Functional Ecology

British Ecological Society

Functional Ecology 2010, **24**, 434–443 doi: 10.1111/j.1365-2435.2009.01625.x

Manipulating the jasmonate response: How do methyl jasmonate additions mediate characteristics of aboveground and belowground mutualisms?

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Summary

1. Plants use a range of sophisticated strategies to protect themselves against herbivores and pathogens, such as the production of jasmonates, a group of plant hormones that prime the plant's defense system upon attack. However, defense-related mechanisms, such as the jasmonate response, play a more diverse role than previously appreciated. Jasmonates also regulate key mutualist relationships, leading to a suit of potential conflicting selection pressures as a single response is employed to mediate multiple species interactions.

2. Here, we experimentally manipulate the host's jasmonate response and document the impact on two key plant mutualisms: (i) changes to arbuscular mycorrhizal symbionts belowground (ii) modifications to floral traits affecting pollinator mutualists aboveground. By exogenously applying a range of methyl jasmonate solutions to cucumber plant roots grown with and without mycorrhizal fungi, we are able to examine the potential costs of the jasmonate response to both above and belowground mutualists.

3. We demonstrate that the negative effect of jasmonates on floral traits depends on whether the plant is mycorrhizal or nonmycorrhizal. Mycorrhization had a positive effect on floral traits, but benefits were lost with jasmonate application. While low levels of jasmonate decreased floral traits, these same jasmonate levels increased colonization by the mycorrhizal symbiont threefold, but only under high phosphorus conditions.

4. Our results highlight potential conflicts for the host in the regulation of their mutualists under different conditions and suggest that the overall impact of the jasmonate response depends on the plant mycorrhizal status and its nutrient context.

5. These findings suggest that increasing the jasmonate response may lead to differential costs and benefits for plants and their mutualists, and highlight potential conflict *in planta*, with mycorrhizal symbionts benefiting from intermediate levels of jasmonates while certain floral traits can be depressed at this same level.

Key-words: community ecology, cooperation, defence, mutualism, multi-trophic interactions, mycorrhizae, pollination, trade-off

Introduction

Plants employ sophisticated strategies to perceive and respond to a diversity of stresses and environmental cues. Jasmonates [jasmonic acid (JA) and methyl jasmonate (MeJA)] are naturally occurring plant hormones biosynthesized in response to wounding, abiotic stresses, flowering, senescence, regulation of microbes and a wide range of other processes (Wasternack & Hause 2002; Pozo, Van Loon & Pieterse 2004; Balbi & Devoto 2008). The jasmonate response is most well documented in relation to its role in defense (Ryan & Moura 2002; Thaler, Owen & Higgins 2004; Cui *et al.* 2005; Liu *et al.* 2005). Induction of the JA response by pathogens and herbivores leads to a cascade of

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complex biochemical changes within the host plant that can provide resistance against a diversity of attackers (Agrawal 2002; Kaplan *et al*. 2008).

Jasmonate responses may also help regulate mutualist interactions both above and belowground, but their role in these interactions is much less explored. Rhizosphere symbioses, such as those between host plants, nitrogen-fixing bacteria and arbuscular mycorrhizal fungi (AMF), rely on a diversity of complex mechanisms to control resource partioning to symbionts (Kiers *et al*. 2003; Bücking & Shachar-Hill 2005; Kiers & Van Der Heijden 2006; Javot *et al*. 2007; Kiers & Denison 2008). There is emerging evidence that plants employ the JA response as a host control mechanism to regulate resource partitioning in the mycorrhizal symbiosis (Hause *et al*. 2002, 2007; Vierheilig 2004; Mabood, Zhou & Smith 2006; Seo *et al*. 2007; Herrera-Medina *et al*. 2008). For example, when a root system is colonized by mycorrhizae, concurrent rises in root JA levels have been found in cucumber (Vierheilig & Piche 2002), barley (Hause *et al*. 2002), medic (Stumpe *et al*. 2005), and soybean (Meixner *et al*. 2005). Genes coding for enzymes that regulate sink/source relationships within a host plant are JA responsive (Blee & Anderson 2002; Isayenkov *et al*. 2005), and when a JA biosynthesis-related gene is suppressed, fungal root colonization and arbuscular formation is delayed (Isayenkov *et al*. 2005). These lines of evidence suggest that plants may use JA mechanisms to control carbon (C) partioning to AMF symbionts (Hause *et al*. 2002; Isayenkov *et al*. 2005; Tejeda-Sartorius, De La Vega & Delano-Frier 2008).

From a plant's point of view, there may be an 'optimal' mycorrhizal jasmonate response depending on numerous biotic and abiotic factors. For instance, both positive and negative mycorrhizal colonization responses have been found when JA and MeJA were applied exogenously to plant leaves. Generally, AMF colonization was stimulated at low jasmonate concentrations (0·05–5·0 μM) and reduced at higher levels (5 mM) (Regvar, Gogala & Zalar 1996; Regvar, Gogala & Znidarsic 1997; Ludwig-Müller *et al*. 2002), suggesting key concentration-dependent effects of jasmonates on AMF growth (see Hause *et al*. 2007 for review).

The role of jasmonates in regulating mycorrhizal fungi is of particular interest because mycorrhizae are not always beneficial for host plants. From the plant's perspective, colonization by mycorrhizae is a costly process, as up to 20% of plant assimilates are allocated to AMF (Douds, Pfeffer & Shachar-Hill 2000). Under certain conditions, such as high phosphorus (P) soils, AMF can behave more like a parasite than a mutualist and actually reduce plant growth (Francis & Read 1995; Johnson *et al*. 2003; Jones & Smith 2004; Kiers & Van Der Heijden 2006). Thus in high P soils, a defensive response may be induced by the host plant against the symbiont. The jasmonate response may be one such mechanism to suppress excessive mycorrhizal colonization (Vierheilig 2004). Elevated JA levels found in arbusculated cells have been hypothesized to enhance the defense status of the host plant, not only against possible pathogens (Hause *et al*. 2002), but also against costly mycorrhizal colonization (Strack *et al*. 2003). Recent experiments found that mutant tomato plants insensitive to jasmonic acid failed to regulate mycorrhizal colonization, prompting a hypothesis that the disruption of JA signalling will enhance mycorrhization (Herrera-Medina *et al*. 2008). This suggests a new defensive function of jasmonates, and adds a level of complexity to our understanding of how jasmonates mediate interactions that range from mutualistic to parasitic, such as the AMF symbiosis. Up-regulation of jasmonates in the roots could be both a sink-driven mechanism to increase host C supply to support the mycorrhizal symbiont (positive for symbiont because of higher C availability), as well as a defense response to control costly mycorrhizal colonization (negative for symbiont leading to reduction in colonization) (Fig. 1).

Responses to interactions belowground can have indirect consequences for interactions aboveground. The jasmonate response is an excellent example, since it is involved in growth processes that affect mutualist pollinators aboveground as well as mycorrhizae belowground. JA is important in regulating pollen development and fruit ripening (Yildiz & Yilmaz 2002; Wang *et al*. 2005), anther development and pollen fertility (Nagpal *et al*. 2005) and in inducing defences to protect flowers from floral herbivores (McCall & Karban 2006). All of these processes may influence pollinator behaviour.

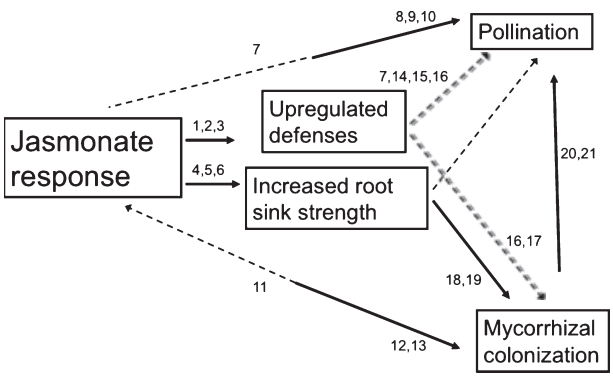

Fig. 1. Schematic drawing of the jasmonate response and its effect on two classes of mutualists, mycorrhizal fungi and pollinators. Solid arrows represent positive effects, broken arrows represent negative effects on response variables, and arrows with shadows represent findings from this study. Lines with both solid and broken arrows indicate both positive and negative effects. Pollination can be directly affected by the jasmonate response or it can be indirectly affected via changes in the host plant's defense resfponse or changes in resource allocation within the plant, such as increased root sink strength. The effect of mycorrhization on pollination is generally positive, but may also be negative if mycorrhization increases the jasmonate response (double headed arrows). Subscript numbers denote supporting references. $_{1}$Thaler, Owen & Higgins 2004; $_{2}$Liu *et al*. 2005; $_{3}$Cui *et al*. 2005; $_{4}$Hause *et al*. 2002; $_{5}$Babst *et al*. 2005; $_{6}$Tejeda-Sartorius, De La Vega & Delano-Frier 2008; $_{7}$Baldwin & Hamilton 2000; $_{8}$Nagpal *et al*. 2005; $_{9}$Wang *et al*. 2005; $_{10}$McCall & Karban 2006; $_{11}$Ludwig-Müller *et al*. 2002; $_{12,13}$Regvar, Gogala & Zalar 1996; Regvar, Gogala & Znidarsic 1997; $_{14}$Tadmor-Melamed *et al*. 2004; $_{15}$Adler & Irwin 2005; $_{16}$Data presented here; $_{17}$Strack *et al*. 2003; $_{18}$Isayenkov *et al*. 2005 ; $_{19}$Hause *et al*. 2007; $_{20}$Wolfe, Husband & Klironomos 2005; $_{21}$Gange & Smith 2005.

There are multiple ways that jasmonate responses could alter traits related to pollinator attraction. For example, leaf damage can induce increased levels of defensive compounds in flowers (Euler & Baldwin 1996; Ohnmeiss & Baldwin 2000; Strauss, Irwin & Lambrix 2004) and nectar (Adler *et al*. 2006), and defensive compounds in flowers may deter pollinators or change their behaviour (e. g. Tadmor-Melamed *et al*. 2004; Adler & Irwin 2005; Kessler & Baldwin 2007). Induced defences could also result in decreased allocation to floral traits if defences are costly. For example, Baldwin & Hamilton (2000) found that aboveground jasmonate addition significantly reduced floral stalk length, the number of flowers produced and lifetime seed production, and attributed this reduction to higher N demand to offset defense costs after JA induction.

To date, there is little known about how jasmonate responses belowground may alter interactions with mutualists aboveground. Mycorrhization can lead to dramatically more attractive plants to pollinators (Gange & Smith 2005; Wolfe, Husband & Klironomos 2005). However, direct links between belowground jasmonate responses and pollinators have yet to be explored. Induction of the JA defense-pathway could negatively affect pollinators if it is costly to host plants and results in reduced allocation to attractive floral structures (Fig. 1) or if it increases defense compounds in flowers.

Our aim was to examine how the JA response mediates interactions between arbuscular mycorrhizal fungi belowground and traits that affect pollinators aboveground, and the degree to which these interactions are influenced by host nutrient status. Although endogenous JA responses are not directly comparable to exogenously applied MeJA, it has been established that exogenous applications of JA or MeJA to leaves or roots can closely mimic the temporal and quantitative characteristics of endogenous JA responses (Zang & Baldwin 1997; Henkes *et al*. 2008; Bruinsma *et al*. 2009; Matsuura *et al*. 2009). Only a few studies to our knowledge have added jasmonates to roots and soil (Bower *et al*. 2005; Huber *et al*. 2005; Henkes *et al*. 2008), even though under normal conditions JA can be exuded in large quantities from the roots of plant seedlings (Dathe *et al*. 1994). We applied a range of MeJA concentrations to the soil of mycorrhizal and nonmycorrhizal cucumber plants (Fig. 2) grown under high and low phosphorous (P) concentrations. We then measured effects on (i) plant growth, (ii) mycorrhizal symbionts and (iii) floral traits that affect pollinator behaviour to determine the potential for jasmonates to affect mutualist interactions above and belowground.

Materials and methods

Cucumber [*Cucumis sativus* –'Straight 8' (Burpee & Co., Warminster, PA, USA)] seeds were soaked in 10% sodium hypochlorite solution and planted in 15 cm pots containing steam sterilized soil. The soil was mixed with either 10 g of *Glomus intraradices* (15 active propagules per g; Premier Biotechnologies, QC, Canada) on a perlite carrier, or a sterilized perlite inoculum control. Three seeds per pot were germinated in a growth chamber (Percival Series 36, Percival Scientific, USA) with 10 h of daylight at 24 °C, and thinned to one seedling per pot 1 week after planting (Fig. 2). After 2 weeks, mycorrhizal seedlings with intact root balls were transplanted into 21-cm pots containing steam sterilized sand : vermiculite : perlite (80 : 10 : 10 v : v : v) and an additional 50 g of *G. intraradices* perlite-based inoculum. Nonmycorrhizal seedlings were transplanted into the same soil mixture but with 50 g of sterilized inoculum. Plants were grown in a greenhouse maintained at 25 °C with supplemental greenhouse light for five hours provided with alternating 1000 W sodium and metal halide lights.

Fig. 2. Cucumber *Cucumis sativus* seedlings, variety 'Straight 8'.

Pots were then randomly divided into high and low phosphorous fertilizer treatments. Twice per week, plants received 100 mL of modified Hoagland's solution of either ¾ P concentration (high P treatment) or ¼ P concentration (low P treatment) (Scheublin, Van Logtestijn & Van Der Heijden 2007), equivalent to *c*. 75 and 25 kg P ha^{-1} $year^{-1}$, respectively. Mycorrhizal and nonmycorrhizal plants were assigned to two levels (0 and 1·0 mM) of methyl jasmonate treatments (TCI America, Portland, OR, USA) with a total of 10 replicate plants per treatment in a 2 × 2 × 2 factorial design (mycorrhizae × fertilizer × MeJA) for a total of 80 pots. To investigate an even greater range of methyl jasmonate treatments on fungal colonization, growth and floral traits of mycorrhizal plants, two additional MeJA levels were added (0·5 and 5 mM), with 10 replicates per treatments, at both high and low P levels for mycorrhizal plants only.

In total, the experiment thus consisted of 40 nonmycorrhizal and 40 mycorrhizal plants [two jasmonate levels (0 and 1·0 mM) and two fertilizer levels] and an additional 40 mycorrhizal plants [two additional jasmonate levels (0·5 and 5·0 mM), and 2 fertilizer levels]. Methyl jasmonate solutions were prepared in 0·25% ethanol as described by Kim *et al*. (2006). Cucumber plants were treated with 100 mL of MeJA solutions (or control solution) four times over a 2-week period, with solutions poured into the soil 5 cm away from the base of the plant, as described previously (Huber *et al*. 2005). After solutions were poured, the soil was covered with plastic film to avoid volatilization. Plants were watered daily to keep soil moist.

Methyl Jasmonate concentrations were chosen based on previous work with these concentrations on cucumber. The upper-level concentration (5·0 mM) was previously shown to exhibit severe negative effects on AMF colonization, while the lowest level (0·5 mM) was shown to exhibit slightly positive to no response (Regvar, Gogala & Zalar 1996; Regvar, Gogala & Znidarsic 1997; Ludwig-Müller *et al*. 2002). It is crucial to note that endogenous JA levels are not directly

comparable to exogenously applied MeJA. However, exogenous MeJA applications can induce responses within the physiological range of endogenous jasmonate dynamics of plants (Zang & Baldwin 1997). By applying MeJA to the soil we are able to evoke a jasmonate response without applying a stress such as leaf damage that would normally cause the synthesis of this hormone. Thus, we are able to look at potential costs of the jasmonate response in terms of plant growth and fitness indices without confounding costs of biotic damage.

Male and female cucumber flowers were counted every day during flowering period. The diameters of a random subsample of 10 male flowers per plant were measured each week and averaged per plant. Four and 5 weeks after transplanting, nectar volume was measured using a 1 μL microcapillary tube inserted near the corolla base on a minimum of three male flowers per plant. To estimate pollen production, anthers were collected from a minimum of three male flowers per plant using forceps that were cleaned with ethanol between flowers to avoid pollen contamination. Each anther was immediately placed in a clean micro centrifuge tube that was left open in a cabinet for 2 weeks while anthers dehisced. We counted pollen per flower using a hemacytometer by adding 1 mL ethanol to each anther, sonicating, and counting eight subsamples of 5 μL each per anther.

Plants were harvested 45 days after transplanting. Roots, shoots and flowers of cucumber plants were separated. Aboveground biomass was dried at 60 °C for 48 hours and weighed. Ovaries were separated from flowers and weighed. A subsample of roots were cleared and stained for mycorrhizal colonization following Koske & Gemma (1989), and the remaining roots were dried for biomass data. To measure AMF colonization, we soaked root subsamples in 2·5% KOH at 10 °C for 8 days and then in 1% HCl at 25 °C overnight. Roots were then placed in 0·05% trypan blue stain solution for 1·5 h at 50 °C. To destain, roots were stored in acidic glycerol. Within 4 weeks, roots were scored for colonization following a modified magnified intersections method (McGonigle *et al.* 1990) in which we used a dissecting microscope (63×) to count the presence of hyphae, arbuscules, and vesicles at 60–100 points where a root crossed the intersection of two grid lines. All nonmycorrhizal treatments were confirmed to be free of mycorrhizal colonization.

SPSS version 13.0 (Chicago, Illinois, USA) was used for all statistical analyses, with a significance level set at 0·05. Above and belowground plant growth were analyzed in a three-way factorial ANOVA with +/− AMF, two fertilizer levels (high and low P), and two MeJA levels (0 and 1·0 mM) as treatment factors, and mean separation calculated using a Tukey's HSD *post hoc* test. Trait values were averaged within plants to create one value per response per plant. Floral traits (number of male and female flowers, flower diameter, ovary weight, and nectar and pollen per flower) were analyzed in a three-way MANOVA with the same independent factors since the traits were collectively considered measures of potential pollinator attraction. Significant MANOVAS were followed by ANOVAS on individual floral traits to determine which floral traits were most affected by treatments. Fungal traits (total colonization and percentage vesicles and arbuscules) were analyzed in a three-way MANOVA for mycorrhizal plants only, including the additional two jasmonate treatments. Using MANOVA allowed us to consider fungal traits as a collective measure of AMF response. Significant MANOVAS were followed by ANOVAS in which individual fungal traits, growth and floral traits were analyzed using two fertilizer levels and four MeJA levels as the fixed variables. Levene's test confirmed the homogeneity of variances of all variables. Percent colonization and pollen data were *ln* transformed to meet homogeneity criteria.

Results

BELOWGROUND

Root dry weight was significantly affected by mycorrhizae, fertilizer level, and MeJA, with a significant interaction between mycorrhizae and MeJA treatment (Table 1, Fig. 3). MeJA significantly reduced root dry weight in mycorrhizal plants ($F_{1,38} = 5{\cdot}12$, $P = 0{\cdot}029$), but had no effect in nonmycorrhizal plants ($P > 0{\cdot}05$). Root:shoot ratios were significantly increased by the presence of mycorrhizae ($F_{1,72} = 7{\cdot}72$, $P = 0{\cdot}007$), but were unaffected by fertilizer and MeJA treatments (Table 1).

Fungal colonization traits (total colonization, vesicular and arbuscular colonization) were affected by both MeJA (Wilks' $\lambda = 0{\cdot}737$, $F_{9,170} = 2{\cdot}53$, $P = 0{\cdot}009$) and fertilizer treatments (Wilks' $\lambda = 0{\cdot}628$, $F_{3,70} = 13{\cdot}82$, $P < 0{\cdot}0001$). As expected, high P levels reduced total AMF colonization ($F_{1,72} = 29{\cdot}9$, $P < 0{\cdot}001$, Fig. 4a). The MeJA treatment, tested at four concentrations, also significantly altered mycorrhizal colonization ($F_{3,72} = 7{\cdot}7$, $P < 0{\cdot}001$), with highest total AMF colonization at 0·5 mM MeJA concentrations (Fig. 4a). The positive effects of MeJA on AMF colonization were most pronounced in the high P fertilizer treatment, where the addition of 0·5 mM MeJA significantly increased AMF colonization 3-fold compared to MeJA controls

Table 1. ANOVAS for effects of mycorrhizae (presence or absence), fertilizer (high or low P), methyl jasmonate (0 or 1·0 mM) and their interactions on above and belowground growth in cucumber hosts

		Root dry weight		Shoot dry weight		Root : Shoot ratio	
	d.f.	SS	*F*	SS	*F*	SS	*F*
Mycorrhizae	**1**	**8·51**	**11·66****	**7·288**	**8·59****	**0·01**	**7·72****
Fertilizer	**1**	**3·89**	**5·34***	**62·61**	**73·78 *****	0·001	0·1
MeJA	**1**	**3·27**	**4·47***	**24·34**	**28·69*****	0·006	0·52
Myco × MeJA	1	**3·47**	**4·75***	**2·98**	**3·51†**	0·05	3·57
Myco × Fertilizer	1	1·18	1·62	2·76	3·25	0·01	0·98
MeJA × Fertilizer	1	0·009	0·124	0·05	0·06	0·002	0·14
Myco × MeJA × Fert	1	0·951	1·303	0·366	0·432	0·03	2·11
Error	72	52·56		61·1		0·93	

Bold denotes significant effects at $P < 0{\cdot}05$.
†$P = 0{\cdot}06$; *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.

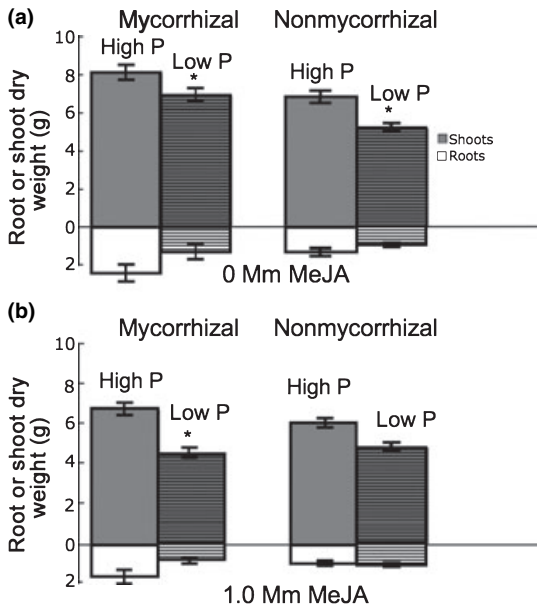

Fig. 3. The effects of high and low P concentration (solid vs. striped bars) and mycorrhizae on shoot and root (dark vs. white) dry weight of cucumber plants in (a) 0 MeJA or (b) 1·0 mM MeJA treatments. Bars indicate standard errors. Asterisks indicate that low P reduced shoot weight ($P < 0{\cdot}05$, Tukey's HSD). MeJA significantly reduced root dry weight in mycorrhizal plants ($F_{1,38} = 5{\cdot}12$, $P = 0{\cdot}029$), but had no effect in nonmycorrhizal plants ($P > 0{\cdot}05$).

($P = 0{\cdot}019$, Tukey's HSD; $P > 0{\cdot}05$ for all other pair wise comparisons). However, this additional colonization did not appear to benefit the plant in terms of increased plant biomass ($P > 0{\cdot}05$, Tukey's HSD, Fig. 4d) or floral traits ($P > 0{\cdot}05$, Tukey's HSD). Flower diameter and ovary weight were actually significantly reduced in the 0·5 mM compared to control MeJA treatment under high P ($P < 0{\cdot}0001$ and $P < 0{\cdot}043$ respectively, Tukey's HSD, Figs 4e, f). The MeJA treatment also influenced the abundance of AMF structures formed, with highest percentage of arbuscules ($F_{3,72} = 3{\cdot}8$, $P = 0{\cdot}014$) and vesicles ($F_{3,72} = 4{\cdot}4$, $P = 0{\cdot}007$) formed at 0·5 mM MeJA levels (Figs 4b, c). Thus, it appears that mycorrhizal colonization was highest at intermediate MeJA levels, but growth and floral traits were highest in treatments without MeJA. In both cases, effects of MeJA were often most pronounced in the high compared to low P treatment.

ABOVEGROUND

Shoot dry weight was significantly affected by mycorrhizae, fertilizer and MeJA treatments (Table 1). Both mycorrhizae and high P increased shoot dry weight, while MeJA decreased shoot dry weight (Fig. 3). The MeJA by mycorrhizal treatment interaction term was marginally significant for shoot dry weight ($F_{1,72} = 3{\cdot}51$, $P = 0{\cdot}06$, Table 1). MeJA reduced shoot dry weight in mycorrhizal plants ($P = 0{\cdot}023$ and 0·006 for high and low P treatments respectively, Tukey's HSD) but had no effect on shoot weight in nonmycorrhizal plants ($P > 0{\cdot}05$ for both high and low P, Fig. 3).

Mycorrhizae, fertilizer and MeJA all significantly affected floral traits (MANOVA: Table 2). Aside from significant main effects of treatments, there were also significant two-way interactions between mycorrhizae and both the MeJA and fertilizer treatment, and a significant three-way interaction (Table 2). Examination of individual responses with ANOVA showed that mycorrhizae significantly increased the number of male flowers (68 ± 2·8 SE vs. 47·3 ± 2·4 SE flowers per plant), flower diameter, and nectar per flower, but decreased pollen per flower (Table 3, Fig. 5). Mycorrhizae did not have an overall main effect on the number of female flowers or ovary weight (Table 3).

Methyl jasmonate had contrasting effects on floral traits in mycorrhizal vs. nonmycorrhizal plants. In mycorrhizal plants, the MeJA treatment significantly reduced floral traits including flower diameter ($F_{1,36} = 33{\cdot}61$, $P < 0{\cdot}001$, Fig. 5a), nectar amount ($F_{1,37} = 9{\cdot}47$, $P = 0{\cdot}004$, Fig. 5b), ovary weight ($F_{1,38} = 5{\cdot}42$, $P = 0{\cdot}025$, Fig. 5c), and pollen per flower ($F_{1,35} = 6{\cdot}33$, $P = 0{\cdot}017$, Fig. 5d). The negative effect of MeJA in mycorrhizal plants was strongest in the high P treatment (Fig. 5). By contrast, the MeJA treatment had no significant effect on any floral trait in nonmycorrhizal plants ($P > 0{\cdot}05$ for all, Figs 5e–h). Thus, there appear to be costs of MeJA induction for floral traits in mycorrhizal but not nonmycorrhizal plants.

Discussion

The importance of jasmonates as regulators of mutualist interactions both aboveground and belowground is emerging (Baldwin & Hamilton 2000; Pozo, Van Loon & Pieterse 2004; McCall & Karban 2006; Hause *et al.* 2007). Two major results illustrate the conflicting selection pressures on host plants as they use the jasmonate response to mediate multiple species interactions. First, we found that MeJA had strong and consistently negative effects on floral traits in mycorrhizal plants, but no significant effect on any floral traits in nonmycorrhizal plants (Fig. 5). This suggests that the impact of the jasmonate response on floral traits and potential pollinator behaviour may depend on the mycorrhizal status of the plant. Second, we found that addition of 0·5 mM MeJA increased mycorrhizal colonization three-fold compared to MeJA controls (Fig. 4a). However, neither host plant growth nor floral traits benefited from this increase in AMF colonization (Figs 4d–f). These results suggest that increasing the jasmonate response could lead to differential costs and benefits for plants and their mutualists; the mycorrhizal symbiont benefited at intermediate MeJA but floral traits were reduced by MeJA, in the presence of AMF.

Generally, we found a negative effect of MeJA on plant growth (Fig. 3). This is in agreement with others who found a reduction in both root growth (Staswick, Su & Howell 1992; Uppalapati *et al.* 2005) and shoot growth (Van Kleunen, Ramponi & Schmid 2004; Walls *et al.* 2005; Cho *et al.* 2007) under exogenous JA treatments, and is consistent with previous studies in cucumber hosts specifically (Ludwig-Müller *et al.* 2002).

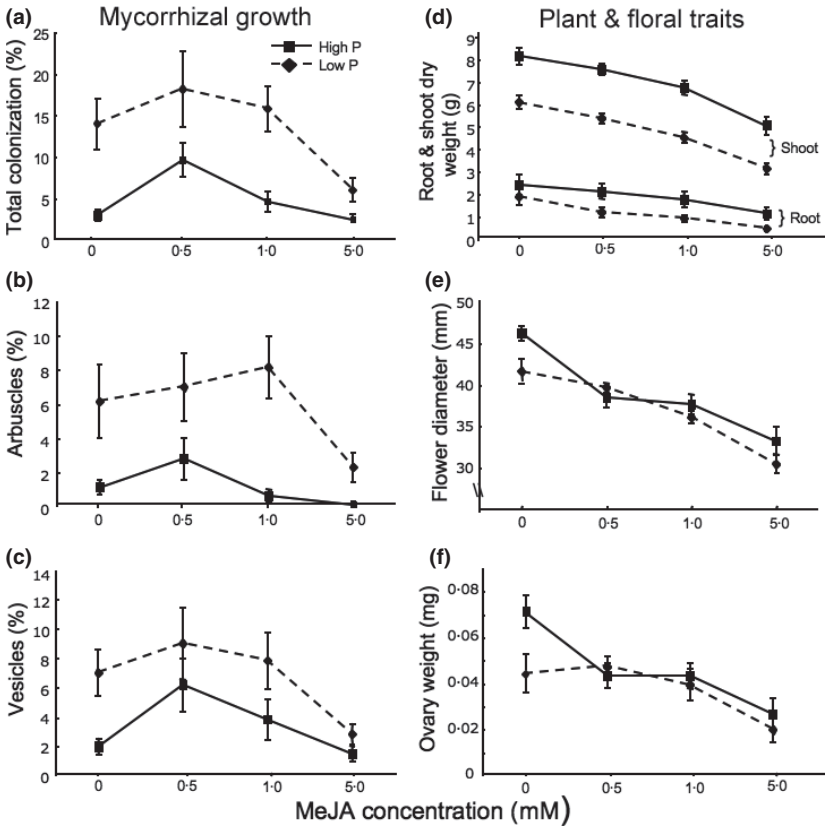

Fig. 4. Effects of four MeJA treatments on mycorrhizal growth, plant growth and floral traits in AMF-inoculated plants grown under high (squares with solid line) and low (diamonds with dotted lines) P regimes: (a) percent mycorrhizal colonization, (b) percent arbuscules, (c) percent vesicles, (d) shoot and root growth, (e) flower diameter, and (f) ovary weight. Values were backtransformed. Bars indicate standard errors.

Table 2. MANOVA for effects of mycorrhizae (presence or absence), fertilizer (high or low P), methyl jasmonate (0 or 1·0 mM) and their interactions on floral traits in cucumber hosts

Factors	Wilks' λ	Num d.f.	Denom d.f.	*F*
Mycorrhizae	**0·49**	**6**	**65**	**10·18*****
Fertilizer	**0·72**	**6**	**65**	**3·76****
MeJA	**0·61**	**6**	**65**	**6·43*****
Myco × MeJA	**0·77**	**6**	**63**	**2·83***
Myco × Fert	**0·75**	**6**	**63**	**3·35****
Fert × MeJA	0·89	6	63	1·16
Myco × MeJA × Fert	**0·78**	**6**	**59**	**2·72***

Bold denotes significant effects at $P < 0{\cdot}05$.
$*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

BELOWGROUND

As expected, we found that high P reduced mycorrhizal colonization compared to low P (Figs. 4a–c). We also found that MeJA affected colonization levels (Fig. 4a), and that colonization was significantly increased under 0·5 mM MeJA levels in the high P, but not low P treatment. We propose that because plants grown under low P had substantially higher mycorrhizal colonization, they also had higher basal JA levels that could not be further induced. It is known that JA levels can increase up to 14-fold upon mycorrhization in cucumber plants (Vierheilig & Piche 2002). High basal JA levels due to high colonization in the low P treatment could mean that additional MeJA did not induce further colonization. Additional work is needed to test this hypothesis, but this is in agreement with the work of Tejeda-Sartorius, De La Vega & Delano-Frier (2008), who found that AMF colonization could not be increased in a tomato phenotype with a constantly activated JA signalling pathway, nor in wild type tomatoes with higher JA basal levels. However, AMF colonization could be significantly increased in a JA-deficient mutant tomato when leaves were supplied with exogenous MeJA. In the high P treatments where mycorrhization, and presumably JA levels, were lower, we found that MeJA significant increased AMF colonization (Fig. 4). These results are consistent with Cipollini (2007), who suggested that the strength of response to exogenous JA treatment will depend on endogenous plant levels.

Interestingly, the MeJA-mediated increase in AMF colonization did not provide any benefits for plant growth or floral traits (Figs 4d–f). This suggests that lower levels of AMF were actual more 'optimal' from the plant's point of view, but we were able to override the plant's control of AMF colonization with MeJA additions. Jasmonates and/or other phytohormones (see Meixner *et al.* 2005) have been implicated in the so-called 'autoregulatory effect' in mycorrhizal plants. These mechanisms are thought to enable host plants to control excessive mycorrhizal colonization (Vierheilig 2004). Here it was possible to increase AMF colonization under conditions when colonization is generally suppressed (e.g. high P soils). However, caution must be taken when relating the effects of exogenously applied MeJA as a root drench to endogenous JA signalling effects elicited by biotic events. Additional work

Table 3. ANOVA for effects of mycorrhizae (presence or absence), fertilizer (high or low P), methyl jasmonate (0 or 1·0 mM) and their interactions on floral traits in cucumber hosts

		Number of male flowers		Number of female flowers		Male diameter		Ovary weight		Nectar per flower		Pollen per flower	
	d.f.	SS	*F*	SS	F	SS	*F*	SS	*F*	SS	*F*	SS	*F*
Myco	1	**8632**	**33·95*****	1·01	0·31	**65·20**	**4·59***	< 0·001	1·02	**18·68**	**19·28*****	**0·70**	**19·59*****
Fertilizer	1	**2656**	**10·45****	**70·31**	**21·64*****	49·12	3·45	**0·003**	**6·12***	3·056	3·15	0·005	0·126
MeJA	1	32·51	0·13	3·61	1·11	**432·3**	**30·40*****	0·001	1·64	**5·58**	**5·76***	**0·29**	**8·10****
Myco × MeJA	1	0·6	0·002	1·01	0·31	**117·7**	**8·38****	**0·002**	**4·47***	**5·59**	**5·77***	0·027	0·75
Myco × Fertilizer	1	25·31	0·10	0·313	0·096	44·95	3·16	< 0·001	0·605	**7·23**	**7·46****	**0·359**	**10·07***
MeJA × Fertilizer	1	43·51	0·171	7·81	2·41	0·761	0·54	< 0·001	0·038	1·21	1·25	0·001	0·039
Myco × MeJA × Fert	1	78·01	0·301	1·51	0·47	**60·21**	**4·23***	**0·002**	**4·29***	1·16	1·19	**0·195**	**5·46***
Error	72	18308		233·9		1023		0·033		68·80		65·0	

Bold denotes significant effects at $P < 0{\cdot}05$.
*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.

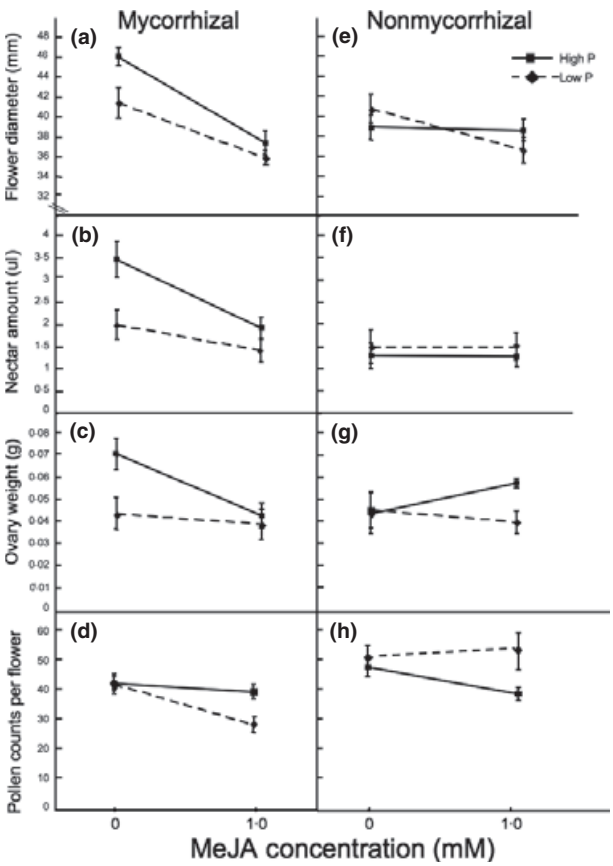

Fig. 5. Effects of MeJA, mycorrhizae and high (squares with solid line) or low (diamonds with dotted lines) P fertilizer on (a, e) flower diameter, (b, f) nectar amount, (c, g) ovary weight and (d, h) pollen production per flower in mycorrhizal and nonmycorrhizal plants. Bars indicate standard errors. Data for total male and female flowers are not shown since there were no significant interaction terms between treatments ($P > 0{\cdot}05$); means for main effects for these responses are given in Results section.

is needed to verify the influence of our treatments on endogenous phytohormone signalling. Jasmonate mutants in which endogenous JA signalling can be controlled genetically will be useful in further exploring our findings (Herrera-Medina *et al.* 2008). Use of such mutants can begin to tease apart the differential effects of true endogenous signalling vs. the host's reaction to an exogenous MeJA treatment.

ABOVEGROUND

We found that inoculation with mycorrhizae enhanced floral traits (Fig. 5), consistent with other studies (Poulton *et al.* 2002; Gange & Smith 2005; Wolfe, Husband & Klironomos 2005). We also found that in the absence of MeJA, floral traits generally benefited most from AMF at high P levels (Fig. 5). It is possible that if we increased P levels even higher, we would begin to see a parasitic effect of AMF leading to a decrease in floral traits, but we saw no such effect at the P levels tested here.

Most importantly, we found that MeJA influenced plant growth and several floral traits, but the effect depended on whether the plant was mycorrhizal or nonmycorrhizal (Table 3). In mycorrhizal cucumber plants, MeJA had a negative effect on floral traits, including flower diameter, ovary weight, and nectar and pollen production. In contrast, MeJA had no significant effect on floral traits in nonmycorrhizal plants (Fig. 5). These results suggest that the overall impact of the jasmonate response depends on the mycorrhizal, as well as the nutrient status of the plant. This is interesting given that in nature the majority (*c.* 80%) of plants are mycorrhizal, suggesting that the impact of MeJA on floral traits may be largely negative in wild plants. In fact, the benefits of mycorrhization for floral traits and pollination behaviour documented in various greenhouse studies (Gange & Smith 2005; Wolfe, Husband & Klironomos 2005) and in the field (Cahill *et al.* 2008) were lost in our experiment after MeJA application. Mycorrhizal plants treated with MeJA had floral traits comparable to nonmycorrhizal plants (Fig. 5), suggesting that (i) the positive role of mycorrhizae in enhancing plant-pollinator interactions may be negated by increases in the jasmonate response and (ii) the cost to the host of inducing a jasmonate response is higher in mycorrhizal than in nonmycorrhizal plants. The mechanism of this result is unknown and requires further study; MeJA could have direct effects on the AMF

itself or indirectly affect AMF and floral traits through higher plant endogenous JA levels.

The physiological costs of inducing a jasmonate response are presumably the result of allocation tradeoffs (Heil & Baldwin 2002; Cipollini, Purrington & Bergelson 2003; Cipollini 2007). These could be tradeoffs in allocation to defense vs. growth or tradeoffs in allocation above and belowground. For example, as found here and elsewhere (Uppalapati *et al.* 2005; Walls *et al.* 2005), jasmonate application generally leads to a reduction in plant biomass. One consequence of reduced biomass could be reduced floral traits. In mycorrhizal plants, such allocation tradeoffs may be more apparent due to the additional costs of supporting a mycorrhizal symbiont belowground. Further stimulation of root sink strength by MeJA, for example, may come at a cost to reproductive structures. This was particularly pronounced in mycorrhizal plants grown under high P conditions, where allocation to reproductive structures was highest (Fig. 5). In contrast, nonmycorrhizal plants, which had lower allocation to most reproductive structures under control conditions, experienced less severe allocation tradeoffs from MeJA root induction than their mycorrhizal counterparts.

One caveat in our study is that tradeoff effects of the jasmonate applications could be the result of negative cross talk between hormones, rather than a direct effect of JA (Koornneef & Pieterse 2008). It is well known that jasmonate application can elicit a cascade of hormone signalling that includes many associated hormones [e.g. salicylic acid (SA) and ethylene (ET), abscisic acid (ABA), brassinosteroids and auxins], as well as responses in bacterial and fungal rhizosphere communities of the rhizosphere (Pozo, Van Loon & Pieterse 2004). Thus, caution must be taken in interpreting our results as reflecting only a JA-mediated 'tradeoff' response, as there are many components potentially interacting to produce the resulting phenotype.

Conclusions

We found that manipulating the jasmonate response via exogenous MeJA additions had the potential to alter mutualist interactions on the whole plant level. The magnitude of this change depended on nutrient availability, mycorrhizal status and concentration of MeJA added. We found that intermediate levels of MeJA stimulated mycorrhizal colonization under high P conditions, while having no benefit for plant growth and even reducing certain floral traits. Although many studies have found that flower size and nectar amount affect pollinator behaviour (reviewed in Kaczorowski, Gardener & Holtsford 2005), experiments are now needed to link MeJA-induced changes in floral traits with actual changes in pollination behaviour in this system. We also found that floral traits were most strongly reduced by MeJA when plants were mycorrhizal and grown under high P conditions. If plants use jasmonates to aid in controlling C partitioning to the mycorrhizal symbiont, as is suspected (Hause *et al.* 2007), host plants may pay an ecological cost aboveground in terms of reduced growth and allocation to reproductive structures. Similar dynamics have been recently documented in *Vicia faba,* in which inoculation with mycorrhizae reduced the size of aboveground extrafloral nectaries and therefore rewards to mutualist ants that protect the plant against herbivores (Laird & Addicott 2007). This was identified as one of the 'indirect costs' of mycorrhizal colonization. Similarly, inoculation with mutualistic leaf endophytes aboveground has negative consequences for mycorrhizal colonization belowground (Muller 2003; Mack & Rudgers 2008), although significant impacts of mycorrhizal treatments on endophytes aboveground have not yet been found (Mack & Rudgers 2008).

Our experiment is among the first to study ecological costs of the jasmonate response to traits potentially affecting mutualists. The challenge for the plant host of balancing multiple mutualisms requires further study. Here we only considered mycorrhizae and several floral traits that can affect pollinators. The overall effect of the jasmonate response will depend on interactions with numerous organisms, spanning multiple trophic levels, as well as the resources and environment under which the plants are grown (Cui *et al.* 2005; Schmidt & Baldwin 2006). Manipulating host control mechanisms such as the jasmonate response, and using plant genotypes in which the jasmonate pathway is enhanced or knocked out, will yield greater insight into the dynamics of how multiple mutualisms are maintained by hosts plants over evolutionary time.

Acknowledgement

Many thanks to Bettina Hause, Yair Shachar-Hill, Erik Verbruggen, Linda Doedens and Hannes Gamper for constructive comments that greatly improved this manuscript and to Kristen Hladun for lab assistance. Research was supported by a Darwin Fellowship from the University of Massachusetts at Amherst (ETK), a VENI grant from the NWO Dutch Science Foundation (ETK), and USDA NRI 2008-02346 (ETK and LSA). This is Kellogg Biological Station contribution number 1489.

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Received 23 December 2008; accepted 1 July 2009
Handling Editor: John Klironomos