur on the stipules that grow in pairs at the base of leaf petioles (Mondor and Addicott 2003). Each stipule pair can bear up to four EF nectaries, but >99% of the stipule pairs in this experiment bore 0–2 EF nectaries. To estimate EF nectary production, we chose to count the number of EF nectaries, rather than measure the volume of EF nectar, thereby avoiding difficulties associated with diurnal and internectary variation in the volume of nectar produced (e.g., Heil et al. 2000). Furthermore, variation in the number of EF nectaries may be more important than nectar volume or concentration in terms of EF nectary function, which from a plant's perspective is not feeding ants per se, but rather attracting them and ensuring that they are distributed across all vulnerable tissues rather than clumped at a small number of "feeding stations."

Under some environmental conditions, the number of EF nectaries produced by *V. faba* is increased by plant damage (Mondor and Addicott 2003; Mondor et al. 2006), suggesting that EF nectaries are an inducible indirect defence (but see Engel et al. 2001), being constructed in increased numbers during times of enhanced risk of herbivory in order to recruit more ant attendants (reviewed in Agrawal and Rutter 1998; Arimura et al. 2005; also see Koptur 1989 for a similar effect of damage on nectar volume in *V. sativa*). This feature allows us to test the hypothesis that AMF can also affect the inducibility of EF nectaries, in addition to constitutive EF nectary production.

### Experimental set-up and design

In order to determine whether the AMF–plant association can influence the constitutive and inducible production of EF nectaries, we used a full-factorial repeated-measures design involving two experimental factors, each with two levels to which individual plants were randomly assigned. The first factor (*mycorrhiza*) was the inoculation of selected plants with mycorrhizal fungi, the two treatments being "inoculated" (M+) and "not inoculated" (M−). First, we surface-sterilized *V. faba* seeds by washing them in a 2% sodium hypochlorite solution for 20 min, followed by five rinses in sterile water, with each rinse lasting 5 min. The seeds were germinated on filter paper that was moistened with sterile water. After six days, we prepared a 150 × 180 mm pot for each seedling, with the pots containing a soil-free growth medium composed of peat moss, perlite and crushed clay (4:3:3 by volume). Immediately prior to planting, we dipped the emerging radicles of M+ seedlings in a granular mycorrhizal inoculant containing the spores of eight common and weedy AMF suspended in finely-ground rock powder at a density of $\geq 50$ ml$^{-1}$—*Glomus aggregatum*, *G. clarum*, *G. deserticola*, *G. intraradices*, *G. monosporus*, *G. mosseae*, *Gigaspora margarita* and *Paraglomus brasilianum* (Bio/Organics, Inc., LaPine, OR, USA). At least three of these species, *G. clarum*, *G. intraradices* and *G. mosseae*, are known to form mycorrhizal associations with *V. faba* (Rabie and Almadini 2005; Vieweg et al. 2004; Kucey and Paul 1982, respectively). We made a 2 cm depression in the surface of the growth

medium in each pot. For each M+ seed, we placed 5 ml of inoculant into the depression (i.e., <0.2% of the pot, by volume), planted the seed, and covered it with a thin layer of growth medium. We treated M− plants similarly to M+ plants, but they did not receive any inoculant. We did not add autoclaved inoculant to M− plants, because heating inoculants can lead to phytotoxic effects (Rovira and Brown 1966). One option would have been to use irradiated inoculant in M− plants and then attempt to reintroduce any unknown non-AMF microorganisms that may have been present by adding sieved inoculant washes. However, we considered that the risk that this attempted reintroduction would be incomplete—or worse, result in the contamination of M− plants by live AMF spores—was unacceptably high compared to the small risk in our method of having M+ plants colonized by non-AMF microorganisms whose presence/absence in the inoculant was uncertain. Following planting, we randomly arranged the plants in a growth chamber. Each day, the plants received 13 h of light at 20 °C and 11 h of darkness at 16 °C, and were watered with deionized water. We took care to avoid contamination of soil of M− plants by the soil of M+ plants by gently top-watering each pot, without allowing splashing between pots. Also, the grooved floor of the growth chamber allowed the pots to drain excess water, but not to exchange water with one another.

The second factor (*damage*) also had two treatments: "damaged" (D+) and "undamaged" (D−). Although Mondor and Addicott (2003) and Mondor et al. (2006) have already shown that EF nectaries can be inducible, we included damage as a factor in this experiment in order to determine whether this inducibility was affected by AMF (i.e., a mycorrhiza × damage interaction). We damaged D+ plants on the 18th day after germination by excising the distal third of all fully expanded leaves. We handled D− plants similarly, but left them undamaged. Our treatments were meant to simulate the presence/absence of damage by herbivores. Artificial damage has the advantage of allowing precise control of the timing and intensity of "herbivory"; however, it may also have its costs in terms of reduced realism compared to damage by herbivores (see Schat and Blossey 2005). Notwithstanding these costs, artificial damage has been used profitably in the study of EF nectary construction in *V. faba*: in the study by Mondor and Addicott (2003), the same level of damage that we employed in D+ plants was sufficient to induce a >100% increase in EF nectary production compared to D− plants, although the magnitude of this increase may depend on the availability of soil nutrients (Mondor et al. 2006).

Initially, there were 20 plants in each mycorrhiza × damage combination. Four plants were excluded from the experiment because they did not have any fully expanded leaves at the time the damage treatment was applied, and therefore could not have been assigned to the D+ treatment (note that these plants were excluded regardless of which damage treatment they were actually assigned to). A fifth and sixth plant were excluded because one died and one was accidentally damaged mid-experiment. Thus, at the end of the experiment there were 19 M−/D− plants, 17 M−/D+ plants, 18 M+/D− plants and 20 M+/D+ plants, giving a total of 74 *V. faba* plants.

Immediately before applying the D+ and D− treatments, we measured each plant's main shoot height, and counted its number of lateral shoots, leaf pairs, and EF nectaries. We repeated these measurements/counts one, two, three, four, and five weeks following the application of the damage treatments, thereby creating a repeated measures factor (i.e., *date*). We used the differences in the measurements between subsequent sampling dates to attain weekly, post-damage changes in plant height and the numbers of lateral shoots, leaf pairs and EF nectaries. We used weekly (as opposed to cumulative) EF nectary production as our dependent variable because once an EF nectary has been constructed it cannot be withdrawn by the plant, meaning that any differences in EF nectary production related to the main effects and their interactions—especially those involving date—will be most clearly revealed in terms of the number of *new* EF nectaries, rather than in the cumulative number of EF nectaries produced. We used multiple sampling dates in order to (1) increase the statistical power of our analyses, and (2) assess how the effects of mycorrhiza and damage change over time.

Following the experiment, we performed root staining and microscopy procedures that allowed us to confirm whether M+ plants were mycorrhizal and M− plants were not, following the methods of Brundrett (1994) and Brundrett and McGonigle (1994) (also see Vierheilig et al. 2005 for a recent overview of root staining techniques). We gently washed the roots of 20 randomly chosen plants, five per mycorrhiza × damage combination, and stored them in 50% ethanol. A vial containing one M−/D+ root sample was accidentally broken, leaving 19 root samples. At a later date, subsamples of the roots were rinsed with distilled water, cleared for 15 min at 121 °C with 10% KOH, rinsed again, stained for 15 min at 121 °C with 0.03% Chlorazol Black E, and stored in 50% glycerol. After allowing the roots to destain for several days, they were oriented horizontally and in parallel on microscope slides. We examined the slides at 400× with a compound microscope whose field-of-view contained a vertically oriented linear micrometer (i.e., oriented perpendicular to the roots). Using a series of vertical transects spaced 2 mm apart, we inspected between 20 and 77 root-micrometer intersections per plant (depending on the total root length of each subsample) for evidence of colonization by AMF.

### Analyses

We performed two analyses to determine whether our mycorrhiza treatments worked as we intended. First, we used Fisher's exact test to test whether the proportion of plants with arbuscules varied between the M+ and M− treatments. We then used two-way analysis of variance to test for differences between the percentage of root-micrometer intersections with evidence of AMF in M−/D−, M−/D+, M+/D−, and M+/D+ plants.

We used *F* tests to test for "pre-damage" differences between M+ and M− plants in the height of the main shoot, and the numbers of EF nectaries, lateral shoots, and leaf pairs. We did not include the damage treatment or the mycorrhiza × damage interaction in these tests, because plants that would eventually become D+ and D− plants had been treated identically up to that point.

Next, we tested the effects of mycorrhiza and damage on weekly EF nectar production over the five consecutive one-week intervals (date) using repeated-measures analysis of variance. All the two- and three-way interactions between the main effects were also included in the model. We ran this ANOVA using several candidate variance–covariance matrix structures, in order to find the structure that most parsimoniously reflected our data (i.e., yielded the lowest $AIC_c$), and to account for correlated responses across sampling dates. Afterwards, we performed post hoc mean contrasts to compare the mean number of EF nectaries produced by M− versus M+ plants, and D− versus D+ plants, within the different sampling intervals, using the Dunn–Šidák procedure to adjust the Type I error rate ($\alpha$) for multiple comparisons. Similar repeated-measures ANOVAs were performed on the other plant traits we measured (i.e., "plant growth" traits: height of main shoot, number of lateral shoots and number of leaf pairs).

Finally, in order to determine whether any effects of mycorrhiza, damage and date on EF nectaries were due to the effects of these independent variables per se, or due to their effects on plant growth, we ran a repeated-measures ANCOVA, with the first principal component (PC1) from the three plant growth traits used as a covariate. PC1 explained 52.8% of the total variance, had positive component loadings with all three variables (all $r > 0.55$), and was the only component with an eigenvalue greater than one. In order to attain the final ANCOVA model, we used sequential elimination, starting with the highest order interactions, of nonsignificant terms involving PC1 that were not part of higher order, significant interactions. As with the repeated-measures ANOVA, we tested several candidate variance–covariance matrix structures for each model in the sequence. We retained the ANOVA, in addition to the more information-rich ANCOVA, because from a foraging ant's perspective, factors that promote variation among individual plants in the absolute number of EF nectaries (as evinced by ANOVA) are still important even if they operate only indirectly through their effects on plant growth. Thus, the twin analyses allowed us to determine both the patterns of EF nectary construction, and to investigate the role of plant growth in shaping these patterns.

We performed all analyses using SAS software (SAS Institute Inc., Cary, NC, USA), except for the Fisher's exact test, for which we used a calculator.

## Results

### Efficacy of the mycorrhiza treatments

Our mycorrhiza treatments worked as we intended. Arbuscules, the hallmark of AMF colonization and the site of plant–fungus carbon and nutrient exchange, were found and confirmed exclusively in inoculated (M+) plants (Fisher's exact test $P \leq 0.05$). The maximum percent total colonization by AMF (i.e., the percentage of intersections with arbuscules, vesicles or hyphae) was approximately four times greater in M+ than in M− plants (Fig. 1). This four-fold difference was a conservative estimate of the difference in the actual percent total colonization by AMF between M− and M+ plants, because the hyphae in arbuscule-colonized M+ roots were much more likely to be mycorrhizal than the hyphae in arbuscule-free M− roots. In any case, significant reductions, as opposed to complete exclusions of AMF from M− plants, would still be suitable for studying the effects of AMF on plants and insects (e.g., Gange and West 1994; Gange et al. 2003), because reduced colonization leads to reduced opportunities for carbon and nutrient exchange with the host plant.

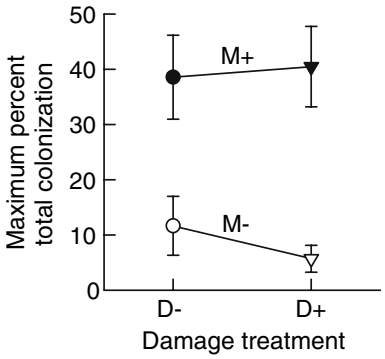


**Fig. 1** Maximum percent total colonization by AMF (mean ± 1 SEM) as a function of the mycorrhiza and damage treatments. *Open symbols* represent M− plants; *closed symbols* represent M+ plants. *Circles* represent D− plants; *triangles* represent D+ plants. The main effect of mycorrhiza was significant ($F_{(1,15)} = 23.51$, $P \leq 0.05$), but there was neither an effect of damage ($F_{(1,15)} = 0.10$, $P > 0.05$), nor an effect of the interaction between mycorrhiza and damage ($F_{(1,15)} = 0.38$, $P > 0.05$)

### Effects of AMF prior to plant damage

Prior to the application of the damage treatments, there were no significant differences between M− and M+ plants in terms of the number of EF nectaries, the height of the main shoot, or the number of lateral shoots and leaf pairs (Fig. 2).

### Effects of AMF and plant damage on construction of EF nectaries and plant growth traits

On average, M+ plants had a significantly lower net EF nectary production than M− plants over the five weeks following the application of the damage treatment (Fig. 3a; Table 1). There was also a significant effect of damage, with D+ plants on average producing significantly fewer EF nectaries per week than D− plants (Fig. 3a; Table 1). The combined effects of mycorrhiza and damage were additive, as there was no significant interaction between mycorrhiza and damage (Fig. 3a; Table 1). Additionally, there was a significant effect of date, with plants producing the most EF nectaries in the third week after the damage treatment was applied. However, none of the interactions involving date were significant (Fig. 3a; Table 1). The significant differences in EF nectary production between M− versus M+ plants, and between D− versus D+ plants, were apparent only when all the weekly data were analyzed together. After applying the Dunn–Šidák procedure to adjust α for multiple comparisons, none of these contrasts within weeks indicated a significant difference in means.

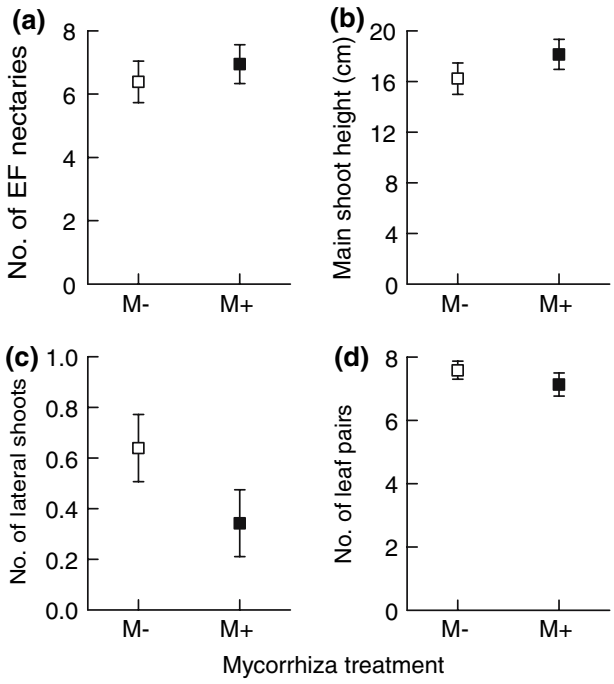


**Fig. 2a–d** Means values (±1 SEM) for **a** number of EF nectaries, **b** height of the main shoot, **c** number of lateral shoots, and **d** number of leaf pairs, as a function of the mycorrhiza treatments prior to the application of the damage treatments. *Open symbols* represent M− plants and *closed symbols* represent M+ plants. The mycorrhiza treatment had no significant effect on any of the variables (**a**–**d**) $F_{(1,72)} = 1.25$, $F_{(1,72)} = 2.50$, $F_{(1,72)} = 0.95$, $F_{(1,72)} = 0.95$ (all $P > 0.05$)

The results for the "plant growth" traits (i.e., weekly change in the height of the main shoot, and the weekly production of lateral shoots and leaf pairs) were qualitatively different from those for the weekly production of EF nectaries (Fig. 3b–d; Table 1). There was still a significant effect of date on all three plant growth traits, and a significant effect of damage on two growth traits, with D− plants experiencing a greater change in the number of lateral shoots and leaves per week compared to D+ plants. However, there were no significant effects of mycorrhiza. These results suggest that the reduction in the weekly production of EF nectaries in D+ compared to D− plants could have been due to the general reduction in growth in D+ plants, since new EF nectaries only occur when new nodes are produced. However, the reduction in EF nectary production in M+ compared to M− plants cannot be explained by differences in plant growth due to mycorrhizal status, because there were none (Fig. 3b–d; Table 1). Rather, after controlling for any differences in plant growth, there was still a reduction in EF nectary production in M+ compared to M− plants (Table 2). When the first principal component of the three plant growth traits (PC1) was added as a covariate to the previous analysis, mycorrhiza remained a significant factor, while damage ceased to be significant (Table 2).

## Discussion

### Effects of AMF on EF nectary production

AMF colonization negatively affected weekly EF nectary production in *V. faba* (Fig. 3a; Table 1), an effect that was due to reduced EF nectary construction per se (Table 2), and not because there was any variation in plant growth correlated with mycorrhizal status (Figs. 2b–d, 3b–d; Table 1). This result suggests a "carbon limitation hypothesis": by paying the carbon costs associated with maintaining the mycorrhizal symbiosis, M+ plants may not be able to afford to construct as many EF nectaries as M− plants. In this respect, AMF may be similar to shade in their effects on plant defences. For example, in an experiment involving several species of *Cecropia*, plants grown in comparatively low light (i.e., plants with reduced access to carbon) produced a lower mass of food rewards (Müllerian bodies) and several other carbon-based defences, compared to high light plants (Folgarait and Davidson 1994).

However, it is important to note that the study of the effects of AMF on EF nectary construction is currently at the pattern-description stage, and determining whether the

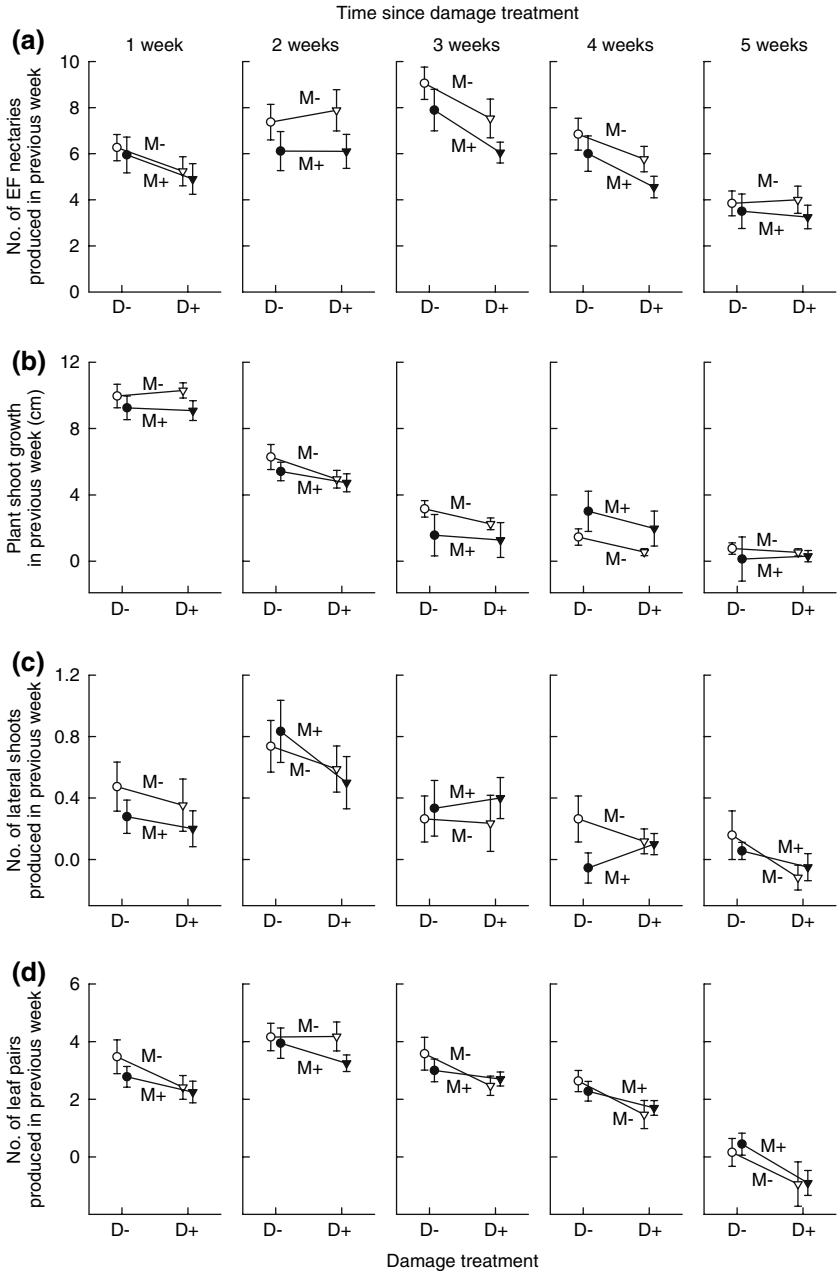


**Fig. 3a–d** Means values (±1 SEM) for **a** the number of EF nectaries produced per week, **b** weekly growth of the main shoot, **c** the number of lateral shoots produced per week, and **d** the number of leaf pairs produced per week, as a function of the mycorrhiza and damage treatments and date (i.e., time since damage treatment). *Open symbols* represent M− plants; *closed symbols* represent M+ plants. *Circles* represent D− plants; *triangles* represent D+ plants. Negative values (e.g., in week 5 of **b**–**d**) reflect plant senescence. Statistical results are provided in Table 1

carbon limitation hypothesis explains the mechanism behind our results will require additional research. In particular, the idea that the carbon limitation hypothesis provides an explanation for our observed negative effect of AMF on EF nectaries relies on the reasonable but as yet untested assumption that the total carbon cost of EF nectary production is closely related to EF nectary number. Further, before accepting the carbon limitation hypothesis, one would eventually have to rule out alternative explanations. For instance, if AMF promote the production of chemical defences (e.g., Gange and West 1994), then the rewards (EF nectaries) used to attract indirect defences (ants) could be rendered redundant and hence, unnecessarily costly. In other words, we cannot yet rule out the possibility that M+ plants can actively reduce their EF nectary construction when other defence options are available. Nevertheless, the carbon limitation hypothesis is more consistent with the acquisition of photosynthates by AMF and the increase in root respiration during mycorrhizal symbioses (e.g., Pang and Paul 1980; Buwalda and Goh 1982; Finlay and Söderström 1992; Bryla and Eisenstat 2005), and should be subjected to future research.

Many studies have reported a positive effect of AMF on plant growth and photosynthesis (see Lekberg and Koide 2005). While we did not find a positive effect on plant growth (Figs. 2b–d, 3b–d; Table 1), plant growth itself had a strong, positive effect on EF nectary production (Table 2), a scenario that will almost certainly be true for any plants

**Table 1** Repeated-measures ANOVAs of the weekly changes in the number of EF nectaries, main shoot height, the number of lateral shoots, and the number of leaf pairs, as a function of the mycorrhiza and damage treatments, and date

| Source of variation | $F_{(NDF,DDF)}$ Values for weekly changes in four plant traits | | | |
|---|---|---|---|---|
| | Number of EF nectaries[a] | Main shoot height (cm)[b] | Number of lateral shoots[b] | Number of leaf pairs[a] |
| Mycorrhiza | $\mathbf{F_{(1,70)} = 6.43}$ | $F_{(1,70)} = 1.25$ | $F_{(1,70)} = 1.87$ | $F_{(1,70)} = 1.71$ |
| Damage | $\mathbf{F_{(1,70)} = 4.07}$ | $F_{(1,70)} = 2.66$ | $\mathbf{F_{(1,70)} = 8.43}$ | $\mathbf{F_{(1,70)} = 23.03}$ |
| Date | $\mathbf{F_{(4,280)} = 21.61}$ | $\mathbf{F_{(4,67)} = 119.1}$ | $\mathbf{F_{(4,67)} = 11.68}$ | $\mathbf{F_{(4,280)} = 47.48}$ |
| Mycorrhiza × damage | $F_{(1,70)} = 0.19$ | $F_{(1,70)} = 0.11$ | $F_{(1,70)} = 1.48$ | $F_{(1,70)} = 0.35$ |
| Mycorrhiza × date | $F_{(4,280)} = 0.61$ | $F_{(4,67)} = 1.38$ | $F_{(4,67)} = 0.60$ | $F_{(4,280)} = 0.41$ |
| Damage × date | $F_{(4,280)} = 1.61$ | $F_{(4,67)} = 0.72$ | $F_{(4,67)} = 0.71$ | $F_{(4,280)} = 0.48$ |
| Mycorrhiza × damage × date | $F_{(4,280)} = 0.02$ | $F_{(4,67)} = 0.14$ | $F_{(4,67)} = 0.37$ | $F_{(4,280)} = 0.49$ |

DDF was calculated using the Kenward–Roger method

Significant $F$ values ($P \leq 0.05$) are shown in boldface

*NDF* Numerator degrees of freedom, *DDF* denominator degrees of freedom

[a] Variance–covariance matrix structure = compound symmetry

[b] Variance–covariance matrix structure = unstructured

**Table 2** Repeated-measures ANCOVA of the weekly change in the number of EF nectaries as a function of the mycorrhiza and damage treatments, and date, with "PC1" as a covariate

| Source of variation | $F_{(NDF,DDF)}$ |
|---|---|
| Mycorrhiza | $\mathbf{F_{(1,132)} = 7.63}$ |
| Damage | $F_{(1,138)} = 1.75$ |
| Date | $\mathbf{F_{(4,155)} = 15.96}$ |
| PC1 | $\mathbf{F_{(1,303)} = 54.13}$ |
| Mycorrhiza × damage | $F_{(1,132)} = 0.59$ |
| Mycorrhiza × date | $F_{(4,142)} = 1.56$ |
| Damage × date | $F_{(4,144)} = 2.05$ |
| Date × PC1 | $\mathbf{F_{(4,167)} = 5.35}$ |
| Mycorrhiza × damage × date | $F_{(4,142)} = 0.05$ |

PC1 is the first principal component of the weekly changes in plant height, number of lateral shoots and number of leaves. Throughout the steps in the sequential elimination process (see the "Analyses" section), the most parsimonious variance–covariance structure in the repeated-measures ANCOVA model was "heterogeneous autoregressive"

Significant $F$ values ($P \leq 0.05$) are shown in boldface

DDF was calculated using the Kenward–Roger method

*NDF* Numerator degrees of freedom, *DDF* denominator degrees of freedom

that, like *V. faba*, can only produce a limited number of EF nectaries per node. Hence, we expect that any EF nectary-producing plant species whose aboveground growth is enhanced by AMF will experience a positive effect of AMF on EF nectaries, in contrast to the negative effect reported here (Fig. 3a; Table 1). A positive effect could even occur in *V. faba* under different environmental conditions or when colonized by different species of AMF (El-Ghandour et al. 1996; Jia et al. 2004). Furthermore, AMF can increase *V. faba*'s photosynthetic rate under some conditions (Jia et al. 2004). We did not measure photosynthetic rate; however, we expect that such effects could also contribute to an AMF-mediated increase in EF nectary production.

Thus, analogous to their range of effects on the tissues consumed by herbivores, the effects of AMF on rewards for insect mutualists in different fungus–plant reward systems should be variable, and we foresee that more examples will be found of both positive effects (Wolfe et al. 2005; Gange and Smith 2005) and negative effects (this study) of AMF on reward size. This should, in turn, translate into variation in the sign and magnitude of the indirect effects of AMF on the mutualistic insects that are attracted to host plants' rewards. In the particular case of *V. faba* under similar conditions to those in this experiment, we predict that AMF will have a negative indirect effect on ant "bodyguards" by competing with one of their food sources (EF nectaries) for photosynthates. Further, since a reduced reward size/quality can lead to reduced ant attendance (e.g., Ness 2003; Rudgers 2004; Katayama and Suzuki 2004), plants with relatively high AMF colonization may attract fewer ants than plants with relatively low AMF colonization. Depending on the functional form of the relationship between plant protection and ant density (e.g., Ness et al. 2006), and on the abundance and species composition of herbivores, reduced ant attendance could represent a previously unappreciated cost of AMF to their host plants. More broadly, it is clear that disentangling the costs and benefits of the mycorrhizal symbiosis to plants in natural systems will require a multitrophic perspective.

## Effects of damage on EF nectary production

Mondor and Addicott (2003) showed that increased EF nectary production can be an induced response to plant damage

in *Vicia faba* (also see reviews by Agrawal and Rutter 1998; Arimura et al. 2005). Yet here, using the same cultivar of the same species, and applying an identical damage treatment, we found that damaged (D+) plants produced significantly *fewer* EF nectaries per week compared to undamaged (D−) plants, and that the size of this effect persisted for at least five weeks after the damage treatments were applied (i.e., no significant damage × date interaction; Fig. 3a; Table 1). In further contrast to both this study and Mondor and Addicott (2003), Engel et al. (2001) found no significant effect of aphid damage on the EF nectar volume, sugar concentration or sugar composition of *V. faba* (they did not report EF nectary number). Clearly a variety of responses to damage are possible, and an important challenge for future research will be to determine the source of this variation. Environmental conditionality is a common feature of induced responses (e.g., Bidart-Bouzat et al. 2005 and references therein). Thus, one hypothesis for why we found a negative effect of plant damage on EF nectary production, whereas Mondor and Addicott (2003) reported a positive effect using the same level of damage, is related to the different environmental conditions experienced by the *V. faba* plants in the two studies. In order to enhance the potential effect of AMF in the M+ treatment, we chose not to fertilize our plants, whereas Mondor and Addicott (2003) fertilized their plants once per week with 20:20:20 fertilizer. Plants that are usually negatively affected by damage/herbivory may exhibit overcompensation when supplemental nutrients are available (e.g., Maschinski and Whitham 1989), and the induced construction of food rewards under nutrient-enriched, as opposed to nutrient-poor, conditions could represent an analogous scenario (also see Folgarait and Davidson 1995). In a very recent study, Mondor et al. (2006) found strong support for this hypothesis. They found that unfertilized *V. faba* plants had a slight (nonsignificant) reduction in the number of EF nectaries constructed when damaged, whereas plants fertilized with 14:14:14 fertilizer exhibited a significant induction of EF nectaries.

## Conclusion

In this study, we showed that AMF can affect the production of rewards that plants use to lure protective insects, lending further credence to the idea that there are indirect interactions between belowground AMF–plant mutualisms and aboveground plant–insect mutualisms (Wolfe et al. 2005; Gange and Smith 2005). Given the ubiquity of both AMF–plant and plant–insect mutualisms, indirect interactions between AMF and mutualistic insects are likely to be common in natural communities. Moreover, we predict that the outcomes of these interactions will depend on how each "terminus" of the interaction chain (i.e., AMF or insect) modifies the costs and benefits of the other's relationship with their shared host plant (see Bronstein 1994). Because of the diverse ways that AMF and mutualistic insects can modify their host plants, and the myriad combinations of fungus, plant and insect species that coexist in a typical community, these outcomes should be variable, and contingent on the environmental context in which they take place, including both the abiotic context (e.g., nutrient status), and the biotic context (e.g., damage by herbivores). Our results amplify the importance of steps towards the increased unification of the historically separate subdisciplines of belowground and aboveground ecology (Porazinska et al. 2003; van Dam et al. 2003; Wardle et al. 2004). More broadly, it is clear that the attention given to indirect effects involving nonmutualistic interactions (reviewed in Wootton 1994, 2002), should also be afforded to "multiple mutualist" indirect effects.

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