L--DOPA functions as a plant pheromone for belowground anti-herbivory communication

Pasquale Cascone[1] | **Jozsef Vuts**[2] | **Michael A. Birkett**[2] | **Sarah Dewhirst**[3] | **Sergio Rasmann**[4] | **John A. Pickett**[5] | **Emilio Guerrieri**[1,6]

[1]Institute for Sustainable Plant Protection, Consiglio Nazionale delle Ricerche, Naples, Italy

[2]Biointeractions and Crop Protection Department, Rothamsted Research, Harpenden, UK

[3]Arctech Innovation Keppel St, London, UK

[4]Institute of Biology, University of Neuchatel, Neuchatel, Switzerland

[5]School of Chemistry, Cardiff University, Cardiff, UK

[6]Institute for Sustainable Plant Protection, Consiglio Nazionale delle Ricerche, Torino, Italy

Correspondence
Emilio Guerrieri, Istituto per la Protezione Sostenibile delle Piante, Consiglio Nazionale delle Ricerche, P.le Enrico Fermi 1, 80055 Portici, Italy. Email: emilio.guerrieri@ipsp.cnr.it

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Abstract

While mechanisms of plant–-plant communication for alerting neighbouring plants of an imminent insect herbivore attack have been described aboveground via the production of volatile organic compounds (VOCs), we are yet to decipher the specific components of plant–-plant signalling belowground. Using bioassay--guided fractionation, we isolated and identified the non--protein amino acid L-DOPA, released from roots of *Acyrtosiphon pisum* aphid-infested *Vicia faba* plants, as an active compound in triggering the production of VOCs released aboveground in uninfested plants. In behavioural assays, we show that after contact with L-DOPA, healthy plants become highly attractive to the aphid parasitoid *(Aphidius ervi)*, as if they were infested by aphids. We conclude that L--DOPA, originally described as a brain neurotransmitter precursor, can also enhance immunity in plants.

KEYWORDS
aphids, parasitoids, plant immunity, plant–-plant signalling, root exudates, VOC

INTRODUCTION

Plant communication with other organisms mainly relies on the release of constitutive or stress--induced chemical signals that travel both through the air head-space or the soil matrix (Bruin & Dicke, 2001; Erb et al., 2015; Karban, 2008). In the rhizosphere, compris-ing the complex soil environment in close contact with plant roots, plants contribute a steady production of root exudates, including ions, free oxygen and water, enzymes, mucilage, and a variety of other secondary metabolites (Rovira, 1969). Once released, root exu-dates can function as signals regulating plant–-microbe (Badri & Vivanco, 2009), plant–-animal (Johnson & Rasmann, 2015) and plant–-plant interactions (Bais et al., 2006). Belowground plant–-plant communication has been proven to mediate key ecological interactions, such as competition and facilitation, in both natural and applied systems, and several molecules have been identi-fied as key agents of chemical communication (van Dam & Bouwmeester, 2016).

Emerging evidence indicates that belowground plant–-plant communication can also serve to signal neigh-bouring plants of a recent aboveground insect herbivore attack. For instance, it was shown that a warning sig-nal can run through the common mycelial network of the arbuscular mycorrhizal fungi to alert neighbour-ing healthy plants of current aphid attack (Babikova et al., 2013). It was also previously demonstrated that un-infested *Vicia faba* (Fabaceae) plants maintained in the

same pot together with plants infested by the pea aphids *Acyrthosiphon pisum* (Homoptera: Aphididae) became more attractive towards the aphid parasitoids *Aphidius ervi* (Hymenoptera: Braconidae) than when placed in the same pot with healthy plants (Guerrieri et al., 2002). This change in attractiveness was not observed when root contact was prevented among plants that had their aerial parts in close proximity, and thus freely exchanging abo-veground volatile organic compounds (VOCs) (Guerrieri et al., 2002). These results were further confirmed using hydroponic growing conditions. Uninfested *V. faba* plants placed in hydroponic solution that was previously used to grow aphid--infested plants became attractive to *A. ervi* parasitoids, whereas placing them in the hydro-ponic solution of uninfested plants did not change their attractiveness (Guerrieri et al., 2002).

Accordingly, as shown in the *Vicia*--aphid-parasitoid system, plant–-plant signalling can also occur within the rhizosphere. Since it only works when roots are in con-tact, we hypothesised that such belowground plant–-plant signalling is mediated by a systemically translocated root--borne elicitors. We therefore predicted that insect herbivore--damaged plants would be induced to produce a unique blend of molecules that elicits a response in neighbouring plants if in contact through the soil ma-trix. Because herbivore--damaged plants can modify their internal chemistry (Karban & Baldwin, 1997) to either directly become more toxic to herbivores (Farmer & Ryan, 1992) or indirectly by attracting herbivore nat-ural enemies via the emission of VOCs above and be-lowground (Dicke & Baldwin, 2010; Heil, 2008; Kost & Heil, 2006), we also predicted that response elicitation in neighbouring plants could be observed in the form of changes in leaf chemistry aboveground (Bezemer & van Dam, 2005). Here, we report on a series of plant–- plant communication bioassays and bioassay--guided fractionation analyses that ultimately characterised the amino acid l--DOPA, a known neurotransmitter precur-sor, as one of the elicitors released by the roots of aphid-- damaged *V. faba* plants. We show that root contact with l--DOPA altered the aboveground headspace chemical profile of healthy plants, which then attracted more aphid parasitoids than plants not treated with l-DOPA.

MATERIALS AND METHODS

Insects

The parasitoid *Aphidius ervi* was reared on its natural host, the pea aphid *Acyrthosiphon pisum* maintained on potted broad bean (*Vicia faba*) plants, cv. Aquadulce (Guerrieri et al., 1993). Aphid and parasitoid cultures were kept in separate environmental chambers at 20 ± 1°C, 75 ± 5% relative humidity, and 18 L: 6D pho-toperiod. Insect parasitoids used in the bioassays were reared as synchronised cohorts by exposing heavily infested plants for 24 h to 1--day--old mated females; after a week, the resultant mummies were clipped from the plant and isolated in glass test tubes (60 × 8 mm) plugged with cotton wool. Experimental females were used within the first day after emergence, mated, and fed with a 50% honey solution. All experiments were conducted 3 h from the onset of the photophase.

Plants

Plant material in hydroponic solution: Broad bean seeds (*Vicia faba* L., cultivar Aquadulce) were soaked in water for 24 h, then potted in vermiculite and kept in a con-trolled environment room at 20°C. After 5 days, the seedlings were gently removed from the vermiculite, the seed coats were discarded and the roots were rinsed with water, carefully removing any vermiculite residue. Two seedlings were then placed in a glass beaker con-taining a hydroponic solution made with Murashige and Skoog basal salt mixture (2 g L^{-1}, Duchefa Biochemies, The Netherlands) and placed in a glasshouse (20°C, L:D 16:8 h). Each beaker was wrapped in aluminium foil to hold the plants in position and to prevent the light from reaching the roots. Every 2–-3 days, the hydroponic solu-tion was renewed. For further experiments, specifically after identification 0.1 ppm or 0.01 ppm of the active compounds in the attractive root exudate blends (see methods below), each pure compound (l-DOPA or d--DOPA) was added to the beakers with clean hydroponic solution and two seedlings were transferred into it and kept as described above for 24 h before testing them in the wind-tunnel.

Plant material in soil: Broad bean seeds (*Vicia faba* L., cultivar Aquadulce) were soaked in water for 24 h, then potted (2 plants/pot) in sterile soil and kept in a glass-house at 20 ± 2°C. The distal end of a Teflon tube (20 cm, 1 cm diameter) covered with parafilm and pinched with a nail to make holes along 5 cm was inserted in each pot and as close as possible to plant roots. After 14 days, 0.1 ppm of each pure compound (l-DOPA or d-DOPA) were syringed through the apical end of the Teflon pinched tube emerging from the soil and left for 24 h be-fore testing them in the wind--tunnel.

Collection and bioassay--guided fractionation of root exudates and identification of L--DOPA in the final active fraction

After a renewal of hydroponic solution, half of the beak-ers, containing 2--week--old plants, were infested with 100 mixed-age *A. pisum* (P+A). In our experiments, we considered an infestation well above the calculated thresholds of 50 aphids feeding for 72 h needed to re-cord a change in the behaviour of the aphid parasitoid *A. ervi* (Guerrieri et al., 1999). Nonetheless, the aphid

population tested corresponds to an initial state of in-festation considering that a single female aphid colonis-ing a plant reproduce by telytokous parthenogenesis and viviparity resulting in the production of tens of nymphs each starting reproducing in a few days. After 3 days, the hydroponic solution from uninfested (P) and infested (P+A) plants was collected and filtered using filter paper to remove any debris. Organic compounds present in the solutions were extracted by solid--phase extraction (SPE) from P and P+A solutions (~10 beakers equalling ~2 L per replicate). The SPE columns were 6 ml cartridges containing Evolute C18 sorbent (500 mg, Biotage). The cartridges were conditioned prior to extraction using HPLC grade methanol (2 ml), followed by displacement by distilled water (2 ml). The extractions were performed using a VacMaster--10 SPE manifold (IST, UK). The car-tridges were then extracted with methanol (2 ml). This was repeated 40 times. Ten replicates (~100 beakers) were combined and the resulting solution was rotary evapo-rated to dryness. The compounds were re--dissolved into HPLC water or ethanol (5 ml, 50 µl per beaker) for bioassay or further fractionation and chemical analysis. For the identification of the DOPA enantiomer, chi-ral separation was achieved on an ACE 5 C18 column (250 × 4.6 mm; 5 µm particle size; Thermo Scientific). The mobile phase was 1 mM CuSO4, 3 mM phenylala-nine, 0.01% trifluoracetic acid, 1% acetonitrile in HPLC H2O. The flow rate was maintained at 1 ml min--1 or 0.5 ml min--1 and isocratic conditions for 20 min (Husain et al., 1994; Wu et al., 2006). Detection was at 280 nm, injected volume was 10 µl. 1 mg/ml DOPA standard con-centrations were used. C18 root exudate extracts were analysed and fractionated on an ACE 5 C18 column (250 × 10 mm; 5 µm particle size; Thermo Scientific) by HPLC (Shimadzu prominence, Shimadzu Corporation). The mobile phase A was 5% formic acid in HPLC H2O, and mobile phase B was acetonitrile. The flow rate was maintained at 1 ml min--1, starting with isocratic condi-tions at 5% B for 10 min, then linear gradient program to 60:40 (A:B) at 25 min, to 30:70 at 40 min, to 5:95 at 41 min and isocratic for 5 min, then to 95:5 at 45 min and isocratic for 5 min. Three fractions were collected at 0–-15 min (Fraction 1), 15–-40 min (Fraction 2) and from 40–-55 min (Fraction 3). Fraction 1 was then fractionated into four sub--fractions 0–-6 min (Fraction 1a), 6–-12 min (Fraction 1b), 12–-24 min (Fraction 1c) and 24–-55 min (Fraction 1d). Detection was at 280 nm, injected volume was 10 µl.

Wind tunnel bioassays

For each experimental condition, a total of 10 plants grown hydroponically or in soil as described above were used and tested in a wind--tunnel (see Guerrieri et al. (1999) for details) daily in a random order to re-duce any bias related to the time of the experiments.

One hundred parasitoid females were tested singly for each target in no--choice experiments, and observed for a maximum of 5 min. The percentage of response (oriented flights, landings on the target) to each tar-get plant was calculated. The parameters of the bioas-say were set as follows: temperature, 20 ± 1°C; 65 ± 5% RH; wind speed, 25 ± 5 cm s--1; distance between releas-ing vial and target, 50 cm; PPFD at releasing point, 700 µmol m^2 s^{-1}.

Air entrainment of plants treated with synthetic L-DOPA and D-DOPA

After bean plants were grown in hydroponic solution for 10 days, the hydroponic solution was replaced (200 ml) and treated with L--DOPA (10 µg), D--DOPA (10 µg) or HPLC water (control, 10 µl) (n = 15 replicates/treatment). After 24 h, the bean plants were enclosed in Multi-- Purpose Cooking Bags [poly(ethyleneterephthalate)] or PET, volume 3.2 L, ~12.5 µm thickness, max. 200°C, Sainsbury's Supermarkets Ltd., London, UK. The bot-tom of the bag was enclosed around the top of the beaker containing the hydroponic solution. The inlet was fitted to the open end of the bag, and the outlet was fitted to a corner of the bag after cutting off with scissors. Air that had been purified by passage through an activated charcoal filter (BDH, 10–-14 mesh, 50 g) was pushed into (750 ml min^{-1}) and pulled (700 ml min^{-1}) out of the bags. Volatiles were trapped onto Tenax (50 mg; Supelco) held in glass tubing (5 mm outer diameter) by two plugs of si-lanised glass wool. The Tenax was conditioned by wash-ing with dichloromethane (2 ml), followed by redistilled diethyl ether (2 ml) and heating at 132°C for 2 h under a stream of purified nitrogen. After 24 h, the Tenax tubes were sealed in glass ampoules in an atmosphere of nitro-gen and stored at −20°C until analysis. Volatile sample analysis Tenax tubes were inserted into the OPTIC PTV unit of a GC (30- > 250°C ballistically at a rate of 16°C/s) connected to a Micromass Autospec Ultima magnetic sector mass spectrometer (Waters). The GC (Agilent 6890 N) was fitted with a 50 m × 0.32 mm i.d. × 0.52 µm film thickness HP--1 column (Agilent). Ionisation was performed by electron impact (70 eV, 220°C). The GC oven temperature was maintained at 30°C for 5 min and then programmed to increase at 5°C/min to 250°C, with a 70--min run time. The identity of peaks was confirmed by comparison of their GC and GC–-MS properties with those of authentic standards (see Sasso et al. (2007) for details), and by GC peak enhancement using authen-tic samples. The enantiomeric composition of linalool was already determined as (*R*)--linalool for this plant by (Webster et al., 2008). Quantification of compounds was achieved by the single--point external standard method with a series of C7--C22 alkanes, where the amount of an analyte was estimated using the peak area of the nearest alkane peak, the amount of which was known.

Statistical analysis

The number of parasitoids responding to each target was compared with a G--test for independence with William's correction using the RVAideMemoire package (Hervé, 2018) in R (R Development Core Team, 2020). The resulting values of G were compared with the criti-cal values of Chi--square. To assess differences in VOCs across DOPA treatments, we first performed a Distance-- based redundancy analysis (*dbRDA*) after pareto-- transformation of the data and based on Gower distance (*capscale* function in *vegan*, (Oksanen et al., 2013)). The amount of DOPA and other peaks in the P and P+A ex-tracts was compared using ANOVA ($p = 0.05$) investigat-ing the effect of 'treatment', 'peak number' and 'treatment × peak number'. Peak area/weight values were square root--transformed for the analysis. We visualised the clusters of species across the three treatments (control, d-DOPA, and l--DOPA) using linear discriminant analy-sis on the VOCs data matrix (*lda* function in the *mass* package (Ripley et al., 2013)). Next, to measure the in-teractive effect of treatment and VOCs identity on VOCs production, we run a two--way generalised linear model (function *glm* in R stats) on $\log_{10}$--transformed data using a Poisson family distribution. Model fit results were fol-lowed by Fisher's Least Significant Difference (LSD) test for detecting treatment effects across individual VOCs ($p < 0.05$).

RESULTS

Bioassay-guided fractionation

To measure the activity of the root exudates released by damaged plants, we sampled *V. faba* root exudate ex-tracts using reverse--phase (C_{18}) solid--phase extraction (SPE) from uninfested plants (plants without aphids: Plant only: P), and pea aphid (*A. pisum*)-infested plants (Plant+Aphid: P+A). Using wind--tunnel bioassays, we show that about four times more *A. ervi* oriented to (G test, $\chi = 44.800$, $p < 0.001$) and landed on (G test, $\chi = 10.303$, $p = 0.001$) *V. faba* plants grown in hydro-ponic solution treated with P+A extract compared to those treated with P alone (Figure 1a,b). The chemical signal present in P+A root exudate was then identified by bioassay--guided fractionation giving three fractions of different polarity. Seven times more *A. ervi* oriented to and landed on *V. faba* plants treated with *fraction 1* (the most polar fraction) from P+A, compared with the similar HPLC fraction of P (Figure 1c; G test, $\chi = 45.297$, $p < 0.001$; G test, $\chi = 11.514$, $p < 0.001$). No sig-nificant synergistic effects of combining fractions were observed for oriented flights and landings (Figure 1c; G test, $\chi = 3.306$, $p = 0.069$; G test, $\chi = 0.471$, $p = 0.492$). *Fraction 1* was then further fractionated into four sub-fractions (Figure 1a–d) of different polarities, of which the *a* and *d* subfractions showed the most significant effect in eliciting the indirect defence in terms of ori-ented flights (Figure 1d; G test, $\chi = 38.339$, $p < 0.001$, G test, $\chi = 43.625$, $p < 0.001$, respectively) and in terms of landings (Figure 1d; G test, $\chi = 20.723$, $p < 0.001$, G test, $\chi = 14.748$, $p < 0.001$, respectively). Thus, by fur-ther analysing *fraction 1a* using peak enhancement by co--injection with enantiomerically pure authen-tic standards, we identified l-DOPA (RT = 4.276 min under our HPLC conditions) (Figure 1e) as one key ac-tive compound mediating plant–-plant communication. The estimated amount of exuded l--DOPA by infested plants was 5.67 µg/g/day and by uninfested plants was 4.95 µg/g/day (ANOVA, df = 1, $p = 0.001$). Subsequent bioassays using pure compounds showed that about five times more *A. ervi* oriented to (G test, $\chi = 48.643$, $p < 0.001$) and about three times more landed on (G test, $\chi = 16.794$, $p < 0.001$), *V. faba* plants grown in hydro-ponic solution treated with l--DOPA relative to when treated with d--DOPA (at both concentrations of 0.1 and 0.01 ppm) and relative to untreated *V. faba* plants (Figure 1f), indicating enantiomers--dependent activ-ity. No dose--dependent effect was noted for l-DOPA in terms of oriented flights (Figure 1f; 0.01 ppm: 35.4% vs. 0.1 ppm: 48.4%; G test, $\chi = 3.378$, $p = 0.066$) and land-ings (Figure 1f; 0.01 ppm: 18.7% vs. 0.1 ppm: 24.7%; G test, $\chi = 0.656$, $p = 0.418$). These response patterns were subsequently confirmed by performing experiments with plants grown in soil and treated with synthetic l-- DOPA at a dose of 0.1 ppm (Figure 1f; G test, $\chi = 27.496$, $p < 0.001$; G test, χ 11.121, $p < 0.001$). While we found that fraction 1d was also attractive, we were not able to fully elucidate the molecular structure of each molecule in that faction. We therefore opted to only focus on the activity of l--DOPA in this study, but we acknowledge that other compounds in the root exudate extract might also activate neighbouring plant's defences.

Induction of VOCs in neighbouring plants

By means of gas chromatography coupled to mass--spectrometry (GC–-MS) analysis of the leaf headspace of *V. faba* plants grown in hydroponic solution with l-- DOPA, or d--DOPA isomers, we found a total of nine compounds which varied significantly across treat-ments (Figure 2; ANOVA based on 999 permutations, $F_{2,24} = 2.08$, $p = 0.034$). Across all VOCs, we also found that some compounds were more induced than others by l--DOPA (treatment effect; LR $\chi = 10.306$, $p = 0.006$; and VOCs by treatment interaction; LR $\chi = 11.601$, $p = 0.771$). Specifically, we show that l-DOPA-treated plants released 10 times and five times more methyl sa-licylate, three times and four times more of the sesquit-erpene (*E*)--ocimene, three times and seven times more (*E*)--caryophyllene than control (untreated) and d--DOPA treated plants, respectively (Figure 3).

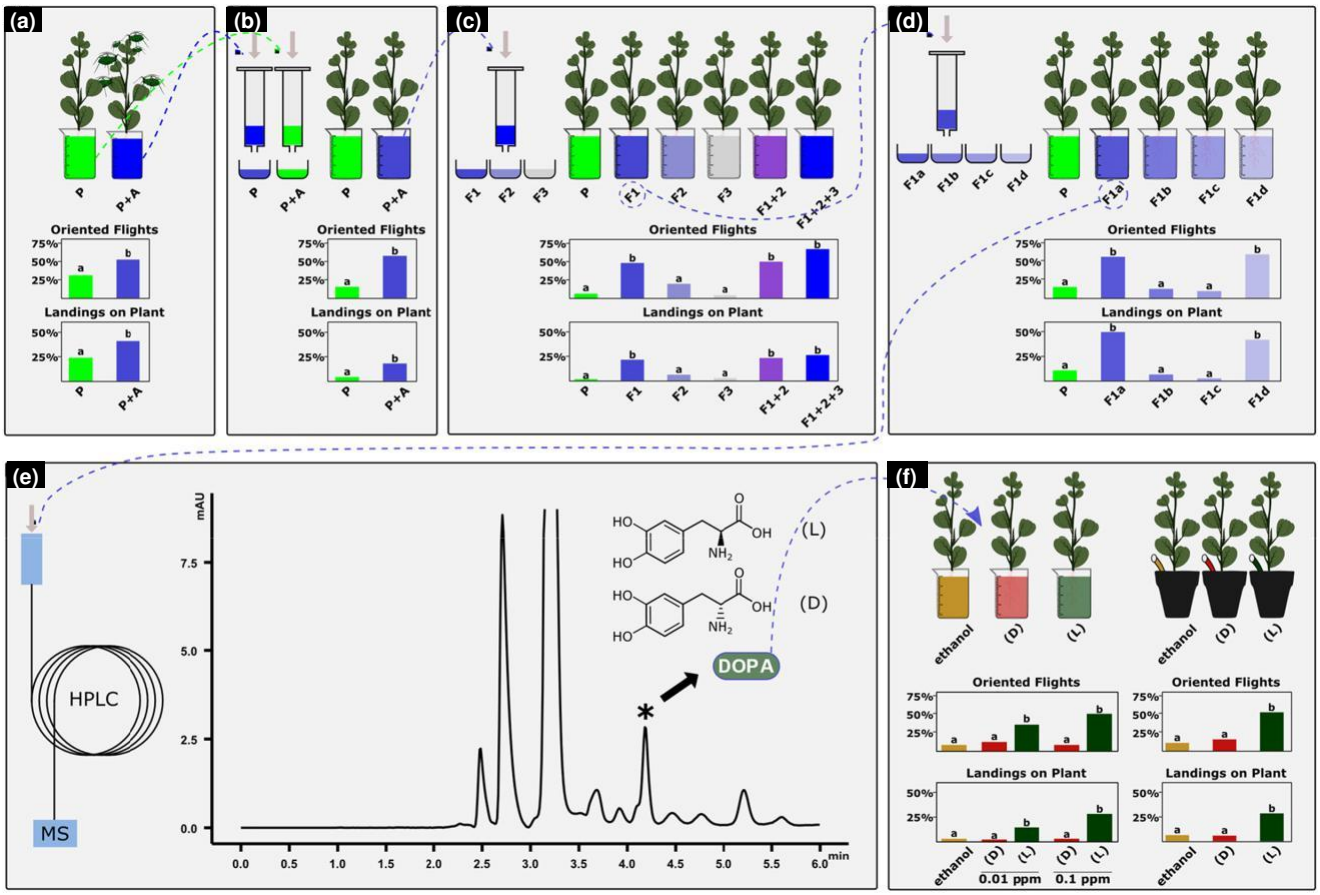

FIGURE 1 Workflow for identifying root exudates for mediating plant–-plant communication. Bar show results (in %) of the oriented flights and landings of the aphid parasitoids (*Aphidius ervi*) towards bean plants (*Vicia faba*) grown in hydroponic medium (Murashige and Skooge). Behavioural assays for (a) pea aphid (*Acyrthosiphon pisum*)-infested (P+A, blue bar), and uninfested (P, green bar) *V. faba* plants; (b) *V. faba* plants treated with C_{18}--SPE collected root exudate extracts from uninfested (P, green bar) and from *A. pisum*-infested (P+A, blue bar) plants; (c) (P+A) *V. faba* plants treated with LC Fractions (F1, F2, F3) of the roots exudates of the P+A treatment; (d) *V. faba* plants treated with LC F1 subfractions (F1a, F1b, F1c, F1d); (e) peak identification of DOPA; (f) Behavioural assays for *V. faba* plants treated with synthetic DOPA (L or D) in hydroponic solution (left panels), or in the soil (right panels). Different letters above bars indicate significant differences ($p < 0.05$) among treatments.

DISCUSSION

The emerging paradigm is that plants may detect chemicals, released from conspecific or heterospecific neighbouring plants, and in response change their physi-ology or chemistry (Arimura et al., 2000; Karban, 2008). Aboveground, the main players of plant–-plant signalling are the volatile organic compounds (VOCs), particularly those released in response to biotic stresses. In this con-text, an ever--growing body of literature is showing that VOCs emitted by herbivore--damaged plants increase resistance of neighbouring undamaged plants (Karban et al., 2014). Responses in the receiving plants include priming, which leads to enhanced defence induction upon subsequent insect attack (Erb et al., 2015), or full induction of direct (Moreira et al., 2016) or indirect (i.e., the attraction of natural enemies of the herbivores) de-fences (Turlings & Erb, 2018).

Belowground, plant–-plant interaction can also rely on the release and perception of chemicals in the form of volatile or non--volatile root exudates (Bais et al., 2006), or those that can travel through the mycelial network connecting neighbouring plants (Babikova et al., 2013; Barto et al., 2012; Song et al., 2010). Among the main functions of plant–-plant signalling belowground is the kin/nonkin recognition, so to alter the development of roots and regulate nutrient and water acquisition. For example, allelopathic rice cultivars generated avoid-ance patterns in the roots of other rice cultivars and several paddy weed species (Yang & Kong, 2017). By far less studied is the role of root exudates in medi-ating plant–-plant communication in response to her-bivore attack (Moreira & Abdala--Roberts, 2019). For example, it was shown that aphid--free plants became repellent to aphids but attractive to aphid parasitoids when they were connected to aphid--infested plants via a common mycorrhizal mycelial network (Babikova et al., 2013). In this example, the mycelia network likely served as conduit for information exchange between the healthy and attacked plants, eliciting in the latter

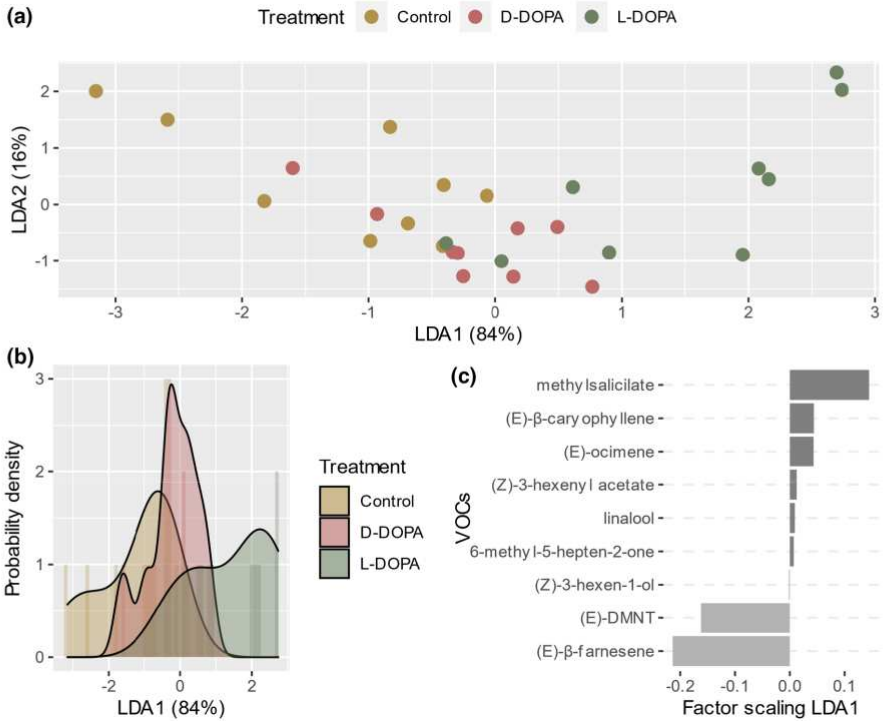

FIGURE 2 Linear discriminant analysis (LDA) of aboveground *Vicia faba* volatile organic compounds (VOCs). VOCs were measured on plants grown in hydroponic medium and treated with Ethanol only (brown colours), or treated with either D--DOPA (red colours) or L--DOPA (green colours) at1 ppm. (a) LDA biplot distribution of discriminant scores of leaf VOCs profiles across the three treatments. The first linear discriminant (LDA1) explains 83% of the between--group variance, and the second linear discriminant (LDA2) explains 16% of the between-- group variance. (b) Histograms and density plots showing the distribution of discriminant scores (from LDA1) of leaf VOCs profiles released by plants under the three treatments. (c) Discriminant coefficients of LDA1 for each VOCs included in the overall volatile blend. Compounds with negative coefficients (in light grey) reflect negative discriminant scores of leaf VOCs (control and D--DOPA treated plants), while compounds with positive coefficient (in dark grey) reflect positive discriminant scores (L--DOPA treated plants).

a change in the production and release of aboveground VOCs, particularly methyl salicylate. We here demon-strated that belowground plant–plant communication, involving changes in aboveground VOC production of healthy plants during ongoing aphid attack on neigh-bouring plants, occurs even in the absence of a fungal connection. Specifically, we found that within the com-plex root exudates blend, a non--volatile compound, the non--protein amino acid L--DOPA, is exuded by the roots of damaged plants and is perceived as an alarm signal by neighbouring plants. In the soil, amino acids have been shown to occur as "free" (i.e., not covalently bound to any other chemical entity), dissolved in the soil aqueous solution, or bound to soil colloids or to soil organic matter (Moe, 2013; Vranova et al., 2011). There is also ample evidence that amino acids can move from the rhizosphere into plant roots (reviewed by Näsholm et al., 2009), and thus move within the soil matrix. Accordingly, we show that by placing L-- DOPA in the rhizosphere, the plants sense it somehow, and activate VOCs production. However, how long L-- DOPA remains in the soil, and how far and how fast this compound can travel in the soil matrix remains an open question that merits future investigations, also by comparing different substrates.

Independent of the mechanism of movement in the soil, we show that neighbouring *V. faba* plants responded to the presence of L--DOPA by inducing methyl salicylate, (*E*)--ocimene and (*E*)--caryophyllene production, all compounds known to attract aphid parasitoids (Babikova et al., 2013; Du et al., 1998; Sasso et al., 2007, 2009) and predators (Zhu & Park, 2005). For instance, tomato plants attacked by the potato aphid *Macrosiphum euphorbiae* also increased significantly the production of methyl salicylate and (*E*)--caryophyllene, which was linked to the increased attraction of the parasitoid *A. ervi* (Sasso et al., 2007, 2009). Similarly, plants treated with *cis*-- jasmone, a plant--derived insect feeding--related signal, were more attractive for *A. ervi*, and this attraction was associated with the induction of (*E*)-ocimene (Birkett et al., 2000), later confirmed in experiments using trans-genic tobacco plants (Cascone et al., 2015). The emis-sion of (*Z*)-3-hexenyl acetate, 6-methyl-5-hepten-2-one and (*Z*)--3--hexenol, which are known to attract *A. ervi* (Du et al., 1998; Sasso et al., 2007, 2009), was enhanced, although not significantly, in L-DOPA-treated plants (Figure 3).

In addition to being exuded from roots, non--protein amino acids such as L--DOPA can be easily translocated within plant tissues and can be reused or diverted to

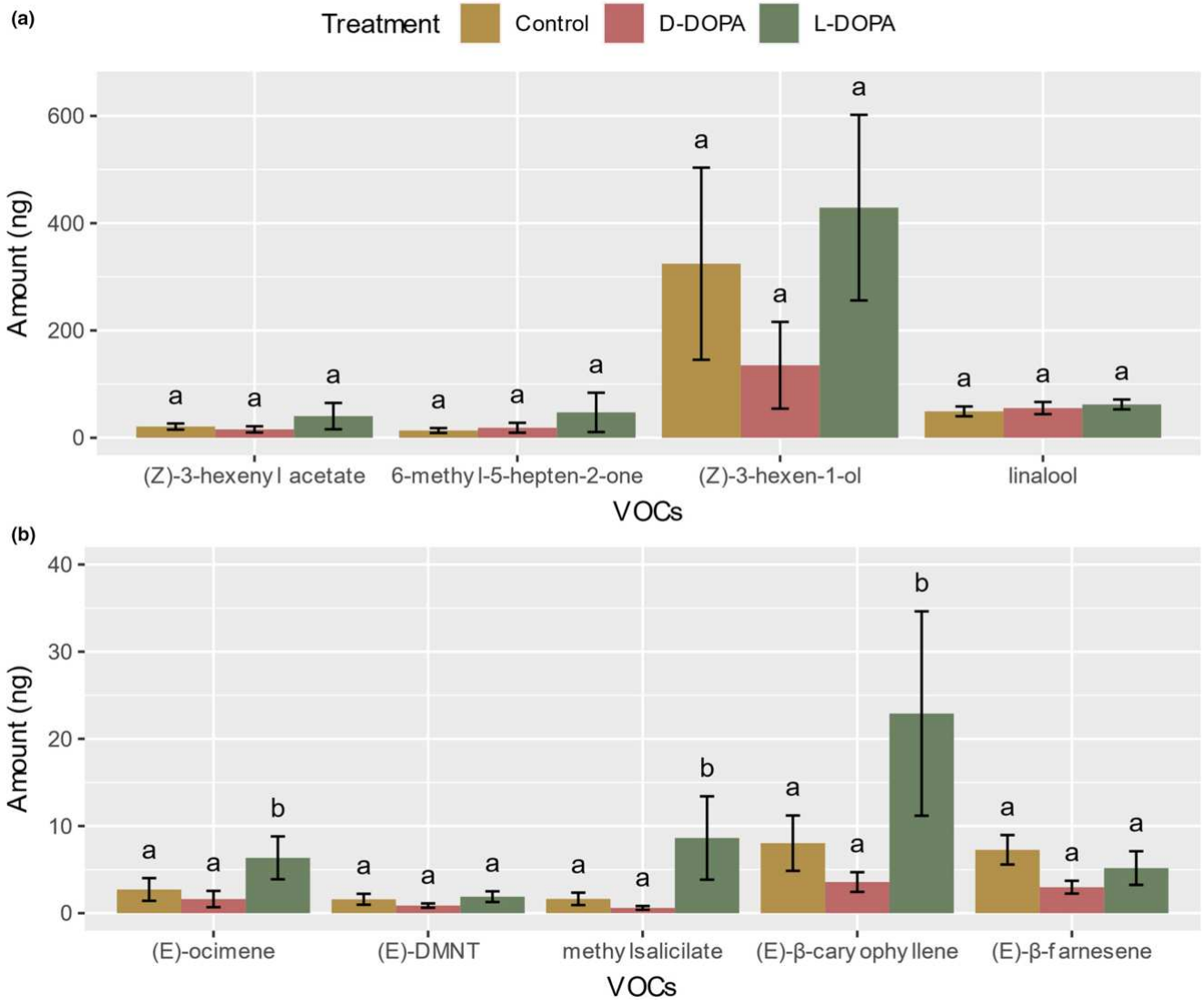

FIGURE 3 Effect of DOPA isomers on aboveground volatile organic compounds (VOCs) production. Shown are the (a) the major and (b) the minor VOCs produced by *Vicia faba* leaves, when plants were grown in hydroponic medium and treated with Ethanol only (control, brown bars), or treated with either D--DOPA (red bars) or L--DOPA (green bars) at1 ppm.

primary metabolism when needed (Huang et al., 2011). The leaves and pods of *V. faba* plants contain high quan-tities of L--DOPA (Burbano et al., 1995), whose presence can affect the community of insect herbivores attack-ing these plants. Accordingly, it has been shown that L--DOPA is detrimental for most generalist herbivores, whilst it is exploited in different ways by specialists. For example, it was shown that *A. pisum* can sequester this compound, which was reported to provide benefits for wound healing and protection against UVA--radiation (Huang et al., 2011). For the other legume specialist aphid, *Aphis fabae*, it was shown that L--DOPA can act as a powerful feeding stimulant (Jördens & Klingauf, 1977). Therefore, L--DOPA can be directly co--opted by insect herbivores for their own benefits. In the perpetual bat-tle between plants and insect herbivores, evolution acts on fostering adaptations and counter--adaptions for at-tacking and defensive strategies (Ehrlich & Raven, 1964).

In this scenario, plants can only escape the attack of an herbivore by developing more potent means of defence, such as the production of novel toxic secondary metab-olites. In response, the herbivores can continue feed-ing on the plant if they develop means of tolerating or overcoming the novel toxic agent. Conversely, the subtle action of indirect defences, associated to the release of specific VOCs that facilitate the foraging behaviour of predators or parasitoids of the herbivore, is, evolution-arily speaking, invisible to the targeted pest on which no immediate selective pressure is posed (Kessler & Heil, 2011). Therefore, broad bean plants seem to have counter--balanced the selective pressure of the specialist aphid *A. pisum* to cope with a toxic compound (L--DOPA) by diverting the function of this compound so to deliver an indirect effect of resistance induced in neighbour-ing plants. Plant–-plant communication regulated by specific elicitors such as L--DOPA amplifies the indirect

resistance response to a biotic stress from a single individual to community level. We know that in the same system the release of specific VOCs regulating the attraction of natural enemies is associated to a specific infestation threshold, in terms of number of feeding aphids and duration of their feeding activity (Guerrieri et al., 2002). We here show that at the same time the broad bean plant responds to aphid infestation aboveground, as well as belowground, by conveying a specific signal to conspecific neighbours eliciting the release of similar VOCs. The efficiency of parasitoid foraging behaviour relies on the reliability and detectability of plant semiochemicals (Vet & Dicke, 1992). The amplification of plant responses, from individuals to the entire community, seems to better ful-fil both requirements. In fact, herbivore--induced VOCs reliably indicate to parasitoids the presence of their tar-get victim. Moreover, it is worth noting that the VOCs released in response to aphid attack can also function as direct defences. For example, methyl salicylate reduced the number of fixed aphids and the reproductive rate of fixed ones by more than two thirds (Digilio et al., 2012). Therefore, to summarise, *V. faba* plants have evolved the ability to perceive stress signals in neighbouring plants both above-- and belowground. Independently of the mode of communication, the healthy perceiving plants induce the production of key volatile compounds that can directly inhibit future aphid infestation, and at the same time, these VOCs can also attract natural enemies of the aphids in their surroundings. However, evolu-tionarily speaking, why do plants alert their conspecific neighbours of an imminent herbivore attack remains a matter of debate (Kessler & Heil, 2011). In this case, we can argue that within an extended and densely packed crop field, the successful detection of an herbivore on a damaged plants by a parasitoid should be very limited. Therefore, by allowing the signal to be amplified by their neighbours, a set of individual plants should facilitate the foraging success of parasitoids (Vet & Dicke, 1992), whose impact on the aphid population is usually visible with some delay in respect to the action of a predator. In fact, the enhanced release of methyl salicylate induced in our system by L--DOPA, has been shown to be also effec-tive in attracting insect predators such as ladybugs (Zhu
& Park, 2005), hence more broadly boosting the biologi-cal control of aphid pests.

The discovery of L--DOPA, a neurotransmitter pre-cursor in animals, acting in the rhizosphere as a plant defensive pheromone supports the paradigm of diver-gent evolutionary outcomes for the activity of the same molecule, spanning the plant and animal kingdoms. Similarly, GABA, another non--protein amino--acidic neurotransmitter found in animal brains, was discov-ered to function as signalling molecule for plant devel-opment and stress response activation against biotic attack (Zimmerli et al., 2000). Plants can therefore co-- opt broad--spectrum molecules for their own defence response against insect herbivores, whose activity could be exploited to enhance natural crop resistance against insect pests (Bown & Shelp, 2016; Conrath et al., 2006).

AUTHOR CONTRIBUTIONS

EG, JAP and MAB conceptualisation; EG, JAP, MAB and PC designed the research; PC, JV, SD, AS performed bioassay; PC, JV, MAB, JAP, SR and EG analysed the data; EG, JAP, SR and MAB wrote the paper.

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DATA AVAILABILITY STATEMENT

All data, and codes used in the analysis are deposited in Zenodo public database: https://doi.org/10.5281/zenodo.7145272

ORCID

Sergio Rasmann https://orcid.org/0000-0002-3120-6226 *Emilio Guerrieri* https://orcid.org/0000-0002-0583-4667

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