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LETTER

Underground signals carried through common mycelial networks warn neighbouring plants of aphid attack

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Abstract

The roots of most land plants are colonised by mycorrhizal fungi that provide mineral nutrients in exchange for carbon. Here, we show that mycorrhizal mycelia can also act as a conduit for signalling between plants, acting as an early warning system for herbivore attack. Insect herbivory causes systemic changes in the production of plant volatiles, particularly methyl salicylate, making bean plants, *Vicia faba*, repellent to aphids but attractive to aphid enemies such as parasitoids. We demonstrate that these effects can also occur in aphid-free plants but only when they are connected to aphid-infested plants via a common mycorrhizal mycelial network. This underground messaging system allows neighbouring plants to invoke herbivore defences before attack. Our findings demonstrate that common mycorrhizal mycelial networks can determine the outcome of multitrophic interactions by communicating information on herbivore attack between plants, thereby influencing the behaviour of both herbivores and their natural enemies.

Keywords

Arbuscular mycorrhizal fungi, broad bean (*Vicia faba*), common mycelial networks, induced defence, multitrophic interactions, parasitoid wasp (*Aphidius ervi*), pea aphid (*Acyrthosiphon pisum*), plant volatiles, plant-to-plant communication.

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INTRODUCTION

Arbuscular mycorrhizal (AM) fungi form symbioses with many herbaceous plants, including important crop species, have a near global distribution and are among the most functionally important soil microorganisms (Smith & Read 2008). AM fungi often significantly improve mineral nutrient uptake (Smith & Read 2008) and can enhance tolerance to root and shoot pathogens (Whipps 2004), nematodes (De La Peña *et al.* 2006; Vos *et al.* 2012) and drought (Smith & Read 2008). In return, plants supply AM fungi with carbohydrates (Johnson *et al.* 2002) that are used in part to develop extensive mycelial networks (Leake *et al.* 2004), which act as conduits for carbon (Johnson *et al.* 2002) and mineral nutrients (Johnson *et al.* 2001). Due to a lack of specificity of AM fungi to their host plants (Smith & Read 2008), external mycelia can produce so-called 'common mycelial networks' that connect the roots of different species, as well as individuals of the same species (Simard & Durall 2004). Common mycelial networks facilitate seedling establishment (Van Der Heijden 2004), influence plant community composition (Van Der Heijden & Horton 2009) and are the primary pathways through which many species of non-photosynthetic plants acquire their energy (Bidartondo *et al.* 2002).

Evidence is emerging that mycelial networks have potential to transport signalling compounds. Barto *et al.* (2011) demonstrated that allelochemicals released by marigold (*Tagetes tenuifolia* Millsp) could be transported through AM fungal networks to inhibit the growth of neighbouring plants. Song *et al.* (2010) found that interplant connections via common mycelial networks led to increased disease resistance, defensive enzyme activities and defence-related gene expression in healthy tomato plants (*Lycopersicon esculentum* Mill) connected to plants infected with leaf early blight (*Alternaria solani*). This finding suggests that interplant transfer of pathogenic fungal disease resistance signals via these networks could be occurring.

If common mycelial networks can act as conduits for signalling compounds, there clearly is considerable potential for mycorrhizal fungi to mediate plant responses to herbivores. There could also be effects on other trophic levels such as herbivore enemies, because both insect herbivores and their parasitoid enemies respond to volatile organic compounds (VOCs) emitted by plant leaves albeit in different ways. Sap-sucking herbivores such as aphids use VOCs as cues for locating host plants (Bruce *et al.* 2005) but, following the attack, the composition of VOCs released changes and becomes repellent to subsequent herbivores (Bernasconi *et al.* 1998) and attractive to their natural enemies, such as parasitoid wasps (Turlings *et al.* 1995). VOCs produced by infested plants are often produced systemically (Pickett *et al.* 2003) and can be transmitted aerially between plants (Dicke & Bruin 2001) as well as being released into the rhizosphere from roots (Chamberlain *et al.* 2001; Rasmann *et al.* 2005).

It has been proposed (Barto *et al.* 2012; Dicke & Dijkman 2001), but so far not tested, that common mycelial networks may facilitate interplant transfer of signalling compounds released by plants under attack by insect herbivores and that such signalling may induce emission of VOCs. If so, this could potentially have profound effects on multitrophic interactions. Here, we test the hypothesis that common mycelial networks act as interplant conduits that provide an early warning system of herbivore attack. We quantify effects on the behaviour of a piercing, sucking aphid herbivore and

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one of its key natural enemies, and identify the chemical signal driving the insect behavioural responses. Specifically, we test how common mycelial networks linking aphid-infested plants with aphid-free plants affect the attractiveness of VOCs to aphids and parasitoid wasps. If AM fungi act as conduits for signalling compounds between aphid-infested plants and uninfested plants, we predict that aphid-free plants connected via common mycorrhizal networks to aphid-infested plants will act as if they themselves are infested, that is, they will share similar VOC profiles and elicit similar responses from insects. We also expect VOCs that are repellent to aphids to be attractive to parasitoid wasps. This is because these VOCs are produced in response to aphid infestation, acting to repel further aphid attack and to attract natural enemies, since they are a signal that the parasitoid's aphid prey is present.

MATERIALS AND METHODS

Mesocosm establishment

Eight mesocosms (30 cm diameter) were established in a greenhouse containing a mix of 10% 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 (Dumas) 0.74%, available phosphorus 64 mg L^{-1}, available potassium 1324 mg L^{-1}, available magnesium 222 mg L^{-1}], 24% sand, 16% terra green and 10% grit all from LBS (Colne, UK) and 40% washed sand from Culbin Forest National Nature Reserve, Morayshire, UK, and an inoculum of the AM fungus *Glomus intraradices* UT118 (INVAM). Seedlings of *Plantago lanceolata* L. were used to establish a mycorrhizal fungal network in each mesocosm for 4 months prior to the experiment, after which all their shoots were removed.

Experimental design

Five eleven-day-old seedlings of bean (*Vicia faba* L.) cultivar 'Sutton dwarf' (Moles seeds, Colchester, UK) were planted in the mesocosms (Fig. 1). The beans were arranged so that a central plant acted as a 'donor', which received aphids in the last 4 days of the experiment, surrounded by four 'receiver' plants that never came into direct contact with aphids (Fig. 1). Two receiver plants were controls whose mycorrhizal fungi were not connected to the donor (achieved by two independent methods), and two receivers were connected to the donor by the common mycelial network (also using two methods). In one control treatment, the receiver plant was grown in a core (6 cm diameter, 20 cm deep) surrounded by 0.5 μm mesh, preventing penetration by fungal hyphae such that external mycorrhizal mycelium from the plant could never form connections with neighbours. The second control receiver plant was grown in a core surrounded by 40 μm mesh. This mesh enabled the plants to form common mycelial networks but, immediately before aphids were added to the donor plants, the core was rotated to snap all fungal hyphae penetrating through the mesh (Johnson *et al.* 2001), thus breaking the connection with the donor plant. The two other receiver plants could form common mycelial networks with the donor plant: one grown with no barrier, allowing the intermingling of both mycorrhizal mycelium and roots with the donor, and one allowing mycelial contact only by means of a 40 μm mesh core that was never rotated. This enabled us to separate any potential plant-to-plant signalling via root contact from signalling via common mycelial networks. A key aspect of this design is that all plants were colonised by AM fungi to avoid issues arising from known differences in composition of volatiles between mycorrhizal and non-mycorrhizal plants (Guerrieri *et al.* 2004). Microscopical examination of trypan blue-stained roots confirmed that all bean plants were colonised by AM fungi.

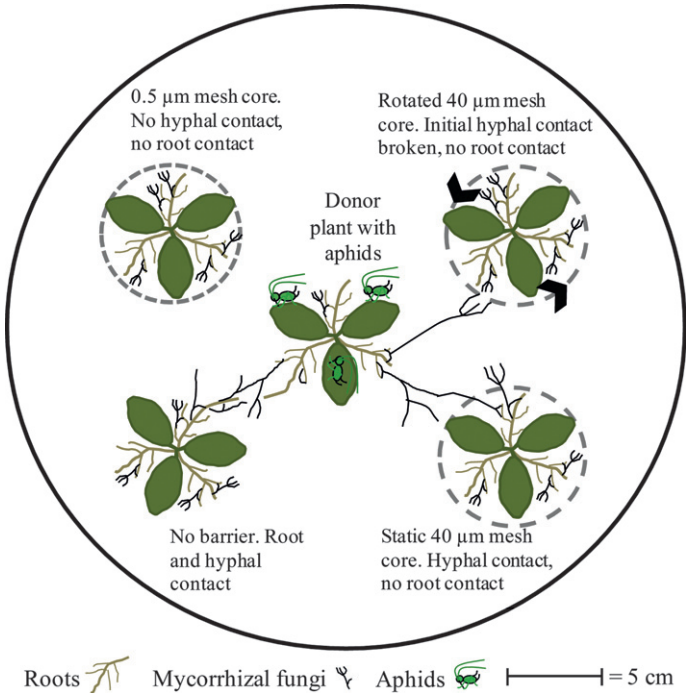

Figure 1 Experimental mesocosm (30 cm diameter; $n = 8$) showing the donor plant, which was colonised by aphids, and four aphid-free receiver plants. All plants were grown in the mycorrhizal condition but one plant was prevented from forming mycelial connections to donor plants (0.5 μm mesh), another was allowed to form connections initially but the connections were snapped after additions of aphids to the donor (rotated 40 μm mesh), and two other plants were allowed to form shared mycorrhizal fungal networks (non-rotated 40 μm mesh allowing fungal contact only; no barrier allowing fungal and root contact) with the donor plant for the duration of the experiment.

Five weeks after transplanting the *V. faba* plants, by which time hyphal connections would have been well established, all receiver plants were placed in polyethyleneterephthalate (PET) bags, which prevented plant-to-plant communication via aerial volatiles, and connected to entrainment apparatus (see section on collection of volatiles below) immediately before the donor plant was infested with 50 adult pea aphids (*Acyrthosiphon pisum* Harris), before itself being sealed with a bag. The aphids were supplied by Rothamsted Research Institute and were reared on broad beans in the laboratory at the University of Aberdeen (20 ± 3 °C; 16 h day: 8 h dark).

Collection of plant volatiles

Collection of volatiles (Bruce *et al.* 2008) was conducted on all plants 96 h after addition of aphids on the donor plant using an air entrainment kit (BJ Pye, Kings Walden, UK). This timing is based on findings that expression of plant defence genes occurs 2–3 days after aphid attack (e.g. De Vos *et al.* 2005). Plant shoots were enclosed in PET bags, heated to 180 °C for at least 2 h before use, which were fastened around the stems using polytetrafluoroethylene (PTFE)

tape. Air, purified by passage through an activated charcoal filter, was pumped into the bag through an inlet port made of PTFE tubing at 600 mL min^{-1}. Volatiles were collected using pre-conditioned (dichloromethane) glass tubes containing Porapak Q polymer (50 mg) inserted into collection ports fitted in the top of the bag. Air was pumped through these tubes at 400 mL min^{-1}, less than the input rate, thus ensuring that unfiltered air was not drawn into the collection bag from outside, but which will have resulted in a capture efficiency of about 66%. The bags and pumped air served to prevent aerial volatiles causing communication between plants, so that our observations were the result of below-ground rather than above-ground conduits. The period of entrainment collection was 24 h. Porapaq Q filters were eluted with 0.5 mL of diethyl ether (spectrophotometric grade, inhibitor free, Sigma Ltd) and stored at −20 °C. Five background headspace samples were obtained using an identical procedure but without any plants. Headspace sampling allowed isolation of the volatiles from plants exposed to different treatments to enable accurate assessment of insect responses to those volatiles in subsequent bioassays. Doses used in bioassays were adjusted so that they were ecologically relevant in terms of the amount in plant equivalents over the duration of the bioassay.

Behavioural responses of aphids and parasitoids

Parasitoids were reared on pea aphids on broad beans in the insectary at Rothamsted Research (22 ± 3 °C; 16 h day: 8 h dark). To test whether aphids and parasitoids were attracted or repelled by headspace samples collected from donor and receiver plants, we conducted bioassays using a four-arm olfactometer (Pettersson 1970; Webster *et al.* 2010) either using alate (winged) morphs of pea aphids starved for 2–4 h prior to the bioassay, or female parasitoid wasps (*Aphidius ervi* Haliday), which had experience of oviposition. Filters paper treated with reagent blanks were attached to three of the arms, while paper treated with 10 μL of VOCs eluted from plant headspace gas samples was attached to the remaining arm. The insect was placed inside the central area and air was pulled through the apparatus by a suction pump (200 mL min^{-1}). Insect movement in the arena was recorded using OLFA (Exeter Software, Setauket, NY, USA) software during the bioassay. Each bioassay was conducted for 16 min, and each 2 min, the olfactometer was turned 45° in one direction to avoid any bias caused by uneven light. The 10 μL VOC sample was 1/35th of the volume collected per 24 h entrainment, and together with the capture efficiency, we estimate the dose in the bioassay was 1.7 times the amount produced by the plants under the experimental conditions. The attractiveness of plant headspace samples to insects was taken as the time spent by the insect in the olfactometer area containing plant headspace samples, minus the time spent in the olfactometer area treated with reagent blanks.

For pea aphids, samples from seven of the eight replicates of each treatment were used, and we performed five bioassays per sample (for statistical analyses, means of these five bioassays were used). For parasitoids, we used VOC samples from between six and eight replicates per treatment, and performed between three and five bioassays per sample. We always used a fresh preparation of VOCs sample on the filter paper and a new insect for each bioassay. This was the highest possible replication of bioassays allowed by the volumes of VOCs samples we collected. We conducted further behavioural bioassays with aphids only, due to limited amounts of remaining headspace samples, to test the effect of authentic standards of the identified chemicals in driving insect responses. Analysis of VOCs eluted from plant headspace samples indicated that methyl salicylate may play a role in insect response to plants. To test this potential chemical mechanism, we added 3 ng mL^{-1} of methyl salicylate (the mean concentration found in samples that were naturally repellent to aphids) to head-space samples that were originally attractive to aphids, and undertook additional bioassays following the protocol described previously.

Gas chromatography (GC) analysis of plant headspace VOCs

Separation of VOCs from each plant headspace sample was achieved on a non-polar (HP-1, 50 m × 0.32 mm inner diameter × 0.5 mm film thickness, J & W Scientific) capillary column using an HP6890N GC (Agilent Technologies, UK) fitted with a cool-on-column injector, a deactivated retention gap (1 m × 0.53 mm inner diameter) and flame ionisation detector (FID). The carrier gas was hydrogen. Samples (2 μL) were injected using an HP 7683 series injector. The amounts of VOCs produced per plant were quantified using external standards.

Identification of electrophysiologically active VOCs

Electroantennography (EAG) recordings from aphid and parasitoid antennae ($n \geq 3$ preparations) coupled to a gas chromatograph (GC-EAG; Wadhams 1990; Sasso *et al.* 2009) were used to identify active VOCs eliciting a response from the insects. EAG recordings were made using Ag-AgCl glass electrodes filled with a saline solution (as in Maddrell 1969, but without glucose). For both aphids and parasitoids, the head was excised and placed within the indifferent electrode, and the tips of both antennae were removed before they were inserted into the recording electrode. The effluent from the transfer line to the antenna was delivered into a purified airstream (1.0 L min^{-1}) flowing continuously over the preparation. Separation of the volatiles from each plant headspace sample was achieved on an AI 93 GC equipped with a cold on-column injector and FID. The carrier gas was helium and the VOCs were passed through a high impedance amplifier (UN-06, Syntech, The Netherlands) and analysed using the software package Syntech. Compounds were assumed to be EAG-active if they caused EAG responses on three or more preparations.

GC coupled mass spectrometry (GC-MS) analysis of electrophysiologically active VOCs

GC-EAG recordings were used to determine which peaks of the GC separation elicited electrophysiological responses from aphid and parasitoid antennae, and identification of the active peaks was achieved by GC on a capillary column (50 m × 0.32 mm i.d., HP-1) directly coupled to a mass spectrometer (GC-MS; AutospecUltima, Micromass, UK). Tentative GC-MS identifications were confirmed by peak enhancement with authentic standards on two GC columns of differing polarity. The stereochemistry of linalool and germacrene D was determined using an HP 5890 GC equipped with a cool-on-column injector and FID, fitted with a β-cyclodextrin chiral capillary column (Supelco, 30 m × 0.25 mm i.d., 0.25 μm film thickness). After confirming that successful separation of synthetic enantiomers was accomplished, co-injections were carried out. Peak enhancement confirmed the presence of the enantiomer in the headspace sample. The

identity of EAG-active compounds was confirmed by repeating the EAG analysis using a synthetic blend of the VOCs (see Appendix S1 in Supporting Information). Chemicals used for peak enhancements were from the same sources as the chemicals used for the synthetic blend (Appendix S1). There were no EAG-active VOCs in the plant-free background control samples.

Statistical analysis

The behavioural response of insects was tested in two ways. First, the attractiveness or repulsion of each EAG-active VOC from headspace samples was tested by paired t-test. In this analysis, we compared the time spent by each insect in olfactometer compartments containing VOCs from headspaces compared to compartments containing reagent blanks. The second approach tested for differences between treatments in the attractiveness to insects. Here, the time spent by insects in olfactometer compartments containing VOCs from headspace samples was subtracted from the time spent in compartments containing reagent blanks, and the resulting data (means per plant) were analysed using a general linear model (GLM) with treatment (i.e. corresponding to the five different plants within a mesocosm) as a fixed factor and mesocosm as a random factor. Least significant difference (LSD) *post hoc* tests were applied to examine pair-wise differences in the attractiveness of plant headspace samples between treatments. We also explored the relationship between aphid and parasitoid responses using linear regression. All analyses were run in PASW (SPSS) 19 package.

The composition of VOCs produced by the plants was analysed by principal component analysis (PCA) on a correlation matrix consisting of log transformed amounts (g dwt^{-1}) of EAG-active VOCs, obtained from all replicates, using the prcomp function in R version 2.3.7.1 (R Development Core Team 2008). This distilled the 17 EAG-active VOCs into a smaller number of groupings, or principal components (PCs). We used two types of output from the PCA: the first was a matrix of 'loadings' for each of the 17 EAG-active VOCs obtained for each of the PCs. These loadings aided biological interpretation of the PCA, because they indicate the strength of correlation between individual VOCs and each PC. The second was a matrix of 'scores', with a single score representing each replicate headspace VOC sample for each PC. To explore potential chemical mechanisms driving the insect behavioural response to treatments, scores from each of the first five PCs associated with each plant's headspace EAG-active VOCs were tested against behavioural responses of both aphids and parasitoids using linear regression. We also used a GLM (using the lm function in R) to test for effects of treatment on the scores of the first five PCs. In this analysis, there were three treatment groups comprising unconnected plants (i.e. plants in 0.5 μm mesh and rotated 40 μm mesh cores), connected plants (i.e. plants in static 40 μm mesh cores and in bulk soil) and donor plants. Pair-wise comparisons were achieved by re-levelling of the order of treatments in the analysis. As a further test of the effect of treatment on plant-emitted VOCs (normalised g dwt^{-1}), we tested for differences in the amounts of individual EAG-active VOCs between donor, connected and unconnected plants using a nonparametric Kruskal–Wallis test in SPSS, because the data did not meet assumptions for homogeneity of variances for parametric tests.

Differential responses of pea aphids to attractive samples before and after addition of methyl salicylate were tested using GLM with methyl salicylate addition (i.e. with or without addition) as a fixed factor and plant as a random factor (SPSS). Assumptions for using GLMs were validated by plotting residuals vs. fitted values, square root residuals vs. fitted values, normal qq plot and constant leverage.

RESULTS

Behavioural responses of aphids and parasitoids

Headspace samples collected from aphid-infested donor plants were significantly repellent to aphids ($P < 0.001$; $t_{1,34} = -5.73$) and attractive to parasitoids ($P < 0.001$; $t_{1,26} = 4.49$; Fig. 2a).

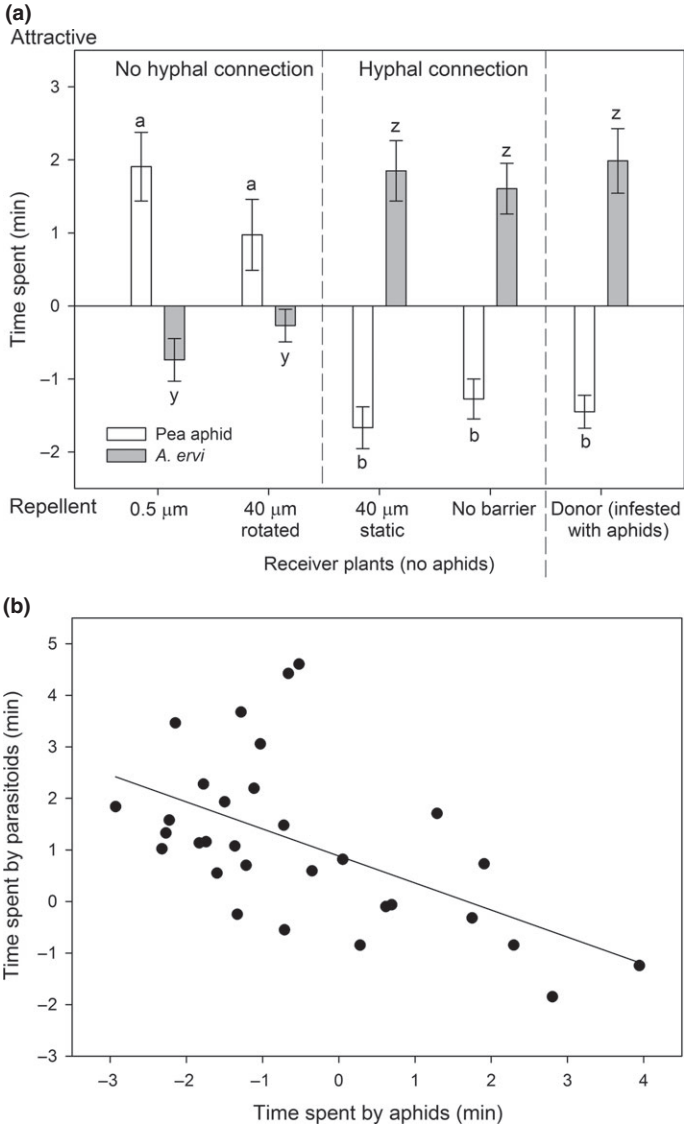

Figure 2 Behavioural responses of pea aphid and the parasitoid wasp *A. ervi* to volatile organic compounds from the headspace of experimental plants. (a) Mean time spent in olfactometer compartments containing volatiles from treated plants minus reagent blanks (± SE). Pea aphid and *A. ervi* responses are compared separately. Bars sharing a letter are not significantly different from each other ($P > 0.05$); (b) Relationship between mean time spent in olfactometer arms by pea aphids and *A. ervi* across all treatments (Pearson coefficient = −0.553; $P = 0.001$).

Crucially, when aphid-free receiver plants were connected to donor plants by common mycelial networks, headspace samples collected from the connected receivers were significantly repellent to aphids, regardless of whether the plants were connected by external mycelium only (non-rotated 40 μm mesh cores; $P < 0.001$; $t_{1,34} = -6.04$) or by root contact and external mycelium (i.e. plants in bulk soil; $P < 0.001$; $t_{1,34} = -4.32$). Parasitoid wasps gave the opposite response and were significantly attracted to VOCs from plants in the non-rotated 40 μm mesh cores ($P < 0.001$; $t_{1,28} = 4.48$) and bulk soil ($P < 0.001$; $t_{1,27} = 4.62$; Fig. 2a). Thus, insect behavioural responses to aphid-free receiver plants with a hyphal connection were similar to those for the aphid-infested plants themselves. In contrast, headspace samples from control receiver plants that had no hyphal connection to donor plants were significantly ($P < 0.001$, $F_{4,24} = 21.1$) attractive to aphids compared to infested donors or to receivers that were connected to infested donors with hyphae (Fig. 2a). This effect occurred regardless of the method used to prevent formation of fungal networks.

The responses of parasitoids were also significantly affected by treatment ($P < 0.001$, $F_{4,24} = 6.67$) and were opposite to the responses of aphids, as often occurs with insect responses to herbivore-induced volatiles. We also found a significant negative correlation between aphids and parasitoids in how attractive they found each sample of volatiles (Pearson coefficient = −0.553; $P = 0.001$; Fig. 2b). In addition, we found that headspace samples from receiver plants that had contact with donors via common mycorrhizal fungal networks only (plants in the non-rotated 40 μm mesh cores) were as repellent to aphids as were headspace samples from plants where roots could also intermingle between donors and receivers ($P = 0.461$; Fig. 2a), implying that direct root contact and soil diffusion were not significant additional conduits of signalling.

Effect of treatments on VOCs and their association with behavioural responses of aphids and parasitoids

We identified 17 VOCs that were EAG-active with aphid and parasitoid antennae (Table 1). The first five PCs from the PCA analysis of VOCs accounted for 79% of variance in the data. There was no effect of treatment on PC1, PC2, PC4 and PC5 scores. However, there was a significant effect of treatment on PC3 scores ($F_{2,39} = 5.15$; $P = 0.011$), which accounted for 12% of variance in the data, with higher scores in connected receiver plants ($P = 0.008$) and donor plants ($P = 0.013$) than unconnected receiver plants; there was no difference in PC3 scores between connected plants and donor plants ($P = 0.747$; Fig. 3). PC3 was not only negatively correlated to aphid behavioural responses ($P = 0.046$, $F_{1,34} = 4.3$; Fig. 4a) but also positively correlated with parasitoid behavioural responses ($P = 0.004$, $F_{1,34} = 9.4$; Fig. 4b). The greatest loadings of PC3 were for methyl salicylate (0.42), naphthalene (−0.40), (*E*)-β-farnesene (0.38) and 6-methyl-5-hepten-2-one (0.35), which indicates that these were the main VOCs contributing to PC3 and to insect behavioural responses. Methyl salicylate was found to be the only compound that was EAG active with both aphids and parasitoids, and had concentrations in plant headspace samples that differed significantly ($P = 0.015$) between connected and unconnected receiver plants (Table 1). Quantitative differences in production of all other VOCs between treatments were not significant. Methyl salicylate, which had greatest loadings with PC3, was also a constituent of VOCs from donor plants and plants connected with common mycelial networks. Addition of methyl salicylate to samples originally attractive to aphids (using the mean quantity that occurred in the repellent samples) made them become repellent to aphids ($F_{1,7} = 118.4$; $P < 0.001$; Fig. 5). This chemical

Table 1 The mean amounts (ng g^{-1} dwt per 24 h ± SEM) of volatile organic compounds collected from the headspace of plant shoots in response to treatments, and electrophysiological activity with aphids and parasitoids

				No hyphal connection		Hyphal connection		
Volatile compounds	Kovats index	Aphid	Parasitoid	0.5 μm	40 μm rotated	40 μm static	No barrier	Aphid-infested donor
(*Z*)-2-hexenal	817	+	-	2.16 ± 0.65	3.02 ± 0.50	3.07 ± 0.23	3.67 ± 0.67	3.26 ± 0.56
(*E*)-2-hexenal	825	+	+	0.47 ± 0.20	1.26 ± 0.41	1.28 ± 0.30	1.18 ± 0.54	0.80 ± 0.33
(*E,E*)-2,4-hexadienal	880	+	-	0.10 ± 0.06	0.25 ± 0.12	0.22 ± 0.07	0.59 ± 0.27	0.46 ± 0.23
(*Z*)-2-heptenal	924	+	-	2.59 ± 1.48	0.93 ± 0.53	0.50 ± 0.27	0.90 ± 0.68	2.00 ± 0.96
benzaldehyde	929	+	+	44.9 ± 22.3	17.4 ± 8.5	76 ± 57.23	117.7 ± 86.8	7.69 ± 2.58
6-methyl-5-hepten-2-one	967	+	#	1.84 ± 0.93	2.46 ± 1.23	1.49 ± 0.88	5.92 ± 2.23	7.26 ± 3.73
(*R,S*)-β-pinene	972	+	-	8.22 ± 5.33	10.49 ± 5.86	6.1 ± 5.15	11.12 ± 5.64	1.47 ± 0.39
(*Z*)-3-hexenyl acetate	986	+	+	34.4 ± 18.2	14.7 ± 9.5	7.7 ± 3.1	22.5 ± 15.7	19.6 ± 11.4
3-carene	1009	-	+	9.26 ± 1.19	8.88 ± 0.85	9.04 ± 0.38	8.52 ± 1.09	8.00 ± 1.18
(*S*)-linalool	1086	+	+	40.8 ± 27.5	15.3 ± 12.2	0.63 ± 0.31	15.1 ± 13.1	4.41 ± 2.86
naphthalene	1168	+	+	11.1 ± 6	4.97 ± 1.83	1.66 ± 0.81	5.60 ± 2.72	2.66 ± 1.33
methyl salicylate*	1172	+	+	0.06 ± 0.06	0.41 ± 0.25	1.85 ± 1.10	1.42 ± 0.90	1.46 ± 1.04
cinnamaldehyde	1232	+	+	27.5 ± 15.3	19.7 ± 10.6	3.72 ± 1.79	5.96 ± 2.99	35.3 ± 18.6
(*E*)-caryophyllene	1424	+	-	210.5 ± 96.5	86.6 ± 47.7	107 ± 92.2	155.5 ± 54.6	57.7 ± 21.3
(*E*)-β-farnesene	1450	+	#	1.38 ± 0.45	0.77 ± 0.27	1.84 ± 1.08	2.75 ± 0.84	3.06 ± 1.07
(*R*)-germacrene D	1486	+	-	63.6 ± 41	29.8 ± 19.3	39.9 ± 38.1	31.3 ± 15.7	9.4 ± 7.5
(*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	1570	+	+	466 ± 81.3	185 ± 39	109.1 ± 28	451.7 ± 164.8	416.9 ± 140.1

+Indicates volatiles electrophysiologically active with either pea aphids or parasitoids in these experiments.
#Indicates compounds that are electrophysiologically active with parasitoids according to published data (Du *et al.* 1998).
-Indicates compounds that showed no electrophysiological activity.
*Significant difference ($P = 0.01$; $H = 6.5$) between receiver plants connected and unconnected to the donor (Kruskal–Wallis test).

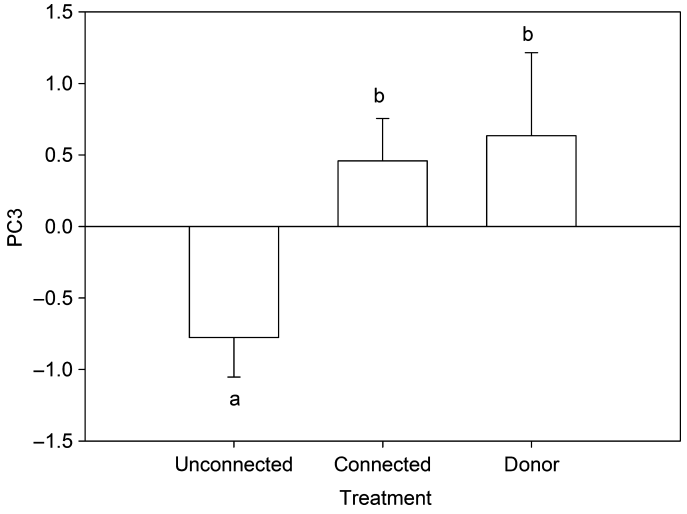

Figure 3 Mean (+SE) principal component 3 (PC3) scores derived from the amounts of electrophysiologically active volatiles produced in donor plants, plants connected to donors (plants in static 40 μm mesh cores and in bulk soil) and plants unconnected to donors (plants in 0.5 μm mesh and rotated 40 μm mesh cores). Bars sharing a letter are not significantly different ($P > 0.05$).

manipulation provides strong support that methyl salicylate is a key aerial compound driving aphid behaviour.

DISCUSSION

We present the first experimental evidence that herbivore-induced signalling molecules can be transferred from plants infested with aphids to uninfested neighbours via a common mycelial network. Our data show that presence or absence of hyphal connections play a vital role in determining the response of receiver plants connected to aphid-infested donors. The use of a mycotrophic plant species, a vigorous AM fungal inoculum for colonisation of roots, and an initially sterile substrate maximises the likelihood that mycorrhizal rather than non-mycorrhizal fungi were the key agents in the transfer of signal molecules between plants. While non-mycorrhizal fungi might have colonised plant roots and contributed to the transfer of signalling compounds, this is unlikely because AM fungi often antagonise soil pathogenic fungi (e.g. Bharadwaj *et al.* 2012; Campos-Soriano *et al.* 2012; Jung *et al.* 2012) and the bean roots were confirmed to contain abundant arbuscules, which are specific to AM fungi. Moreover, bridges between plants formed by AM fungi can be established both by hyphal growth from one plant to another and by anastomosis where two hyphae of the same isolate fuse together and exchange nuclei. Giovannetti *et al.* (2001) demonstrated that anastomosis is very common, with fusion occurring every 2 mm of hypha. Thus, in our study, extensive functional mycorrhizal networks are expected to have established throughout the mesocosms.

Our experimental design also allowed us to tease apart any potential effects of soil diffusion or root-to-root contact from the effects of mycelial contact between plants, and the data suggest that transfer of signalling compounds via rhizosphere deposition is not the major pathway of below-ground plant-to-plant communication under the conditions of our experiment. Nevertheless, we cannot rule out that, in different natural conditions, pathways other than

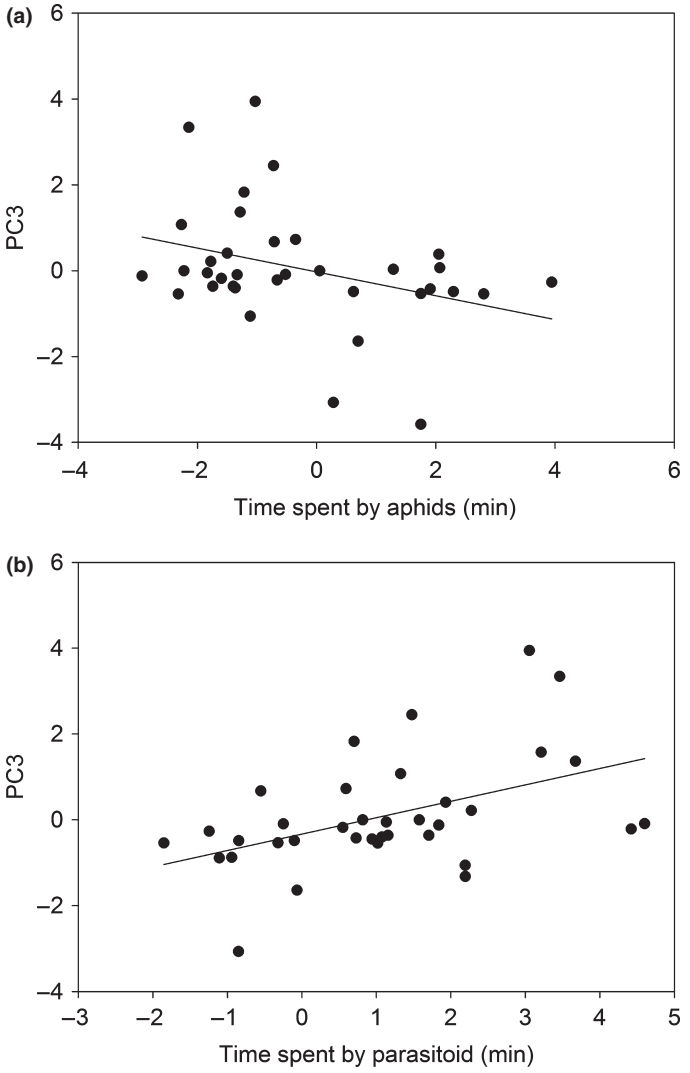

Figure 4 Relationship between principal component 3 (PC3) scores from principal component analysis on the correlation matrix of the amounts of electrophysiologically active volatiles produced by plants and insect host location response in olfactometer bioassays (time spent in area treated with headspace volatiles minus reagent blanks) of (a) pea aphid (Adjusted R-squared: 0.09; $P = 0.046$, $F_{1,34} = 4.29$) and (b) parasitoid wasp (Adjusted R-squared: 0.19; $P = 0.004$, $F_{1,34} = 9.38$).

AM fungal mycelia in the rhizosphere might also act as signal conduits. For example, there is a possibility that the signal might be transferred between plants in a liquid stream, or film layer, and it is also a possibility that formation of these might be greater in the presence of the fungal mycelia. Because the meshes used in our experiment were water permeable, it is unlikely that both of our independent methods of preventing hyphal connections (rotated 40 μm mesh core and non-rotated 0.5 μm mesh core) also prevented formation of liquid streams or film layers. From our experimental design, we therefore have confidence to attribute the signal transfer to fungal mycelium, or possibly some physical phenomenon associated with hyphal connection.

Signalling via common mycelial networks elicited emission of *V. faba* VOCs that are repellent to *A. pisum* aphids but attractive to a key natural enemy, the parasitoid wasp *A. ervi*. Our study shows that AM fungal networks provide a channel for interplant communica-

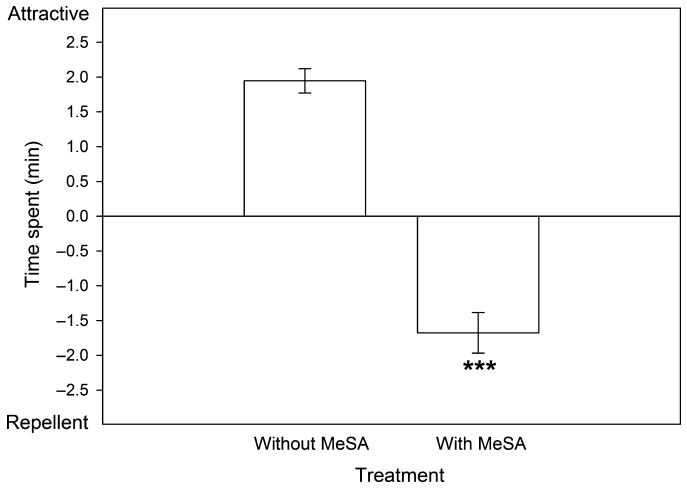

Figure 5 Effect of addition of methyl salicylate (MeSA) to previously attractive volatile organic compound extracts on pea aphid behaviour (± SE). ***Significant difference ($P < 0.001$, t = 10.9, d.f. = 6).

tion and enable plants to prepare for aphid attack with chemical defence mechanisms, without being in direct contact with the herbivore. This finding demonstrates that mycorrhizal fungal networks can function as a messaging system to neighbouring plants and trigger effects on organisms at different trophic levels. Such an early warning system may have profound consequences for the functioning of multitrophic systems, and highlights the need to consider linkages between above- and below-ground organisms (Wardle 2004), even when these organisms do not come into contact with each other.

Although we do not know the identity of the signalling compounds transported via the shared fungal network eliciting production of VOCs in uninfested plants, other work has shown that lipids such as triacylglycerols are actively transported through AM fungal mycelia (Bago *et al.* 2002). Identifying the compound(s) transported through the fungal network is outside the scope of this study, but is an important target for future research to elucidate the mechanisms regulating aphid-induced signal transport through AM fungal mycelia.

Most studies of common mycelial networks have focused on possible nutritional benefits to plants (e.g. Watkins *et al.* 1996; Simard & Durall 2004; Robinson & Fitter 1999). Our work provides evidence of an additional benefit from the formation of common mycelial networks. We do not know the extent to which communication of aphid-induced signalling molecules affect wider ecosystem properties, but there are clear possible benefits to both mycorrhizal plants and fungi. For example, it is known that infestation by aphids has profound impacts on plant allocation of carbon (Girousse *et al.* 2005) which may be detrimental for mycorrhizal fungi, and so it would be advantageous to the fungus if aphid populations were suppressed. Aphid populations can proliferate rapidly even following small-scale infestation of plants (Guerrieri & Digilio 2008), and so prevention of infestation of neighbouring plants is likely to be an effective mechanism to prevent large-scale infestations (Barto *et al.* 2012) and thus maintain a selective advantage to individual plants and fungi. Aphids have clumped distributions that fluctuate rapidly and thus it would be adaptive for plants neighbouring infested ones to prepare their defences before they are attacked.

The composition of VOCs released by leaves often differs between plants grown in the mycorrhizal and non-mycorrhizal condition (Guerrieri *et al.* 2004; Schausberger *et al.* 2012) so our experimental design, which used plants grown only in the mycorrhizal condition, enabled us to identify those compounds elicited specifically in response to common mycelial networks. The key VOC driving insect response to the plants in our system was identified as methyl salicylate. Several lines of evidence showed that release of methyl salicylate from leaves in plants connected to donors via mycelial networks underpins the behavioural responses of aphids in our experiment: First, methyl salicylate was one of the VOCs shown to elicit electrophysiological activity with the antennae of both pea aphids and parasitoids. Second, the quantities of methyl salicylate in headspace samples were significantly less from plants unconnected to donors compared to plants connected to donors and donors themselves. Third, methyl salicylate had the highest loadings with PC3 from the PCA, which was the principal component that correlated with behavioural responses of both aphids and parasitoids. Finally, addition of synthetic methyl salicylate to attractive plant headspace samples at the amount naturally present in repellent samples, made them repellent to aphids, providing clear experimental evidence that this compound is a key driver of aphid behavioural responses. Methyl salicylate has previously been shown to repel other species of aphids (Hardie *et al.* 1994) and attract parasitoids (Sasso *et al.* 2009), and is suggested to be a mobile signal that can be transported throughout plant tissue in phloem sap to induce systemic acquired resistance in tobacco plants (Shulaev *et al.* 1997; Park *et al.* 2007). Nevertheless, it remains a possibility that other VOCs (e.g. those with high loadings in PC3) may also have a role in affecting insect behaviour, either individually or by interaction with other VOCs.

It was found recently that some commercial cultivars of maize have lost their ability to produce herbivore-induced plant volatiles (Tamiru *et al.* 2011). It is therefore important to determine whether selective breeding of other important crops, such as beans, results in loss of their ability to either perceive aphid-induced signals from mycorrhizal fungi, or disrupt downstream signalling for production of VOCs. Moreover, manipulation of VOCs released by crops has considerable potential for pest control in the field (Xiao *et al.* 2012; Khan *et al.* 2010). Given the ubiquity of AM symbioses in herbaceous plants including most major crops (Smith & Read 2008), our data suggest a pressing need to determine the extent to which manipulation of common mycorrhizal mycelial networks can provide sustainable solutions to manage insect pests. The role of mycorrhizal fungi in mediating multitrophic interactions in agricultural ecosystems has largely been overlooked, but our findings suggest that there may be potential to develop fungal treatments to enhance crop protection.

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AUTHORSHIP

LG and DJ conceived the study, and together with TB and JP, acquired the funding and supervised the work; LG, DJ and ZB

designed the experiment; ZB performed volatile collections, bioassays and gas chromatography analyses; ZB and DJ analysed the data; CW performed electrophysiology; TB, MB, JC and ZB identified the volatiles; DJ, LG and ZB wrote the manuscript; TB and JP contributed to the revision.

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