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Babikova, Z., Gilbert, L., Bruce, T. J. A., Dewhirst, S. Y., Pickett, J. A. and Johnson, D. 2014. Arbuscular mycorrhizal fungi and aphids interact by changing host plant quality and volatile emission. *Functional Ecology.* 28 (2), pp. 375-385.

Arbuscular mycorrhizal fungi and aphids interact by changing host plant quality and volatile emission

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Summary

1. Most plants interact with both arbuscular mycorrhizal (AM) fungi, which increase nutrient acquisition, and herbivores such as aphids, which drain nutrients from plants. Both AM fungi and aphids can affect plant metabolic pathways and may influence each other by altering the condition of the shared host plant.

2. This study tests simultaneously the effects of AM fungi on interactions with aphids (bottom-up effects) and the effects of aphids on interactions with AM fungi (top-down effects). We hypothesized that: (i) attractiveness of plants to aphids is regulated by induced changes in production of plant volatile organic compounds (VOCs) triggered by AM fungi or aphids; (ii) aphids reduce AM fungal colonization; and (iii) AM fungal colonization affects aphid development.

3. Broad beans were exposed to AM fungi, aphids and a combination of both. To test for the strength of bottom-up and top-down effects, separate treatments enabled establishment of mycorrhizas either before or after aphids were added to plants. VOCs produced by plants were used to (i) test their attractiveness to aphids and (ii) identify the semiochemicals causing attraction. We also measured plant growth and nutrition, AM fungal colonization and aphid reproduction.

4. AM fungi increased the attractiveness of plants to aphids, and this effect tended to prevail even for aphid-infested plants. However, both attractiveness and aphid population growth depended on the timing of AM fungal inoculation. AM fungi suppressed emission of the sesquiterpenes (*E*)-caryophyllene and (*E*)-*β*-farnesene, and aphid attractiveness to VOCs was negatively associated with the proportion of sesquiterpenes in the sample. Emission of (*Z*)-3-hexenyl acetate, naphthalene and (*R*)-germacrene D was regulated by an interaction between aphids and AM fungi. Aphids had a negative effect on mycorrhizal colonization, plant biomass and nutrition.

5. Our data show that below- and above-ground organisms can interact by altering the quality of their shared host plant even though there is no direct contact between them. Plant interactions with herbivores and AM fungi operate in both directions: AM fungi have a key bottom-up role in insect host location by increasing the attractiveness of plant VOCs to aphids, whereas aphids inhibit formation of AM symbioses.

Key-words: Broad bean *Vicia faba*, herbivores, insect host location, multitrophic interactions, mycorrhizal colonization, pea aphid *Acyrthosiphon pisum*

Introduction

In both natural and agricultural ecosystems, it is possible for below- and above-ground organisms to interact and change each other's fitness, even where they do not come into direct contact, via indirect effects mediated through

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shared host plants. From a below-ground perspective, among the most important functional groups of organisms are mycorrhizal fungi. In particular, arbuscular mycorrhizal (AM) fungi form symbiotic relationships with around 80% of herbaceous plant species, including many important crops, and have a near global distribution (Smith & Read 2008). These fungi can significantly and positively increase mineral nutrient acquisition (Smith & Read 2008), tolerance to root and shoot pathogens (Whipps 2004) and nematodes (De La Peña *et al.* 2006), while ameliorating water and mineral nutrient stress (Smith & Read 2008). In exchange for these benefits, plants supply AM fungi with large amounts of carbohydrates (Johnson, Leake & Read 2001). From an above-ground perspective, aphids are among the most abundant and agriculturally important invertebrate herbivores (Minks & Herrewijn 1989). They feed on plant sap directly from the phloem, thus draining the plant of nutrient resources and greatly reducing plant fitness and biomass (Guerrieri & Digilio 2008). There is therefore considerable potential for interactions between AM fungi and aphids via competition for plant resources.

From a bottom-up perspective, AM fungi generally have positive effects on aphid growth and fecundity (Gange, Bower & Brown 1999; Koricheva, Gange & Jones 2009), by making plants better-quality hosts through improved nutrition or by changes in the morphology of phloem sieves (Koricheva, Gange & Jones 2009). From a top-down perspective, insect herbivores may affect AM fungal colonization either positively (Wamberg, Christensen & Jakobsen 2003; Currie, Murray & Gange 2006) or negatively (Gange, Bower & Brown 2002; Wamberg, Christensen & Jakobsen 2003; Wearn & Gange 2007). Potential mechanisms include induced changes in carbon allocation, increased root exudation from herbivore infested plants (Gehring & Bennet 2009), increased photosynthetic rate or greater use of resources from storage organs (Gehring & Whitham 2002). However, to our knowledge, there are no previous reports of the effects of aphids on AM fungal colonization.

There is also scope for interactions involving changes to volatile organic compounds (VOCs) released from plant shoots. VOCs can act as kairomones, which are beneficial to the receiver but not to the emitter, and are used by insect herbivores, including migrating aphids, to locate their host plants (Bruce, Wadhams & Woodcock 2005; Pickett *et al.* 2012). Insect herbivores induce systemic defence-related signalling in host plants such as the salicylic acid and jasmonic acid signalling pathways (Goggin 2007), which affect the biosynthesis of plant VOCs. Therefore, the type and quantity of VOCs can change significantly when plants are attacked by herbivores (Unsicker, Kunert & Gershenzon 2009; Dicke 2009), becoming less attractive or repellent to subsequent herbivores (Dicke 1999), and attractive to natural enemies of these herbivores, such as parasitoids (Turlings *et al.* 1995). Salicylic acid and jasmonic acid signalling pathways are also regulated by mycorrhizal colonization in order for AM fungi to achieve compatibility with host plants (Pozo & Azcón-

Aguilar 2007). Therefore, AM fungi, via bottom-up activation of these pathways, may also affect the biosynthesis of VOCs and consequently aphid host location.

Indeed, studies have shown altered emissions of VOCs from mycorrhizal plants (Nemec & Lund 1990; Fontana *et al.* 2009), although only two studies have investigated the effect of AM fungi on the attractiveness of plants to insects. Guerrieri *et al.* (2004) found that mycorrhizal tomato plants (*Lycopersicon esculentum* Miller) were more attractive to parasitoids (an enemy of aphids) than were non-mycorrhizal plants. Schausberger *et al.* (2012) found that AM fungi affected the VOCs emitted by bean plants (*Phaseolus vulgaris* L.), making them more attractive to predators of spider mites.

No studies have tested how AM fungi affect the location of host plants by aphids, or any other insect herbivore. Importantly, many agricultural practices negatively affect the presence and effectiveness of AM fungal inoculum in the soil (Lekberg & Koide 2005), which might delay colonization of the crop relative to herbivore infestation. Therefore, if we are to understand how biotic interactions shape ecosystem functioning through changes in nutrition and fitness of plants, fungi and insect herbivores, the impacts of such bottom-up and top-down effects have to be examined together. A crucial factor is likely to be the relative strength of bottom-up and top-down effects, which is likely to depend on a range of factors, including the relative timing of colonization by AM fungi and infestation of aphids, and the activity and abundance of AM fungi and aphids.

This study was designed to test simultaneously the effects of AM fungi on plant interactions with aphids (bottom-up effects), and the effects of aphids on plant interactions with AM fungi (top-down effects) to address the following hypotheses: (i) the attractiveness of plants to host locating aphids is regulated by an interaction between aphids and AM fungi via induced changes in production of plant VOCs. We predict that plants infested with aphids will produce VOCs that repel aphids, whereas mycorrhizal plants will be attractive, and the effects of adding both will depend on the relative strength of the negative effect of aphids and the positive effect of AM fungi on the attractiveness; (ii) aphids have a negative effect on AM fungal colonization due to impacts on plant nutrition; and (iii) AM fungal colonization promotes aphid population development through positive changes in plant nutrition (Bennett, Alers-Garcia & Bever 2006). We manipulate the strength of top-down and bottom-up effects by altering the timing of exposure of plant roots to AM fungal inoculum relative to infestation of leaves by aphids.

Materials and methods

PLANTS, FUNGI, SOIL AND APHIDS

The plant species used was broad bean (*Vicia faba* L.) cultivar 'The Sutton dwarf' (Moles seeds, Colchester, UK); this species is mycotrophic, is an important crop and has previously been used

as a model plant for studying aphid–plant interactions (e.g. Schwartzberg, Böröczky & Tumlinson 2011).

The fungal inoculum used for both inoculations was a mix of two different sources. The source from BioOrganics LLC (Palm Springs, CA, USA) included spores of *Glomus aggregatum*, *G. clarum*, *G. deserticola*, *G. monosporus*, *G. mosseae*, *Rhizophagus irregularis* (syn. *Glomus intraradices*), *Gigaspora margarita* and *Paraglomus brasilianum* in clay powder carrier (*c.* 50 spores mL^{-1}). This was mixed (1 : 3) with inoculum obtained from INVAM (West Virginia University, Morgantown, WV, USA) comprising dried root fragments of *Plantago lanceolata* colonized with *Glomus clarum*, *G. etunicatum*, *G. claroideum*, *G. mosseae* and *Rhizophagus irregularis* in Terra-green. The control inoculum was an identical mix except it contained no spores and dried non-mycorrhizal roots of *Sorghum* spp.

The potting mixture comprised 26% vermiculite, 20% sandy loam top soil (all nutrients solely from the base materials: 9% clay, 17% silt, 74% sand, pH = 7·8, organic matter 24·2%, total nitrogen (N) (Dumas) 0·74%, available phosphorus (P) 64 mg L^{-1}, available potassium 1324 mg L^{-1}, available magnesium 222 mg L^{-1}), 10% grit and 16% sand, all from LBS (Colne, UK), and 28% sand from a local dune grassland system. All sand was autoclaved at 121 °C for 15 mins. A layer of live or control inocula was added underneath the seeds to one-third of the depth of the pot. During week seven, all plants were repotted into 2-L pots with potting mixture made of 40% top soil, 30% autoclaved sand, 15% vermiculite and 15% grit all from LBS. Similarly, AM fungal inoculum was added at one-third of the depth of the pot as described previously.

A clone of the pea aphid (*Acyrthosiphon pisum* L.) from Rothamsted Research (Harpenden, UK) was maintained in the laboratory at 22 ± 3 °C and 16 h light on broad beans of the same variety as the experimental plants 'The Sutton dwarf'. These beans were grown in nonsterile soil and so were likely to have some degree of mycorrhizal association, although this was not measured.

EXPERIMENTAL DESIGN

A glasshouse experiment was established in which plants were grown from seed either with or without mycorrhizal inoculum, and with or without aphids using a factorial design of six treatments. Crucially, plants colonized with AM fungi before aphids were compared with plants colonized with AM fungi after aphids, enabling us to tease apart bottom-up and top-down effects (Table 1). Seven weeks after planting, four adult aphids of the

same weight were added to plants allocated to aphid treatments. For those plants inoculated with AM fungi at planting (termed 'early inoculation'), this achieved the treatment where plants were colonized with AM fungi before aphids. Aphids colonize plants faster than do AM fungi, so to achieve the treatment where plants were colonized by AM fungi after aphids (top-down effect), and its equivalent nonaphid comparison treatment, two groups of the noninoculated plants were repotted with mycorrhizal inoculum at the same time as aphid addition, at week 7 (termed 'late inoculation'). To experimentally control for any effects of repotting, we treated all plants the same by repotting all plants in week 7, providing roots with additional inoculum, which was either free of AM fungi for controls and aphids-only treatments, or included AM fungi. In addition, to prevent spread of aphids to neighbours, all plants (even those without aphids) were enclosed in air-permeable insect screen bags.

The experiment took place between June and August 2010 (average day temperature 20 °C, minimum temperature 12 °C, average day length 16 h). Sample sizes varied from 6 to 9 between treatments due to low seed germination. At the end of the experiment (week 11), selected plants were used for collection of VOCs after which all plants were destructively harvested.

PLANT HEADSPACE SAMPLES

Five plants selected randomly from each treatment were used for collection of headspace samples (Bruce *et al.* 2008) during week 11 using an air entrainment kit (BJ Pye, Kings Walden, UK) as described previously (Babikova *et al.* 2013). Samples were stored at −20 °C, and subsamples for long-term storage were stored in glass ampoules under a nitrogen atmosphere.

We assessed pea aphid response to plant headspace samples using bioassays in a four-way olfactometer (Babikova *et al.* 2013). Each headspace sample was tested in four or more bioassays, each using a different aphid.

Analysis of plant headspace VOCs was achieved using GC as in Babikova *et al.* (2013). This analysis was restricted to 16 VOCs (Tables 2 and 3) previously identified from broad beans and determined to be electrophysiologically active to pea aphids by GC-coupled electroantennography (EAG; Babikova *et al.* 2013). Thus, our analysis quantifies only those VOCs known to affect pea aphid behaviour. The quantification of the amounts of VOCs produced per plant was carried out using external standards (Skelton *et al.* 2010), and the amounts were calculated per unit plant biomass (see Table 3).

Table 1. Treatment codes and timing of experimental manipulations of the six treatments. The beans were planted from seed on day 1. The codes refer to the treatments imposed on the plants both at day 1 (C for control, M for mycorrhizal) and at repotting at week 7 (C or M inoculum; plus A for aphids where applicable)

Treatment	Code	Day 1	Week 7	Week 11	Week 12	N
Control	CC	Control inoculum	Control inoculum	Collection of VOCs; N = 5	Harvest	6
Aphids only	CCA	Control inoculum	Aphids + control inoculum	Collection of VOCs; N = 5	Harvest	7
AM fungi late inoculation	CM	Control inoculum	AM fungal inoculum	Collection of VOCs; N = 5	Harvest	9
AM fungi late inoculation + aphids (colonization after aphids)	CAM	Control inoculum	Aphids + AM fungal inoculum	Collection of VOCs; N = 5	Harvest	8
AM fungi early inoculation	MM	AM fungal inoculum	AM fungal inoculum	Collection of VOCs; N = 5	Harvest	6
AM fungi early inoculation + aphids (colonization before aphids)	MAM	AM fungal inoculum	Aphids + AM fungal inoculum	Collection of VOCs; N = 5	Harvest	6

AM, arbuscular mycorrhizal; VOCs, volatile organic compounds.

Table 2. Results of general linear models for the main effects of aphids (present/absent), arbuscular mycorrhizal (AM) fungi (no AM fungi; inoculation late; inoculation early) and their interaction on attractiveness of plant headspace samples to aphids, nutrition and above-ground dry mass of plants, and amounts of volatile organic compounds (VOCs) produced by plants corrected for unit of dry mass

Dependent variable	Aphids		AM fungi		Aphids*AM fungi	
	F	P	F	P	F	P
Attractiveness to aphids	0·919	0·339	6·768	**0·002**↑↑	5·317	**0·006**
% Root length colonized by AM fungi	24·450	**0·000**↓	87·012	**0·000**↑↑	0·054	0·948
Total leaf N	8·459	**0·006**↓	0·486	0·619	0·411	0·666
Total leaf P	27·613	**0·000**↓	2·743	0·076	0·738	0·485
N to P ratio	1·060	0·309	0·589	0·560	0·318	0·729
Above-ground biomass	43·149	**0·000**↓	3·431	**0·042**↓→	1·299	0·284
Production of VOCs						
Green leaf volatiles total	1·777	0·202	6·845	**0·019**→→	4·251	**0·038**
(Z)-2-Hexenal	3·632	0·076	0·001	0·971	1·886	0·191
(E)-2-Hexenal	0·000	0·996	1·095	0·312	0·339	0·719
(E,E)-2,4-Hexadienal	7·297	**0·016**↑	0·144	0·710	1·668	0·227
(Z)-2-Heptenal	0·718	0·410	1·705	0·211	1·039	0·381
(Z)-3-Hexenyl acetate	2·088	0·169	9·620	**0·007**↓→	6·987	**0·009**
Aromatic hydrocarbons total	3·374	0·086	2·959	0·106	0·322	0·730
Benzaldehyde	3·331	0·088	2·453	0·138	0·259	0·775
Naphthalene	0·668	0·427	6·411	**0·023**↓↓	7·186	**0·008**
Cinnamaldehyde	0·313	0·584	0·075	0·788	0·097	0·909
Ketone						
6-Methyl-5-hepten-2-one	0·991	0·335	3·261	0·091	1·816	0·202
Phenol ester						
Methyl salicylate	0·005	0·946	2·026	0·175	0·062	0·940
Terpenes	4·720	**0·046**↓	3·247	0·092	4·749	**0·028**
(R,S)-β-Pinene (monoterpene)	2·632	0·126	0·092	0·766	3·687	0·054
(S)-Linalool (terpene alcohol)	1·178	0·295	2·365	0·145	3·362	0·067
(E,E)-4,8,12-Trimethyl-1,3,7,11- tridecatetraene (homoterpene)	4·367	0·054	2·042	0·174	2·802	0·097
Sesquiterpenes	2·660	0·124	12·202	**0·003**↓↓	3·033	0·083
(E)-Caryophyllene	4·088	0·061	11·640	**0·004**↓↓	2·847	0·094
(E)-β-Farnesene	0·328	0·575	15·237	**0·001**↓↓	2·108	0·161
(R)-Germacrene D	0·275	0·608	3·487	0·082	5·504	**0·019**
Total production of electroantennography active volatiles	4·003	0·064	8·008	**0·013**↓→	1·806	0·203

P values < 0·05 are highlighted in bold. Direction of effect is indicated by shifts (↑ positive effect; ↓ negative effect; → no effect). For the effect of AM fungi, the first arrow indicates direction of effect between plants with no AM fungi and plants inoculated on day 1; second arrow indicates direction of effect between plants with no AM fungi and plants inoculated at week 7. When the name of the chemical group is used, for example sesquiterpenes, this refers to the compounds specifically identified within this group and not to all possible members of that group.

ARBUSCULAR MYCORRHIZAL FUNGAL COLONIZATION, PLANT BIOMASS, AND LEAF N AND P CONCENTRATIONS

The extent of mycorrhizal colonization in trypan blue-stained root fragments was assessed microscopically using the magnified inter-section method (McGonigle *et al.* 1990), by scoring >100 intersects from at least three slides per sample. Plant above-ground dry mass was measured after drying at 60 °C for 48 h. Subsamples of dried and homogenized leaves were analysed for total N and P by sulphuric acid digest with hydrogen peroxide (Allen 1989) followed by colorimetric analysis by flow injection analysis (FIA star 5000; Foss, Hillerød Denmark).

STATISTICAL ANALYSIS

To test whether each individual treatment produced plant VOCs that were significantly attractive or repellent to aphids, time spent by aphids in the areas of the olfactometer containing plant head-space samples was compared with time spent in control areas (means of three control areas) for each treatment separately using a paired *t*-test (Bruce *et al.* 2008). Then, we calculated the attractiveness of each headspace sample to aphids as the time spent in the area containing solvent blanks (mean of three control areas) subtracted from that containing headspace samples. We used a general linear model (GLM) with the attractiveness estimate as the response variable and treatments as the explanatory variables as follows: aphids (two levels: present or absent), AM fungi (three levels: control, AM fungi early inoculation and AM fungi late inoculation) and an AM fungi*aphid interaction term. Because each headspace sample was tested repeatedly, the plant was entered as a random factor. We also ran these GLMs with each of the following response variables: percentage root length colonized by AM fungi (arcsine transformed percentage data), amount of each individual plant VOCs and amount of VOC functional groups (log-transformed data), total leaf N and P concentrations, leaf N : P ratio, above-ground plant dry mass. As headspace sample collection took place over several days, models of VOCs included entrainment day as a random factor. Fisher's least significant difference *post hoc* test was applied to identify which treatment groups differed.

Table 3. The mean amounts (ng g^{-1} dw 24 h^{-1} ± SEM) of volatile organic compounds (VOCs) collected from the headspace of plant shoots, nitrogen and phosphorus concentrations in plant shoots (mg g^{-1} dw ± SEM) and above-ground biomass of plants (g dw). Refer to Table 1 for treatment codes

Functional groups and VOCs	Kovats index	Treatment					
		1-CC	2-CCA	3-CM	4-CAM	5-MM	6-MAM
Green leaf volatiles		43·37 ± 18·61	10·46 ± 6·73	11·23 ± 5·97	7·23 ± 1·22	13·97 ± 0·30	3·78 ± 0·30
(Z)-2-Hexenal	817	1·14 ± 0·22	1·11 ± 0·30	1·51 ± 0·28	0·47 ± 0·20	1·34 ± 0·31	1·31 ± 0·08
(E)-2-Hexenal	825	1·28 ± 0·40	1·76 ± 0·23	1·62 ± 0·21	1·75 ± 0·50	1·96 ± 0·20	1·78 ± 0·19
(E,E)-2,4-Hexadienal	880	0·15 ± 0·07	0·58 ± 0·22	0·60 ± 0·45	2·59 ± 0·98	0·19 ± 0·05	0·30 ± 0·16
(Z)-2-Heptenal	924	1·41 ± 1·26	0·87 ± 0·76	0·69 ± 0·40	0·04 ± 0·02	1·61 ± 1·30	0·17 ± 0·05
(Z)-3-Hexenyl acetate	986	39·39 ± 18·19	6·14 ± 5·6	6·82 ± 5·93	2·39 ± 0·27	8·87 ± 7·91	0·22 ± 0·05
Aromatic hydrocarbons		563·2 ± 265·0	137·0 ± 131·1	167·6 ± 128·0	8·75 ± 2·77	181·3 ± 143·1	44·4 ± 41·0
Benzaldehyde	929	555·5 ± 262·1	134·2 ± 131·2	164·8 ± 128·0	5·70 ± 2·36	179·8 ± 143·3	41·6 ± 40·3
Naphthalene	1168	7·59 ± 3·7	2·60 ± 0·18	2·46 ± 0·51	1·83 ± 0·39	1·19 ± 0·37	2·52 ± 0·75
Cinnamaldehyde	1232	0·15 ± 0·03	0·21 ± 0·05	0·38 ± 0·15	1·22 ± 1·06	0·26 ± 0·07	0·25 ± 0·05
Ketone							
6-Methyl-5-hepten-2-one	967	11·19 ± 5·25	1·95 ± 1·64	5·04 ± 3·24	2·12 ± 0·36	3·01 ± 2·86	1·21 ± 0·86
Phenol ester							
Methyl salicylate	1172	0·41 ± 0·21	0·48 ± 0·22	0·24 ± 0·16	0·29 ± 0·13	0·21 ± 0·02	0·22 ± 0·08
Terpenes		569·7 ± 357·6	12·92 ± 7·43	185·3 ± 109·3	12·73 ± 3·72	22·60 ± 12·64	9·99 ± 3·67
(R,S)-β-Pinene (monoterpene)	972	0·18 ± 0·09	0·2 ± 0·11	0·23 ± 0·10	1·41 ± 0·59	0·18 ± 0·06	0·1 ± 0·03
(S)-Linalool (terpene alcohol)	1086	499·6 ± 363·46	0·3 ± 0·15	104·2 ± 103·97	7·87 ± 3·06	0·17 ± 0·12	0·19 ± 0·08
(E,E)-4,8,12-Trimethyl-1,3, 7,11-tridecatetraene (homoterpene)	1570	69·91 ± 41·17	12·42 ± 7·43	80·87 ± 41·10	3·46 ± 1·04	22·24 ± 12·70	9·7 ± 3·69
Sesquiterpenes		832·8 ± 306·7	205·0 ± 154·2	185·1 ± 96·9	57·2 ± 19·6	201·8 ± 146·9	37·62 ± 31·62
(E)-Caryophyllene	1424	802·2 ± 293·1	189·1 ± 150·3	174·4 ± 89·1	38·3 ± 13·1	195·1 ± 142·8	36·01 ± 31·11
(E)-β-Farnesene	1450	11·37 ± 5·09	11·54 ± 4·36	0·68 ± 0·47	3·09 ± 0·97	2·16 ± 1·64	0·95 ± 0·68
(R)-Germacrene D	1486	19·22 ± 9·38	4·31 ± 3·37	9·95 ± 8·33	15·77 ± 6·69	4·53 ± 2·54	0·65 ± 0·52
Total production of electroantennography active volatiles		2020 ± 920·5	367·8 ± 297·6	554·5 ± 326·1	88·32 ± 22·75	422·9 ± 310·0	97·22 ± 76·09
Plant nutrition							
Total leaf phosphorus		4·94 ± 0·38	3·29 ± 0·15	3·60 ± 0·44	2·60 ± 0·30	5·89 ± 0·53	3·75 ± 0·27
Total leaf nitrogen		43·4 ± 8·0	35·6 ± 3·7	34·8 ± 3·6	27·6 ± 6·5	46·9 ± 7·4	38·8 ± 3·0
Nitrogen to phosphorus ratio		8·94 ± 1·75	10·91 ± 1·10	10·31 ± 1·95	10·27 ± 1·96	7·79 ± 0·66	10·38 ± 0·41
Plant above-ground biomass		1·84 ± 0·02	1·70 ± 0·04	1·88 ± 0·02	1·69 ± 0·02	1·76 ± 0·05	1·66 ± 0·01

The effect of treatment on aphid fecundity was tested using a generalized linear model with aphid count on week 10 (1 week before collection of VOCs and harvest) as the response variable and treatment (control, AM fungi added initially and AM fungi added at week 7) as the explanatory factor. A poisson distribution and log link function were specified due to the count data distribution.

To explore the chemical mechanisms of the attractiveness of plants to aphids, we used linear regression with attractiveness of headspace samples to aphids (means of bioassays from each headspace) as a response variable and the following explanatory variables: each individual VOC (and their functional groups), percentage root length colonized by AM fungi, total leaf N concentration, total leaf P concentration, N : P ratio and plant above-ground biomass. In addition, as the mechanism of insect host location often depends on the ratio of VOCs (Bruce, Wadhams & Woodcock 2005), we also tested the proportions (arcsine transformed percentage data) of each VOCs (and their functional groups) within the sum of all EAG-active compounds to explain the attractiveness of plants to aphids. All statistical analysis was performed using SPSS (version 20, IBM).

Results

TREATMENT EFFECTS ON ATTRACTIVENESS OF HEADSPACE SAMPLES TO APHIDS

Aphids were significantly attracted to volatiles from CM (AM fungi only) plants (t = 2·8, d.f. = 23; P = 0·009) and MAM (AM fungi, early inoculation + aphids) plants (t = 3·6, d.f. = 22; P = 0·001; Fig. 1). In contrast, volatiles from CCA (aphids only) were significantly (t = −2·27, d.f. = 22; P = 0·033) repellent to them. Aphids were neither significantly attracted to nor repelled from headspace samples collected from CC (control; t = −0·44, d.f. = 23,
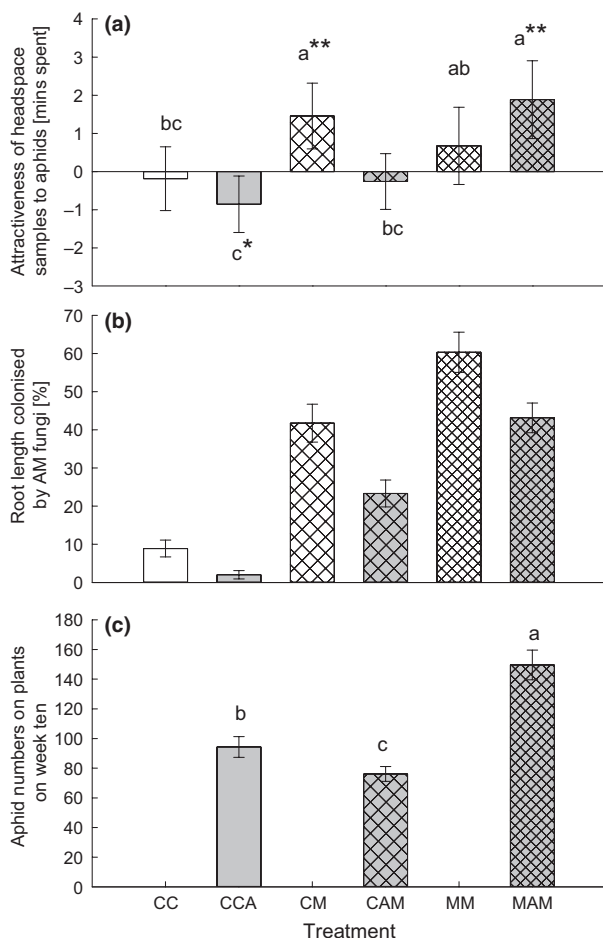
Fig. 1. (a) Response of pea aphids in a four-arm olfactometer to treatments, expressed as time spent in arms treated with volatile organic compounds minus the average time spent in control arms (min); ± 95% confidence intervals. Between-treatment differences are represented by letters; bars sharing a letter are not significantly different ($P > 0.05$). Within a treatment, significant effects (either positive or negative) on attractiveness have confidence intervals that do not overlap with zero and are indicated by asterisks (*$P < 0.05$; **$P < 0.01$). (b) Effect of treatment on percentage root length colonized by arbuscular mycorrhizal (AM) fungi; means ± standard errors. (c) Effect of AM fungi on aphid abundance on plants at week 10; means ± Wald 95% confidence intervals; Pairwise comparison of significant differences in aphid abundance was accomplished using generalized linear model. Treatment codes: CC – aphid-free and AM fungi-free plants; CCA – aphids only; CM – AM fungi only (late inoculation); CAM – AM fungal colonization before aphids; MM – AM fungi only (early inoculation); MAM – AM fungal colonization after aphids.

$P = 0.67$), CAM (AM fungi, late inoculation + aphids; $t = -0.69$, d.f. = 25, $P = 0.45$) and MM (AM fungi only, early inoculation; $t = 1.31$, d.f. = 25, $P = 0.20$) treatments.

There was no significant overall effect of aphid infestation on attractiveness of headspace samples to aphids ($F_{1,138} = 0.34$, $P = 0.64$; Table 2; Fig. 1). In contrast, from the bottom-up perspective, there was a significant positive effect of AM fungi on attractiveness of plant headspace samples to aphids ($F_{2,138} = 6.77$, $P = 0.002$). Control plants were less attractive compared with mycorrhizal plants inoculated late ($P = 0.014$), as well as compared

with mycorrhizal plants inoculated early ($P < 0.001$); there was no difference in the attractiveness of headspace samples from these two mycorrhizal treatments ($P = 0.182$). Furthermore, there was a significant interactive effect between aphids and AM fungal treatments (Table 2) on the attractiveness of headspace samples to aphids ($F_{2,136} = 5.32$, $P = 0.006$), indicating that the timing of AM fungal inoculation with respect to aphid infestation is important (Fig. 1).

There was no significant difference in attractiveness to VOCs collected from aphid-free control plants (CC) and aphid-infested control plants (CCA; $P = 0.31$). In contrast, for mycorrhizal plants, the addition of aphids significantly reduced headspace attractiveness: when plants were colonized by AM fungi late (inocula added at the same time as aphids; CAM), they were significantly less attractive to aphids than the equivalent plants without aphids (CM; $P = 0.006$), whereas, when plants were colonized by AM fungi early (before aphids) (MAM), they were more than twice as attractive to aphids than were the equivalent plants without aphids (MM), although this was not statistically significant ($P = 0.061$; Fig. 1). Thus, plants infested with aphids were repellent, unattractive or attractive, depending on whether plants were colonized by AM fungi and also on the timing of aphid infestation relative to colonization by AM fungi.

TREATMENT EFFECTS ON MYCORRHIZAL COLONIZATION

The percentage root length colonized by AM fungi in inoculated plants ranged from 20 to 60% (Fig. 1). There was a significant positive overall effect of AM fungal treatment group (no inoculum, early inoculation and late inoculation) on percentage root length colonized ($F_{2,45} = 87.12$; $P < 0.001$). Plants inoculated early had the highest colonization ranging 40–60%, which was significantly more than plants inoculated late ($P = 0.001$), which had about 20–40% of their root length colonized. A small proportion of roots were colonized in the noninoculated control plants (2–9%), which was significantly less compared with plants inoculated early ($P < 0.001$) and late ($P < 0.001$).

There was a highly significant overall negative effect of aphids on the percentage root length colonized by AM fungi ($F_{1,45} = 24.45$; $P < 0.001$; Fig. 1). There was a 20% reduction in root length colonized regardless of whether plants were inoculated early or late; however, the absolute extent of colonization was greater in aphid-infested plants inoculated with mycorrhizal fungi early than those inoculated late. There was no effect of interaction between aphids and AM fungi on colonization (Table 2).

TREATMENT EFFECTS ON APHID ABUNDANCE

There was a significant effect of AM fungal colonization on aphid population development as shown by differences in aphid counts at week 10 (Wald $\chi^2 = 207.03$, $P < 0.001$;

Fig. 1). In the control treatment CCA, there were on average 94 (87–101; Wald 95% confidence interval) aphids per plant, whereas on plants inoculated with AM fungi late [hence colonized after the aphid infestation (CAM)], there were on average 76 (71–82; Wald 95% confidence interval) aphids, which is 20% less compared with CCA (Wald $\chi^2 = 15\cdot41$, $P < 0\cdot001$). However, on plants inoculated with AM fungi early [hence colonized with AM fungi before aphids (MAM)], there were on average 150 (140–158; Wald 95% confidence interval) aphids per plant, which is about 40% more than in treatment CCA (Wald $\chi^2 = 86\cdot198$, $P < 0\cdot001$) and about 50% more than in treatment CAM (Wald $\chi^2 = 189$, $P < 0\cdot001$).

TREATMENT EFFECTS ON PRODUCTION OF PLANT VOCS

The effects of aphids, AM fungi treatment groups and their interactions on production of individual VOCs and VOC functional groups are summarized in Table 2, and significant effects are also shown in Fig. S1 (Supporting Information). As there were significant differences in plant biomass between the treatments, production of VOCs was calculated per gram of dry tissue.

Aphids had an effect on production of (E,E)-2,4-hexadienal, which was increased on aphid-infested plants compared with aphid-free plants ($F_{1,29} = 7\cdot30$, $P = 0\cdot016$). AM fungi negatively affected production of sesquiterpenes ($F_{2,29} = 12\cdot20$, $P = 0\cdot003$), particularly the sesquiterpenes (E)-caryophyllene ($F_{2,29} = 11\cdot64$, $P = 0\cdot004$) and (E)-β-farnesene ($F_{2,29} = 15\cdot24$, $P = 0\cdot001$). Compared with control plants, both these compounds were produced in smaller amounts both with plants inoculated with AM fungi late ($P = 0\cdot025$ for (E)-caryophyllene and $P = 0\cdot007$ for (E)-β-farnesene) and with plants inoculated with AM fungi early ($P = 0\cdot028$ for (E)-caryophyllene and $P = 0\cdot002$ for (E)-β-farnesene). There was no difference in production of (E)-caryophyllene and (E)-β-farnesene between plants inoculated by AM fungi late ($P = 0\cdot95$) and early ($P = 0\cdot59$).

There was a large range in total emissions of EAG-active VOCs, which was greatest in treatment CC (2021 ng g^{-1} dw^{-1} 24 h^{-1}) and lowest in treatment CAM (88·32 ng g^{-1} dw^{-1} 24 h^{-1}; Table 3). AM fungi had a significant effect on total production of EAG-active VOCs ($F_{1,29} = 8\cdot00$, $P = 0\cdot013$). Early inoculation with AM fungi decreased total emission compared with control plants ($P = 0\cdot049$); however, decreased emission from late-inoculated plants was not significant ($P = 0\cdot28$), and there was no difference in emission between early- and late-inoculated plants ($P = 0\cdot32$). There was no effect of aphids on total emission of EAG-active VOCs or any effect of the interaction between aphids and AM fungi.

The interaction between AM fungi and aphids affected emission of total green leaf volatiles ($F_{2,29} = 4\cdot251$, $P = 0\cdot038$), the green leaf volatile (Z)-3-hexenyl acetate ($F_{2,29} = 6\cdot99$, $P = 0\cdot009$), the aromatic hydrocarbon naphthalene ($F_{2,29} = 7\cdot19$, $P = 0\cdot008$), total terpenes ($F_{2,29} = 4\cdot75$, $P = 0\cdot028$) and the sesquiterpene (R)-germacrene D ($F_{2,29} = 5\cdot50$, $P = 0\cdot019$) (Fig. S1, Supporting Information).

WHICH FACTORS UNDERPIN THE ATTRACTIVENESS OF PLANT HEADSPACE SAMPLES TO APHIDS?

We found a significant positive relationship between attractiveness of headspace samples to aphids and percentage root length colonized by AM fungi ($F_{1,29} = 5\cdot23$; $R^2 = 0\cdot16$; $P = 0\cdot030$; Fig. 2). Attractiveness of headspace samples to aphids had no relationships with measures of plant nutrition, including total leaf N concentration ($F_{1,29} = 0\cdot365$; $P = 0\cdot551$), total leaf P concentration ($F_{1,29} = 0\cdot538$; $P = 0\cdot469$), N : P ratio ($F_{1,29} = 0\cdot056$; $P = 0\cdot815$) and plant biomass ($F_{1,29} < 0\cdot001$; $P = 0\cdot997$).

To investigate the chemical mechanism of attractiveness of plant headspace samples to aphids via VOCs, we tested for linear regression between the attractiveness and the production of each VOC (and their functional groups) per plant calculated per gram of dry tissue. We observed a significant negative relationship between the attractiveness to aphids and the amount of phenol ester methyl salicylate ($R^2 = 0\cdot23$; $F = 8\cdot31$; $P = 0\cdot007$; Fig. 2).

We further tested for linear regressions between the attractiveness and proportions of each VOC (and VOCs expressed as functional groups) within the sum of all EAG-active compounds (percentage data). The attractiveness was positively affected by proportions of two green leaf volatiles: (Z)-2-hexenal ($R^2 = 0\cdot18$; $F = 6\cdot30$; $P = 0\cdot018$; Fig. 2) and (E)-2-hexenal ($R^2 = 0\cdot14$; $F = 4\cdot73$; $P = 0\cdot038$; Fig. 2). Furthermore, we observed a negative relationship between the attractiveness and proportions of sesquiterpenes in the VOC blend ($R^2 = 0\cdot35$; $F = 14\cdot74$; $P < 0\cdot001$; Fig. 2), particularly proportions of (E)-caryophyllene ($R^2 = 0\cdot29$; $F = 11\cdot35$; $P = 0\cdot002$; Fig. 2).

TREATMENT EFFECTS ON PLANT BIOMASS AND NUTRITION

Mean above-ground biomass ranged from 1·66 to 1·88 g dw (Table 3). There was an overall effect of AM fungal treatment on plant above-ground biomass ($F_{2,45} = 3\cdot431$; $P = 0\cdot042$; Table 2). There was an overall negative effect of aphids on plant biomass ($F_{1,45} = 43\cdot149$; $P < 0\cdot001$). Biomass of aphid-free plants was in average 1·83 g dw, and biomass of aphid-infested plants was in average 1·68 g dw. There was no interaction between AM fungi and aphids on above-ground biomass ($F_{2,45} = 1\cdot29$; $P = 0\cdot28$).

Total N concentrations ranged between treatments from 33·2 mg g^{-1} dw in treatment CCA to 48·1 mg g^{-1} dw in treatment MM (Table 3). There was a significant overall negative effect of aphids on total leaf N ($F_{1,45} = 8\cdot46$; $P = 0\cdot006$) (Table 2), which was on average 45·3 mg g^{-1} dw in aphid-free plants and 34·7 mg g^{-1} dw in aphid-infested plants.
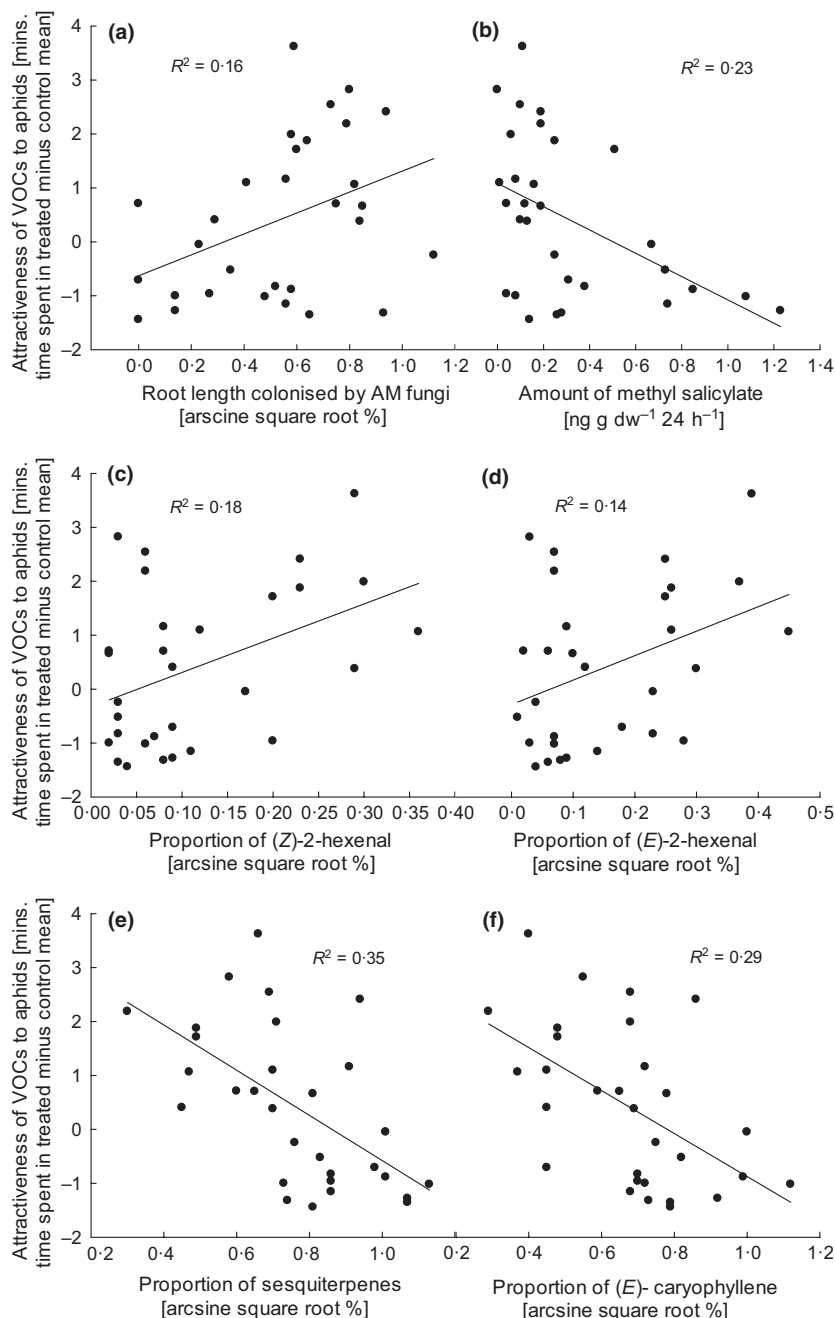
Fig. 2. Association between the attractiveness of headspace samples to aphids and (a) the percentage root length colonized by arbuscular mycorrhizal (AM) fungi ($F_{1,29} = 5.23$; $R^2 = 0.16$; $P = 0.030$) and (b-f) plant volatiles that were electrophysiologically active on antennae of pea aphids (b) – amount of methyl salicylate; $F_{1,29} = 8.31$; $R^2 = 0.23$; $P = 0.007$). (c) Proportion of (Z)-2-hexenal; $F_{1,29} = 6.30$; $R^2 = 0.18$; $P = 0.018$. (d) Proportion of (E)-2-hexenal; $F_{1,29} = 4.73$; $R^2 = 0.14$; $P = 0.038$. (e) Proportion of sesquiterpenes; $F_{1,29} = 14.7$; $R^2 = 0.35$; $P < 0.001$. (f) Proportion of (E)-caryophyllene; $F_{1,29} = 11.35$; $R^2 = 0.29$; $P = 0.002$.

Similarly, total leaf P concentrations, which ranged from 3·1 mg g^{-1} dw in CAM treatment to 5·7 mg g^{-1} dw in treatment MM (Table 3), were negatively affected by aphids ($F_{1,45} = 27.613$; $P < 0.001$; Table 2). The average total leaf P concentration in the aphid-free treatments was 4·9 mg g^{-1} dw, whereas in the aphid-infested treatments it was 3·3 mg g^{-1} dw. There was no overall effect of AM fungi or interaction between AM fungi and aphids on total leaf N concentration and total leaf P concentration. There

was no effect of AM fungi or aphids or their interaction on N : P ratio in the leaves (Table 2).

Discussion

Interactions between aphids and mycorrhizal fungi sharing a common host plant have not been characterized in detail before. Previous studies investigated mainly bottom-up effects of AM fungi on aphids (Koricheva, Gange

& Jones 2009), but no studies have investigated top-down effects of aphids on AM fungi, or how the interactions between AM fungi and aphids regulate production of plant VOCs and therefore attractiveness to herbivores such as aphids. Although aphids and AM fungi are not in direct contact, our study shows that they have profound effects on each other by altering the condition of their shared host plant. Our findings reveal complex interactions between AM fungi and aphids that are dependent on the strength of bottom-up and top-down effects, here manipulated by changing the timing of exposure to AM fungal inoculum. From the bottom up, AM fungi alter plant VOC emissions, attractiveness of plants to aphids and aphid development, whereas from the top down, aphid infestation leads to reduced mycorrhizal development.

ATTRACTIVENESS OF PLANT VOCS TO APHIDS TENDS TO BE DRIVEN MORE BY AM FUNGI THAN APHIDS

We hypothesized that the attractiveness of plants to host locating aphids is regulated by an interaction between aphids and AM fungi via induced changes in production of plant VOCs. We predicted that plants infested with aphids will be repellent, mycorrhizal plants will be attractive and the effects of adding both will depend on the relative strength of the negative effect of aphids and the positive effect of AM fungi on attractiveness. In agreement with our hypothesis, plants exposed to aphids alone (CCA) released VOCs that were repellent to other aphids, whereas plants exposed to AM fungi alone (CM) were attractive. The effect of aphids and AM fungi together depended on the sequence of exposure, which we used as a proxy for the strength of bottom-up and top-down effects: plants where aphids infested before AM fungi colonized (CAM) were not attractive to aphids, whereas plants colonized by AM fungi before aphids (MAM) were significantly attractive to aphids. Thus, whereas aphids infesting non-mycorrhizal plants produce repellent VOCs, these effects were negated even by 'weak' bottom-up effects (i.e. when plants were exposed to AM fungi at the same time as aphids) to produce VOCs that were neither attractive or repellent. When bottom-up effects were 'strong' (mycorrhizal colonization well-established before aphid infestation), plants produced VOCs that were attractive to aphids. This suggests that attractiveness tends to be driven more by AM fungi than aphids.

We also found a positive relationship between percentage root length colonized and attractiveness of plant VOCs to aphids, but this relationship explained only 16% of the variation in our data. The extent of colonization is therefore unlikely to be the only driver of VOC attractiveness to aphids. In nature, the strength of bottom-up effects is likely affected by a number of factors including timing of colonization, plant phenology, and the abundance and activity of AM fungi. Because both AM fungi (Fontana *et al.* 2009; Schausberger *et al.* 2012) and aphids (Cham-

berlain *et al.* 2001) regulate plant signalling leading to emission of VOCs, a further explanation relates to possible interactive effects of timing of AM fungal colonization with respect to aphid infestation (before AM fungi or after AM fungi) on plant signalling pathways.

We hypothesized that aphids have a negative effect on AM fungal colonization due to impacts on plant nutrition. In support of this hypothesis, we observed significant reductions in the extent of AM fungal colonization of all plants infested with aphids. While we do not know the mechanism, some experiments have shown that aphid infestation reduces allocation of carbon below-ground (Gehring & Whitham 1994) and that this can lead to weaker mycorrhizal development (Gehring & Whitham 2002). Moreover, AM fungi require regulation of jasmonic acid- and salicylic acid-dependant pathways (Pozo & Azcón-Aguilar 2007), and aphid induced defence-related signalling likewise involves regulation of these pathways (Goggin 2007), which could have negative effects on AM fungal colonization and ultimately their functioning.

THE TIMING OF AM FUNGAL COLONIZATION AFFECTS APHID ABUNDANCE

We hypothesized that aphids will develop faster on mycorrhizal plants through improved nutrition (Bennett, Alers-Garcia & Bever 2006). In agreement with our hypothesis, aphids developed faster on plants if they were already mycorrhizal when they received the aphids (MAM) compared with controls (CCA). However, on plants colonized with AM fungi after aphid infestation, aphids developed slower compared with controls (CCA). While we did not detect any statistically significant effect of AM fungi on leaf nutrition, plants inoculated with mycorrhizas early (MM) had the greatest leaf P concentration, followed by control plants (CC), while plants inoculated late (CM) had the least leaf P concentrations (Table 3). This suggests that aphid development could be related to plant nutrition.

VOLATILE ORGANIC COMPOUNDS ASSOCIATED WITH PLANT ATTRACTIVENESS TO APHIDS

The attractiveness of plant VOCs to aphids was negatively correlated with the amount of methyl salicylate and proportions of sesquiterpenes particularly (*E*)-caryophyllene, and positively correlated with proportions of green leaf volatiles (*Z*)-2-hexenal and (*E*)-2-hexenal. However, only production of sesquiterpenes (i) was affected by aphids and AM fungal treatments and (ii) showed a direct link with the aphid host location response. Both (*E*)-caryophyllene and (*E*)-β-farnesene were suppressed in plants colonized by AM fungi, regardless of the timing of inoculation. This supports previous work where less sesquiterpenes were detected from plantain (*Plantago lanceolata*) damaged by noctuid moth (*Spodoptera littoralis*) larvae when plants were colonized by AM fungus (*Rhizophagus irregularis* syn. *G. intraradices*), compared with similarly herbivore-damaged

non-mycorrhizal plants (Fontana *et al.* 2009). (*E*)-*β*-farnesene, which can be produced by both plants and aphids, is an aphid alarm pheromone, which repels aphids (Hardie *et al.* 1999). We therefore suggest that suppressed emission of sesquiterpenes in mycorrhizal plants was a key chemical mechanism of attractiveness of mycorrhizal plants to aphids under our experimental conditions.

Aphids had weak overall effects on the production of VOCs by plants, with only the production of (*E,E*)-2,-4-hexadienal being significantly greater in the presence of aphids. This general weak effect of aphids on VOCs supports other work where pea aphids did not induce volatile defence responses from broad bean (Schwartzberg, Böröczky & Tumlinson 2011). Indeed, it is possible that aphids, as stealthy herbivores, have adapted to evade detection by the plant, which would otherwise trigger VOC release and attract predators (Walling 2008). However, we found significant interaction terms between AM fungi and aphids on emission of several VOCs, particularly total green leaf volatiles, (*Z*)-3-hexenyl acetate, naphthalene, total terpenes and the sesquiterpene (*R*)-germacrene D (Fig. S1, Supporting information). Aphids appear to have suppressive effects on emission of all but one of these compounds ((*R*)-germacrene D), but their effect was only apparent if AM fungi were absent or the extent of AM fungal colonization small, a scenario that is unlikely in nature but possible under more intensive agronomic or horticultural settings. Similar interactive effects of aphids, albeit with the beet armyworm caterpillar (*Spodoptera exigua*) rather than AM fungi, also occur (Schwartzberg, Böröczky & Tumlinson 2011). In another multispecies system, simultaneous colonization of cotton plants by beet armyworm caterpillars and whitefly (*Bemisia tabaci*) led to production of VOCs markedly differing from the situation when the plants were under attack from either one of the herbivores separately (Rodriguez-Saona, Crafts-Brandner & Cañas 2003). Our findings therefore contribute to the growing realization that understanding the effects of herbivores on plants requires experiments that represent natural complexity, by considering simultaneously other key organisms that interact with plants in nature.

Conclusions

It is clear that both AM fungi and aphids affect production of plant VOCs, which alters plant attractiveness and insect behaviour. Our work demonstrates that the level of colonization by AM fungi regulates plant VOC emission and thereby has a key role in insect host location. Mycorrhizal plants produced VOCs that were more attractive to aphids than noninoculated plants, while aphid infestation negatively affected AM fungal colonization. This suggests a possible feedback loop whereby the attractiveness of mycorrhizal plants to aphids stimulates aphid infestation, which then negatively affects mycorrhizal development. Our findings provide new insights into how soil microbial communities can affect above-ground processes, but high-light the need to determine the long-term effects of these bottom-up and top-down processes on plant performance and ecosystem functioning.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Effect of treatments on emission of volatiles from broad bean (*Vicia faba*) [ng g dw^{-1} 24 h^{-1}], which elicit electrophysiological activity on antennae of pea aphid (*Acyrthosiphon pisum*).