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Plant volatiles cause direct, induced and associational resistance in common bean to the fungal pathogen *Colletotrichum lindemuthianum*

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Summary

1. Plants that express resistance to herbivores emit volatile organic compounds (VOCs) that can trigger resistance responses in undamaged neighbours. Recent reports indicate that VOCs can also trigger the resistance to pathogens, an effect that might be due to different mechanisms: the priming of an induced expression of resistance genes in the receiver or direct inhibitory effects on microbial pathogens that cause a passive 'associational' resistance in the VOC-exposed plant.

2. We investigated whether VOCs emitted from a resistant common bean (*Phaseolus vulgaris*) cultivar enhance the resistance to the fungus *Colletotrichum lindemuthianum* in a susceptible cultivar and analysed whether specific VOCs are likely to directly affect the pathogen.

3. We found that susceptible plants exposed to the headspace of resistance-expressing plants over 6 h became phenotypically as resistant as the resistant cultivar. Several resistance marker genes (*PATHOGENESIS-RELATED* [*PR*] *1, 2* and *4*) were primed in VOC-exposed susceptible plants. After challenging, these genes reached expression levels at least as high as in the resistant cultivar. Additionally, individual VOCs such as limonene, linalool, nonanal, methyl salicylate and methyl jasmonate at natural concentrations directly inhibited the germination of conidia as did also the headspace of a resistance-expressing plant. This inhibition of conidial germination was dosage-dependent and irreversible.

4. *Synthesis.* We conclude that VOCs are involved in the resistance of bean to fungal pathogens. They can contribute to the direct resistance in the emitter itself, and resistance phenotypes of neighbouring receiver plants can result from induced as well as associational resistance. Plant VOCs play multiple roles in the resistance of plants to microbial pathogens.

Key-words: direct defence, fungal pathogen, *Phaseolus vulgaris*, plant disease, plant–plant interactions, plant–plant signalling, resistance, spore germination

Introduction

In their natural environment, plants continuously have to cope with multiple stressors, including herbivores and pathogens. To survive, they must respond to each attacker in a rapid and effective way (Baldwin & Schultz 1983; Dolch & Tscharntke 2000; Heil & Karban 2010). Besides sensing the direct and concurrent damage of their own organs, plants can also enhance their resistance in response to signals that indicate the presence of enemies in their immediate environment. Plants that are attacked by herbivores or pathogens release volatile organic compounds (VOCs) (Dicke & Sabelis 1987; Karban *et al.* 2000; Yi *et al.* 2009). These VOCs can act as a source of information regarding the status of attack suffered by the emitter and then mediate resistance expression in the neighbouring 'receiver' (which has been described as VOC-mediated 'plant–plant communication', see Baldwin & Schultz 1983) or in systemic parts of a locally attacked plant (which has been described as 'within-plant signalling', see Heil & Silva Bueno 2007). Plant–plant signalling by VOCs has been demonstrated for more than 30 species (reviewed in Heil & Karban 2010) and studies on sagebrush (*Artemisia*

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tridentata), lima bean (*Phaseolus lunatus*), poplar (*Populus deltoides* × *nigra*) and blueberry (*Vaccinium corymbosum*) have demonstrated within-plant signalling (Karban *et al.* 2006; Frost *et al.* 2007; Heil & Silva Bueno 2007; Rodríguez-Saona, Rodríguez-Saona & Frost 2009).

Much less is known about the role of VOCs in plant resistance to pathogens (Boulogne *et al.* 2012). However, *Arabidopsis thaliana* showed resistance to the necrotrophic fungus *Botrytis cinerea* after exposure to certain VOCs (Kishimoto *et al.* 2005), and the VOCs emitted from resistance-expressing *Phaseolus lunatus* plants triggered resistance to the biotrophic bacterial pathogen *Pseudomonas syringae* pv. *syringae* in neighbouring plants under both laboratory and field conditions (Yi *et al.* 2009; Heil & Adame-Álvarez 2010). Most recently, the positive effects of intercropping with *Allium tuberosum* on the resistance in banana (*Musa* spp.) to the necrotrophic fungal disease agent *Fusarium oxysporum* were attributed to VOCs (Zhang, Mallik & Zeng 2013). Thus, it appears likely that VOCs also enhance plant resistance to various types of pathogens.

Resistance caused by VOCs at the phenotypic level is usually considered induced resistance: that is, as a result of the priming or induction of resistance genes in the receiver (Arimura *et al.* 2000; Farmer 2001; Engelberth *et al.* 2004; Ton *et al.* 2007; Yi *et al.* 2009). Indeed, the systemic induction of resistance after a local attack has been discussed as a main 'raison d'être' of the induced VOCs because this effect benefits the emitter plant (Farmer 2001; Heil & Ton 2008; Heil & Karban 2010). However, besides functioning as signals, volatile compounds can also inhibit the development of pathogens, and thereby contribute to the direct resistance of the emitter. For example, *trans*-2-hexenal inhibited the conidial germination of the fungus *Monilinia laxa* (Neri *et al.* 2007), whereas hexenal, heptanal, octanal and nonanal inhibited the production of aflatoxins in *Aspergillus flavus* (Zeringue *et al.* 1996). Finally, VOCs can be adsorbed to the cuticle of a plant and then mediate a passive resistance termed associational resistance (Glinwood *et al.* 2004, 2009; Ninkovic & Åhman 2009; Himanen *et al.* 2010; Zakir *et al.* 2012), although this effect has only been reported to enhance resistance to herbivores to date.

In the present study, we used *Phaseolus vulgaris* to investigate whether volatiles can trigger resistance to the fungal pathogen *Colletotrichum lindemuthianum*, the causal agent of anthracnose in bean (Campa, Giraldez & Ferreira 2009). Besides searching for evidence of VOC-mediated resistance, our aim was to identify the active components of the volatile blend and to elucidate their putative direct effects on the fungus. Specifically, we asked (i) whether VOC emission can explain the differences between a resistant and a susceptible host genotype; (ii) whether susceptible receivers exposed to the headspace of a resistance-expressing emitter become more resistant ('plant–plant signalling'); and (iii) whether VOCs at natural concentrations exert inhibitory effects on the pathogen. Our aim was to understand whether VOCs can contribute to the direct resistance of the emitting plant and whether the enhanced resistance phenotype observed in neighbouring plants is modulated at the genetic level.

Materials and methods

PLANT AND FUNGAL MATERIAL

Seeds of seven cultivated genotypes of common bean (*Phaseolus vulgaris* L.), the cultivars 'Pinto Villa', 'Bayo Madero', 'Flor de Junio Marcela', 'Flor de Mayo Anita', 'Pinto Saltillo' and the landraces 'Negro San Luis' and 'Rosa de Castillo', were obtained from the collection at INIFAP (Celaya, Guanajuato, México). These genotypes had been selected in an earlier screening to represent a broad range in the level of resistance to the fungal pathogen used here (Córdova-Campos 2011). *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara strain 1088 was isolated in the state of Durango in México from climbing-type cultivars of bean. *Colletotrichum lindemuthianum* is a fungus that exhibits the hemibiotrophic infection strategy characteristic for most *Colletotrichum* species (Fig. 1) (Perfect *et al.* 1999).

DETERMINATION OF BASAL RESISTANCE TO *COLLETOTRICHUM LINDEMUTHIANUM* IN DIFFERENT BEAN CULTIVARS

Five plants of each cultivar were challenged by spraying a conidial suspension of the pathogen at a concentration of 1×10^7 conidia mL^{-1}, to quantify their basal resistance to *C. lindemuthianum*. Conidial suspensions were prepared by transferring mycelium to a Petri dish with potato dextrose agar (PDA) medium and incubating at 28 °C in the dark, for 2 weeks. The conidia produced by the mycelium were suspended in distilled water with 0.1% Tween (Sigma, St. Louis, MO, USA), and their concentration was adjusted by counting conidia in aliquots in a Neubauer hemocytometer (Hausser scientific, Horsham, PA, USA). Phenotypic disease severity was assessed

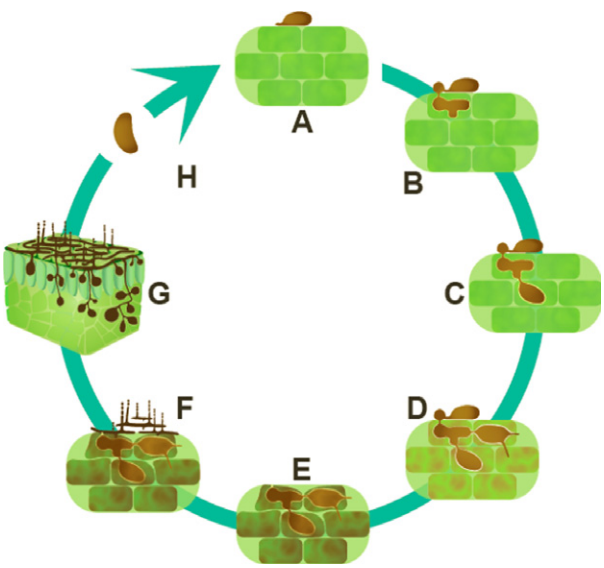

Fig. 1. Infection cycle of the hemibiotroph, *Colletotrichum lindemuthianum*. The cycle begins with the adhesion of a conidium to the leaf surface (A), followed by the formation of melanized appressoria (B) that allow the entry into the host cell. Once having entered the cell, the fungus forms infection vesicles and a broad primary hypha (C). During this stage, the fungus behaves as biotrophic pathogen. Two days after the penetration, secondary hyphae are formed (D) which leads to the necrotic disintegration of the host cells (E), followed by the formation of acervuli and the production of conidia (F). The conidia are spread by rain and wind (G) and the cycle continues (H).

15 days later by quantifying the damaged area from digital images using the UTHSCSA IMAGE TOOL V 3.0 (http://compdent.uthscsa.edu/dig/itdesc.html). The experiments were conducted in a glasshouse under natural light conditions, a day: night period of 12 h:12 h and day and night temperatures of 30 and 26 °C, respectively. Under these conditions, non-inoculated bean plants remain free of culturable fungi, that is, no vertically transmitted or environmentally derived endophytic or pathogenic fungi could be observed in plants grown from surface-sterilized seeds as used in this study (E. Quintana-Rodriguez, R. M. Ádame-Alvarez & M. Heil, unpubl. data, see also Adame-Álvarez, Mendiola-Soto & Heil 2014; Navarro-Melendez & Heil 2014).

VOC-MEDIATED RESISTANCE

To detect a putative VOC-mediated plant–plant signalling response, a cultivar with high basal resistance ('Pinto Villa') was selected as the emitter and a susceptible cultivar ('Negro San Luis') was selected as the receiver. Six plants of 'Pinto Villa' were challenged by spraying a conidial suspension of *C. lindemuthianum* at a concentration of 1×10^7 conidia mL^{-1}. Each plant was then placed in a closed, transparent 40-L box next to a 'Negro San Luis' cultivar (the receiver) for 6 or 24 h, avoiding direct physical contact between the plants (Girón-Calva, Molina-Torres & Heil 2012). To compare the induction mediated by volatiles and by a commercial resistance elicitor, six 'Negro San Luis' plants were treated with benzothiadiazole (BTH; Syngenta, www.syngenta.com) by spraying them with a solution of 300 mg L^{-1} of BTH in deionized water until the entire surface of the plants was wet. Controls were sprayed with deionized water. To avoid plant–plant signalling, plants were separated by transparent plastic curtains to reduce airflow among plants that had been subjected to the different treatments.

After 5 days, the receiver plants were challenged with *C. lindemuthianum* at the same concentration as that used to challenge the emitters. Fifteen days after challenging, leaf tissue was harvested to determine infection levels. We quantified infection levels by counting fungal colony forming units (CFUs) and by quantifying the content of the fungal membrane lipid, ergosterol, which is found almost exclusively in fungi and is frequently used as an indicator of living fungal biomass (Mille-Lindblom, von Wachenfeldt & Tranvik 2004). The two first leaves were collected from every plant, weighed and ground with a mortar and pestle in 1 mL sterilized distilled water. The extracts were diluted 1:10, 1:100 and 1:1000, 10 μL was then plated onto PDA medium and CFUs were counted 3 days later. To quantify ergosterol, the sample was suspended in 1 mL ethanol at 4 °C, extracted for 3 min and then centrifuged for 5 min at 9,520g in an Eppendorf 5804 Centrifuge (Eppendorf, Hamburg, Germany). The supernatant was recovered, the pellet was re-extracted as described above and both solutions were combined. The sample was passed through SEP-PAK C18 filters (Waters Associates Inc., Milford, MA, USA) and subjected to HPLC (Shimadzu class 10A series, Kyoto, Japan) with two LC-10AT pumps and equipped with the software Class LC-10At series, version 5.03 (Shimadzu, Kyoto, Japan) using a Zorbax C-18 column (Agilent Technologies, Wilmington, DE, USA) (Martinez-Soto *et al.* 2013).

DIFFERENTIAL EXPRESSION OF PATHOGENESIS-RELATED GENES

To test for a possible induction or priming of pathogenesis-related (PR) genes in VOC-exposed plants, we repeated the above-described experiment in which plants of the susceptible cultivar ('Negro San Luis') were exposed to VOCs released from challenged plants of the resistant cultivar ('Pinto Villa') and then challenged with the fungus. Challenging and other experimental conditions were as described above. Leaf material was collected from VOC-exposed Negro San Luis plants immediately before challenging with the fungus and at 12, 24, 48 and 72 h after challenging. Challenged resistant emitters (Pinto Villa) and Negro San Luis (NSL) plants that had been exposed to clean air served as positive and negative controls, respectively. Three plants per treatment were used for RNA isolation using Trizol® reagent (Invitrogen, Carlsban, CA, USA) following the manufacturer's instructions. *PATHOGENESIS-RELATED PROTEIN 1* (*PR-1*), *B 1,3-GLUCANASE* (*PR-2*) and *PATHOGENESIS-RELATED PROTEIN 4* (*PR-4*, which codifies for an antifungal chitin-binding protein) were selected as markers. *Phaseolus vulgaris* gene sequences were obtained from http://www.ncbi.nlm.nih.gov/nucleotide/; GenBank numbers for each gene used as template for primer design are: *ACTIN*, DQ159907; *PR-1*, DQ455598; *PR-2*, AY357300 and *PR-4*, X53129. RNA concentration was measured with a NanoDrop® (Thermo Scientific, Wilmington, DE, USA), and its integrity and concentration were determined by electrophoresis in agarose gels. Reverse transcription was performed using 1 μg of DNAase-treated RNA as template. These were incubated with oligo dT and Super-Script II reverse transcriptase (Invitrogen, Carlsban, CA, USA) according to manufacturer's instructions. The oligonucleotides used were the following: *ACTIN*, 5′-GGTCGTCCTCGTCACACTGG3′ and 5′GGCATGTGGGAGAGCATAACC 3′; *PR-1*, 5′AAGACGC CGATACCATCTTCC3′ and 5′CCAGAAGGTATGCCTCTACGG3′; *PR-2*, 5′CGGAGAGCAGTGTGGAAGGC3′ and 5′CCATCCCCC GGTTGTTTCG3′ and *PR-4*, 5′AATGTTGTGGTGAGGGATGGC3′ and 5′CTTTGCATCCTTTGGGCACC3′. PCRs proceeded by incubation of an aliquot of cDNA with specific oligonucleotides and DNA polymerase (Invitrogen) using the following programme: an initial cycle of denaturalization at 94 °C for 2 min followed by 29 cycles as follows: 94 °C for 15 s, alignment at 58 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. The optimal temperature of hybridization and the number of hybridization cycles to obtain results in the linear range of amplification were determined for each gene. PCR products were separated by gel electrophoresis on 1% agarose gels and photographed. Transcript levels were determined with the IMAGE LAB SOFTWARE® (http://www.bio-rad.com/en-ru/product/image-lab-software). The reported results correspond to the average of data from three replicates ± standard error. Data corresponding to *PR-1*, *PR-2* and *PR-4* gene expression were related to the values of the *ACTIN* gene.

VOC PROFILES OF HEAD SPACES

Profiles of VOCs were analysed in two different experiments. Both experiments used six resistant plants ('Pinto Villa') and six susceptible plants ('Negro San Luis') each, which were challenged with a conidial suspension as described above, allowed to dry and then bagged individually in PET foil ('Bratenschlauch': Toppits, www.toppits.de, a material that does not emit detectable amounts of volatiles) for 6 and 24 h.

In the first experiment, the emitted VOCs were trapped on charcoal filters (1.5 mg of charcoal: CLSA Filters, Le Russie de Montbrun, Daumazan sur Arize, France) using closed-loop stripping (Donath & Boland 1995). Organic compounds were eluted from the charcoal traps with dichloromethane (40 μL) containing 1-bromodecane (50 ng mL^{-1}) as an internal standard. The samples were analysed in a gas chromatography–electronic ionization mass spectrometry (GC-EIMS) system (an Agilent 7890 series gas chromatograph interfaced

to an Agilent 5975 electronic ionization mass-selective triple axis detector; Agilent Technologies, Santa Clara CA, USA). The programme used for separation (HP-5MS column Agilent 15 × 0.25, 0.25; Agilent Technologies, Palo Alto, CA, USA) had an initial column temperature of 40 °C (3 min) and then ramped at 5 °C min^{-1} to 240 °C. Identification of compounds was performed by comparison with the spectra of standard substances and with the Nist® 0.5 library.

In the second experiment, VOCs were trapped using Solid Phase Micro-Extraction (SPME) fibres (2 cm, carboxen/Polydimethylsiloxane/Carbowax; Supelco, Bellefonte, PA, USA). The fibres were exposed for a period of 18 h and then desorbed for 30 s directly into the gas chromatograph (GC) injector (180 °C). The VOCs were analysed by GC-mass spectrometry (MS). The temperature programme used for the analysis was as follows: initial temperature at 60 °C, which was increased to 80 °C at 5 °C min^{-1}, then to 210 °C at 8 °C min^{-1} and then maintained at 210 °C for 5 min. Compounds were identified using the National Institute of Standards and Technology (NIST) mass spectral library and, when available, verified by authentic standards (Sigma-Aldrich, St. Louis, MO, USA).

DIRECT EFFECTS OF VOCS ON THE FUNGUS

Putative direct effects of VOCs on crucial phases of the fungal life cycle were investigated both *in vitro* (Petri dish) and *in vivo* (on leaves). We considered the production of conidia as well as their germination and used both pure compounds at defined concentrations and the entire spectrum of VOCs in the headspace of a challenged, resistant plant. The VOCs were selected based on profiles obtained in this study and volatiles detected in profiles of lima bean (Yi *et al.* 2009; Girón-Calva, Molina-Torres & Heil 2012). The volatiles limonene, β-linalool, nonanal, methyl salicylate, *cis*-hexenyl acetate, methyl jasmonate, 1-octen-3-ol, β-pinene, α-terpineol, farnesol and decanal (Sigma-Aldrich, www.sigmaaldrich.com) were dissolved in dichloromethane to reach concentrations resembling the natural concentrations as detected in the headspace of bean plants. For all experiments described in the following, limonene, 1-octen-3-ol, decanal, β-pinene, α-terpineol and farnesene were used at 10 ng μL^{-1}, β-linalool at 6.87 ng μL^{-1}, nonanal at 0.77 ng μL^{-1}, methyl salicylate at 0.62 ng μL^{-1}, *cis*-hexenyl acetate at 0.66 ng μL^{-1} and methyl jasmonate at 13.56 ng μL^{-1}, and pure solvent was used as a control.

Given the importance of spore germination in the process of pathogenesis (Uhm *et al.* 2003), we tested the effect of individual VOCs on conidial germination both *in vivo* and *in vitro* under different conditions, determined the effects of VOCs on the production of conidia and tested whether the headspace of resistance-expressing plants can inhibit the germination of conidia *in vitro*.

1. The *in vivo* effect of individual VOCs was studied using detached bean leaves. The leaf surfaces were superficially disinfected by immersion in a 1% sodium hypochlorite solution for 1 min, rinsed in sterile water and then placed in Petri dishes with 5 mL of 0.7% agar to prevent desiccation. Each individual VOC was pipetted onto a filter paper disc and then placed in the same Petri dish as the leaf, avoiding any direct contact with the leaf. The leaves were inoculated by applying 100 μL of the conidial suspension (1 × 10^5 conidia mL^{-1}). After 36 h of exposure, a leaf disc (2.5 cm in diameter) was cut-out and boiled in a solution of ethanol (70%) for 5 min. The leaf disc was stained with lactophenol blue solution (Merck KGaA, Darmstadt, Germany) and observed under a light microscope (Zeiss K7, Oberkochen, Germany) to determine the proportion of germinated versus non-germinated spores. Conidia were considered germinated when the germ tube length was equal to or greater than the spore length. The proportion of germinated and non-germinated conidia was estimated by observing at least 100 conidia with five replicates per treatment. The entire experiment was performed twice.

2. To investigate the effects of individual VOCs on conidial germination *in vitro*, 100 μL of the conidial suspension (1 × 10^5 conidia mL^{-1}) was placed on a Petri dish with 5 mL of 0.7% agar. A VOC was added to a filter paper placed on the opposite side of the Petri dish. The dishes were sealed with Parafilm® (Pechiney Plastic Packaging Company, West Chester, PA, USA) immediately and then incubated at 28 °C. Solvent added to filter paper served as a control. Five Petri dishes were used for each compound. The germinated conidia were observed at 36 h after inoculation. The agar was cut and placed on a slide. The preparation was stained with lactophenol blue solution (Merck KGaA) and germinated and non-germinated conidia were determined as described above. The entire experiment was performed twice.

3. To investigate the effect of individual VOCs on the formation of conidia by a live mycelium, a mycelial disc (5 mm diameter) was taken from the periphery of an actively growing culture of *C. lindemuthianum* on PDA and was placed in the centre of a Petri dish (70 mL) containing 20 mL of PDA. The VOC (dissolved in 10 μL of dichloromethane) was added to a filter paper placed on the cover inside the dish (avoiding any direct contact between the filter paper and the agar or the mycelium). For each VOC, we used five independent replicates. The control consisted of a Petri dish with a mycelial disc and 10 μL of pure solvent (dichloromethane) added to the filter paper. After 15 days of incubation, the conidia were removed using 10 mL of sterilized distilled water and quantified in a Neubauer counting chamber (Hausser Scientific, Horsham, PA, USA). The entire experiment was performed twice.

4. To investigate whether the VOCs have a fungistatic (i.e. reversible) or a fungicidal (i.e. non-reversible) effect on conidial germination, limonene and methyl jasmonate were selected as the volatiles that had the strongest effect on conidial germination. Glass microscope slides (25 × 75 mm; www.corning.com/lifesciences) were covered with 500 μL of 0.7% agar and placed in Petri dishes next to filter paper containing the volatile; the same concentration was used as that used in the previous experiments. The slides were inoculated with 10 μL of conidial suspension (1 × 10^5 conidia mL^{-1}). After 2 days, half of the slides were removed from the Petri dishes and placed in new Petri dishes without a VOC, whereas the other half remained exposed to the VOC until the end of the 4-day experiment. The slides were stained with lactophenol blue solution (Merck KGaA) as described above and the proportion of germinated versus non-germinated conidia was recorded. Five replicates were performed per VOC, and the entire experiment was performed twice.

5. The effect of the total (natural) volatile profile as emitted from a resistant plant on conidial germination was evaluated by exposing Petri dishes inoculated with conidia to the headspace around the plants. Potted plants of the resistant cultivar 'Pinto Villa' were placed into acrylic boxes, and the pot was covered with aluminium foil to avoid contamination. Glass columns (46 cm in height and 6.5 cm in diameter) with Petri dishes on the top were also placed in the acrylic boxes. Previously, the petri dishes had been filled with a solution of 0.7% agar and inoculated with 100 μL droplets of the conidial suspension (1 × 10^5 conidia mL^{-1}). Two types of VOC emitter plants were used: (i) plants that had been previously challenged with *C. lindemuthianum* and (ii) healthy plants. As a negative control, we also placed Petri dishes on columns in acrylic boxes without any plants. The acrylic boxes were sealed, and after 36 h, the Petri dishes

were removed. The agar was cut and placed on a slide. The preparation was stained with lactophenol blue solution, and the proportion of germinated versus non-germinated conidia was recorded as described above. For each treatment, five replicates were used and the experiment was performed twice.

6 In order to investigate the minimum concentration at which the volatiles inhibit spore germination, we used different concentrations of selected volatiles: limonene, β-linalool and methyl jasmonate. A petri dish was filled with 5 mL of 0.7% agar water and inoculated with 100 μL droplets of the conidial suspension (1×10^5 conidia mL^{-1}). A VOC was added to a filter paper placed directly on the agar, on the opposite side of the Petri dish to the droplet of inoculum. Five replicates were performed per VOC and per concentration, and the entire assay was replicated twice.

DATA ANALYSIS

Significant differences among the different treatments (levels of resistance of different cultivars, the airborne induction of resistance and the effect of volatiles on different parameters of the fungal development) were tested using an ANOVA and *post hoc* comparison with Tukey using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA). Significance was determined by the magnitude of the *F*-value at $P = 0.05$.

Results

RESISTANCE TO *COLLETOTRICHUM LINDEMUTHIANUM* IN DIFFERENT BEAN CULTIVARS

The challenged plants of the seven cultivars differed significantly in their infection levels. For example, the cultivars 'Pinto Villa' (PV), 'Bayo Madero' and 'Pinto Satillo' showed no visible symptoms of infection 15 days after challenging, whereas more than 25% of the leaf area of plants of 'Negro San Luis' exhibited lesions by that time (Fig. 2).

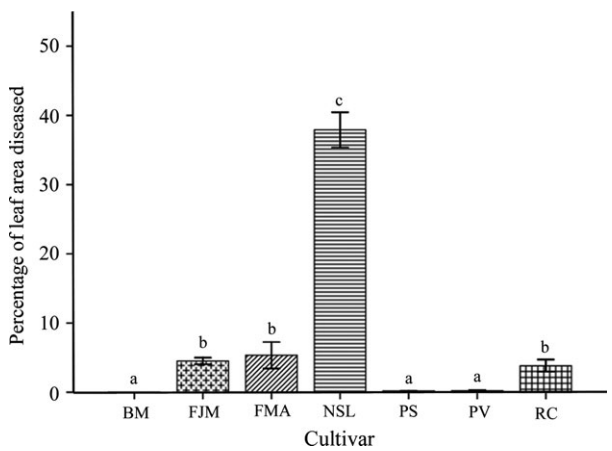

Fig. 2. Disease levels in cultivars of common bean (*Phaseolus vulgaris*) plants challenged with *Colletotrichum lindemuthianum.* Cultivars: BM, Bayo Madero; FJM, Flor de Junio Marcela; FMA, Flor de Mayo Anita; NSL, Negro San Luis; PS, Pinto Saltillo; PV, Pinto Villa; RC, Rosa de Castilla. The values represent the diseased leaf area relative to the total leaf area (means ± SE). Bars with different letters are significantly different ($P < 0.05$, Tukey *post hoc* test).

RESISTANCE MEDIATED BY VOLATILES

We challenged PV plants with fungal conidia and then exposed NSL plants (receivers) to the headspace of these plants over the next 6 or 24 h. Quantifying infection levels as CFUs demonstrated that the receivers showed significantly lower levels of infection (*ca*. one-fifth) as compared with those seen in the controls. Indeed, the infection levels in the VOC-exposed receivers were as low as the infection levels in the resistant (PV) plants or in plants that were directly treated with BTH (Fig. 3a). Time of exposure did not matter, as exposure over 6 or 24 h resulted in very similar infection levels. By contrast, the headspace of healthy PV emitter plants caused no significant reduction in infection levels in exposed NSL plants (Fig. 3a). The same patterns were found when infection levels were determined via the quantification of ergosterol (Fig. 3b).

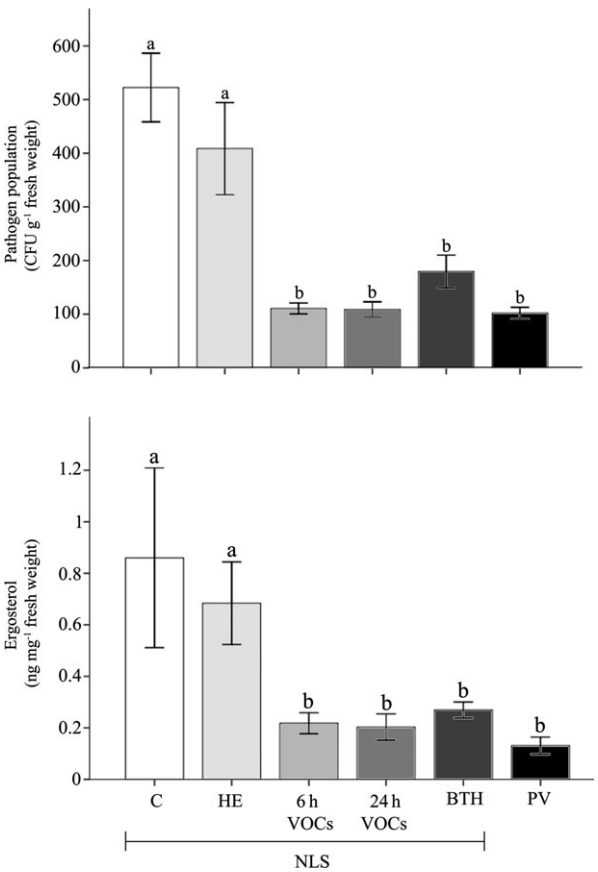

Fig. 3. Airborne versus chemically induced resistance. Plants of the cultivar Negro San Luis (NSL) were exposed to air from the headspace of the challenged, resistant Pinto Villa (PV) plants for six hours (6 h) or twenty-four hours (24 h), to volatiles from the headspace of a healthy PV plant (HE), treated directly with BTH, or exposed to clean air (control, C). All plants were spray-inoculated with *Colletotrichum lindemuthianum* at 5 day after the treatment. We depict infection levels in these plants and in challenged PV plants (mean ± SE of $n = 6$ repetitions). Panel (a) depicts average pathogen populations quantified as numbers of colony forming units (CFUs) per gram leaf fresh weight, panel (b) depicts the concentration of ergosterol in ng per mg leaf fresh mass. Different letters indicate significant differences among treatments ($P < 0.05$, Tukey *post hoc* test). BTH, benzothiadiazole.

VOLATILES TRIGGER THE EXPRESSION OF RESISTANCE-RELATED GENES

The expression of the resistance-related genes PR 1, 2 and 4 in *C. lindemuthianum*-challenged NSL plants was significantly enhanced when these had been exposed to the VOCs emitted from infected PV plants before challenging (Fig. 4a–c). In fact, the expression of *PR-1* and *PR-4* in VOC-exposed NSL plants at 12, 24, 48 and 72 h after challenging was even significantly higher than in (resistant) PV plants at the same times after challenging (Fig. 4a,c).

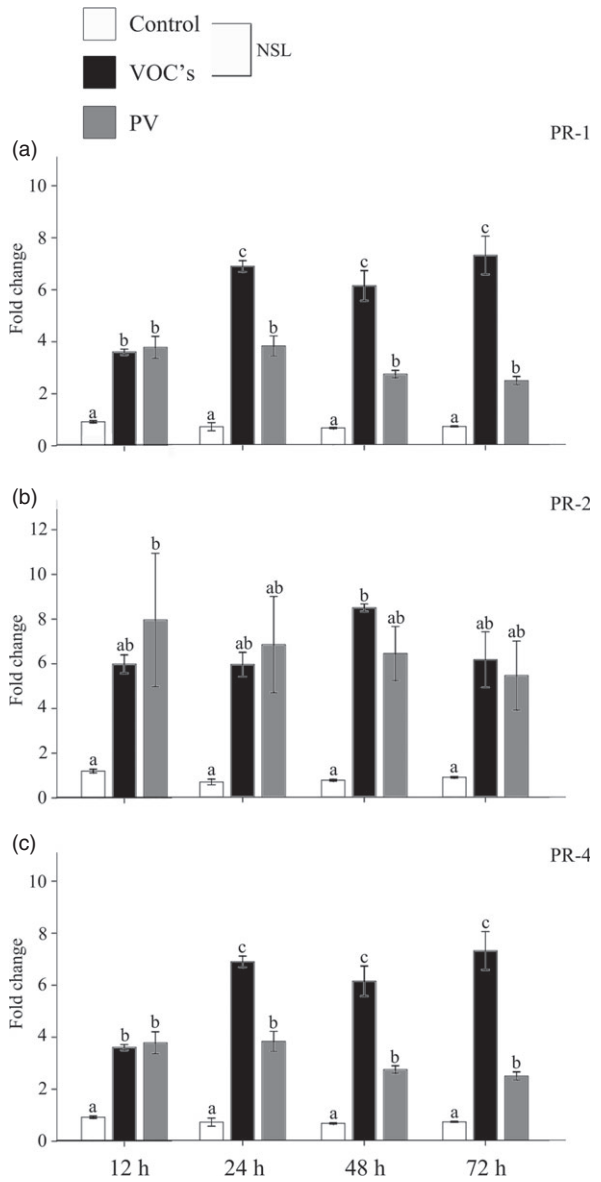

Fig. 4. Exposure to volatile organic compounds (VOCs) enhances the expression of resistance-related genes in challenged plants of the susceptible cultivar, Negro San Luis (NLS). We depict the expression of pathogenesis-related (PR) genes in unexposed plants (control) and plants exposed to volatiles (VOCs) of the susceptible cultivar (NSL) and in resistant Pinto Villa plants (PV). Bars indicate mean ± SE of $n = 3$ replicates of the expression levels of each gene expressed as a ratio relative to the level found directly before inoculation (0 h), which was set to 1. Values with different letters are different at $P < 0.05$. *PR-1*, *PATHOGENESIS-RELATED PROTEIN 1*, *PR-2*, β *1,3-GLUCANASE*; *PR-4*, *PATHOGENESIS-RELATED PROTEIN 4* (codifies for a protein similar to an antifungal chitin-binding protein). NSL, Negro San Luis.

VOLATILE PROFILES

The headspaces of the resistant cultivar (PV) and the susceptible cultivar (NSL) collected over 6 and 24 h after challenging and the headspaces of the unchallenged controls showed few differences when they were collected on charcoal traps. For example, the compounds nonanal and decanal were found in all headspaces, although at different concentrations (Table 1). *Cis*-hexenyl acetate was emitted at much higher concentrations by the susceptible cultivar than by the resistant cultivar. Only β-linalool was exclusive to the headspace of the resistant cultivar and was only detected in the headspace collected over 24 h after challenging PV plants (Table 1). When the volatiles were collected with SPME fibres, we observed that the resistant plants presented limonene and α-terpineol after 24 h post infection and that only the resistant cultivar (PV) emitted farnesene, β-pinene and β-ocimene (Table 2). SPME fibres confirmed the exclusive presence of β-linalool in the headspace of resistant (PV) plants at 24 h after challenging (Table 2).

DIRECT EFFECTS OF VOCS ON THE FUNGUS

When leaves were challenged with conidia of *C. lindemuthianum* and then immediately exposed to single VOCs (each at concentrations resembling natural conditions), significantly fewer conidia germinated (< 50%) after exposure to limonene, β-linalool or nonanal as compared with the proportion of germinating conidia on the control leaves (Fig. 5a,b). All tested compounds except octanal had at least some inhibitory effect that was statistically significant ($P < 0.05$, according to least significant difference *post hoc* tests, Fig. 5b). Effects *in planta* can be caused directly by the VOC or by the induced responses of the plant. Therefore, the experiment was repeated *in vitro*. In Petri dishes, significantly fewer conidia germinated (< 50%) after exposure to limonene, β-linalool, nonanal, methyl salicylate, methyl jasmonate, octanal or decanal (Fig. 5c) compared with the controls ($P < 0.05$, according to least significant difference *post hoc* tests). The strongest inhibitory effects were observed after exposure to limonene, methyl salicylate or methyl jasmonate (conidial germination was only ≤ 30% of that of the control values). Among the tested compounds, *cis*-hexenyl acetate, β-pinene, farnesene and α-terpineol did not detectably inhibit conidial germination (Fig. 5c). Most of the tested VOCs also inhibited the production of conidia by a living mycelium *in vitro* (see Fig. S1 in Supporting information).

Two of the VOCs that significantly inhibited conidial germination (methyl jasmonate and limonene) were used to investigate whether the observed effects were fungistatic or fungicidal (Arroyo *et al.* 2007). Exposure to each of these VOCs significantly inhibited the germination of conidia (germination rates were ≤30% of that observed for the controls;

Table 1. Volatile organic compounds in the headspace of bean (*Phaseolus vulgaris*) plants challenged with the fungus *Colletotrichum lindemuthianum.* Amounts are given in nanogram released per gram leaf dry weight (n = 6 plants per treatment)

Compound	NSL c	NSL 24 h	PV c	PV 6 h	PV 24 h
Cis-hexenyl acetate	9.29 ± 5.48	7.14 ± 1.92	ND	ND	2.20 ± 1.97
Nonanal	4.08 ± 4.00	3.29 ± 1.75	3.20 ± 2.55	1.07 ± 0.37	4.38 ± 3.01
Decanal	0.14 ± 0.10	0.99 ± 0.90	0.51 ± 0.32	1.29 ± 0.68	2.66 ± 2.00
1-octen-3-ol	ND	4.22 ± 4.00	1.35 ± 0.88	1.45 ± 0.42	ND
β-linalool	ND	ND	ND	ND	2.12 ± 1.85

NSL c, susceptible cultivar (Negro San Luis), control; NSL 24 h, susceptible cultivar (Negro San Luis) over 24 h after challenging; PV c, resistant cultivar (Pinto Villa) control; PV 6 h, resistant cultivar (Pinto Villa) over 6 h after challenging; PV 24 h, resistant cultivar (Pinto Villa) over 24 h after challenging; ND, not detected.

Table 2. Volatile organic compounds emitted by *Colletotrichum lindemuthianum* inoculated bean plants as detected by SPME-GC/MS

Compound	NSL c	NSL 24 h	PV c	PV 6 h	PV 24 h
Cis-hexenyl acetate	2.391 ± 1.432	ND	ND	ND	ND
β-pinene	ND	ND	ND	0.699 ± 0.373	0.922 ± 0.743
β-ocimene	ND	ND	9.818 ± 3.657	2.467 ± 2.379	5.852 ± 3.939
L-carveol	ND	17.345 ± 10.061	ND	ND	ND
Nonanal	1.876 ± 0.9687	2.006 ± 0.969	ND	ND	1.538 ± 1.453
1-octen-3-ol	ND	ND	ND	ND	1.894 ± 1.385
Decanal	0.481 ± 0.769	9.601 ± 1.903	ND	ND	2.209 ± 1.424
Limonene	ND	ND	ND	ND	0.469 ± 0.056
α-terpineol	ND	ND	ND	ND	1.076 ± 0.169
Cis-α-bergamotene	ND	1.081 ± 0.334	ND	ND	ND
β-linalool	ND	ND	ND	ND	0.719 ± 0.182
Farnesene	ND	ND	0.396 ± 0.191	1.595 ± 0.385	1.649 ± 1.430

Each value represents the mean peak area of n = 6 replicates.
NSL c, susceptible cultivar (Negro San Luis), control; NSL 24 h, susceptible cultivar (Negro San Luis) over 24 h after challenging; PV c, resistant cultivar (Pinto Villa) control; PV 6 h, resistant cultivar (Pinto Villa) over 6 h after challenging; PV 24 h, resistant cultivar (Pinto Villa) over 24 h after challenging; ND, not detected; SPME, solid phase micro-extraction.

Fig. 6). The germination rates of conidia exposed to the VOCs for the entire duration of the experiment were not significantly different to the germination rates of conidia that were only exposed to the VOCs for the first 2 days of the 4-day experiment (Fig. 6).

We evaluated whether the total profile of VOCs emitted from resistant plants can negatively affect the germination of *C. lindemuthianum* and found that conidial germination was significantly affected by exposure to the headspace of plants (Fig. 7). The germination rate for conidia exposed to the headspace of uninfected PV plants and to infected PV plants was only about 70% and 50%, respectively, of that observed for the unexposed controls. The effects caused by the headspace of uninfected and infected plants were not statistically different, although the VOCs emitted by the infected plants tended to have a stronger inhibitory effect on conidial germination (Fig. 7).

Finally, we tested volatiles at different concentrations to analyse whether the effect in the germination of the fungus was dosage-dependent. In these experiments with individual VOCs, we observed a correlation of the inhibitory effect with the concentration of limonene, β-linalool or methyl jasmonate in the headspace (Fig. 8).

Discussion

The induction of the release of volatiles can affect the direct resistance of plants to herbivores (Arimura *et al.* 2000; Kessler *et al.* 2006; Frost *et al.* 2007), their indirect resistance to herbivores (Heil & Kost 2006; Heil & Silva Bueno 2007; Ton *et al.* 2007) and their resistance to pathogens (Kishimoto *et al.* 2005; Yi *et al.* 2009; Das *et al.* 2013). However, the range of organisms against which VOC-induced resistance can act requires further study, likewise the identity of the active components and their mode of action. VOCs emitted from infected resistant bean plants caused resistance to *C. lindemuthianum* in a susceptible cultivar (Fig. 3). Moreover, direct application of BTH-induced high levels of resistance to *C. lindemuthianum* in the susceptible cultivar (Fig. 3), whereas an earlier study observed that several cultivars of common bean had lost the capacity to respond to BTH with an induced resistance to biotrophic bacteria (Córdova-Campos *et al.* 2012). Induced resistance has been discussed as an enhancement of the basal resistance (Ahmad *et al.* 2010). Our results now demonstrate that basal resistance, VOC-mediated resistance and BTH-induced resistance do not necessarily depend on each other. Certain VOCs with

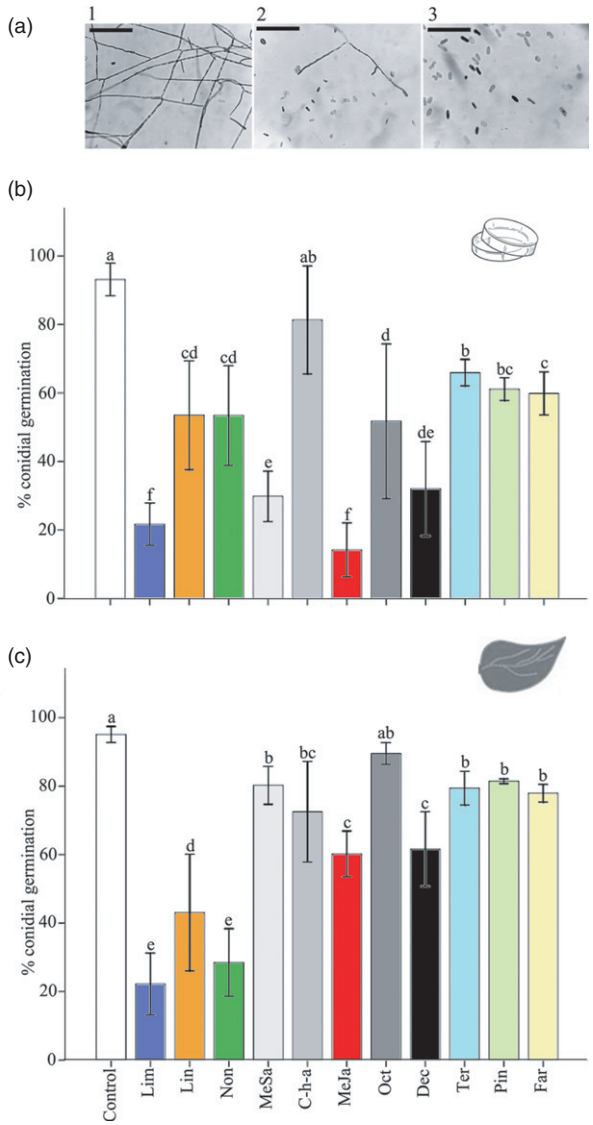

Fig. 5. Direct effects of volatiles on fungal development. The fungal pathogen *Colletotrichum lindemuthianum* was exposed to different volatiles to analyse the effects on fungal development. (a) Phenotypes of germinating fungi in an *in vitro* assay: 1, control; 2, exposed to nonanal; 3, exposed to methyl jasmonate (bars represent 15 μm). (b) Effect of volatile organic compounds (VOCs) on conidial germination *in vivo* [detached leaf of common bean (*Phaseolus vulgaris*)]. The bars represent the percentage of germinated conidia (mean ± SE). (c) Effect of VOCs on conidial germination *in vitro* (Petri dish). The bars represent the percentage of germinated conidia (mean ± SE). Different letters indicate significant differences among treatments ($P < 0.05$, Tukey *post hoc* test). VOC abbreviations: Lim, limonene; Lin, β-linalool; Non, nonanal; MeSa, methyl salicylate; C-h-a, *cis*-3-hexenyl acetate; MeJa, methyl jasmonate; Oct, 1-octen-3-ol, Dec, decanal; Ter, α-terpineol, Pin, β-pinene and Far, farnesene.

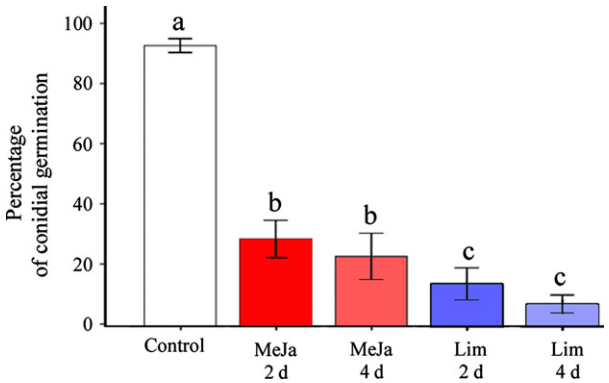

Fig. 6. Volatile organic compounds (VOCs) irreversibly inhibit the germination of conidia of *Colletotrichum lindemuthianum*. The conidia were exposed to methyl jasmonate (MeJA) for 2 days and then moved to a MeJA-free atmosphere (MeJA 2d) or remained exposed to this VOC for the duration of the 4-day experiment (MeJA 4d). The same pattern was followed for limonene (Lim). Bars represent the percentage of germinated conidia (mean ± SE) of $n = 5$ different letters indicate significant differences among treatments ($P < 0.05$, Tukey *post hoc* test).

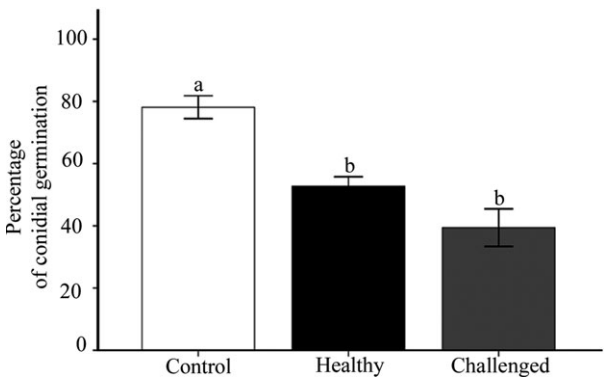

Fig. 7. Effect of the total volatile organic compounds (VOC) profile of Pinto Villa bean plants on the conidial germination of *Colletotrichum lindemuthianum*. Conidia were exposed to the headspace of resistance-expressing common bean (*Phaseolus vulgaris*) plants to evaluate the effect on their germination. 'Control', conidia not exposed to volatiles; 'healthy', conidia exposed to the headspace of healthy resistant plants; 'challenged', conidia exposed to the headspace of plants challenged with the fungus *C. lindemuthianum*. The bars represent the percentage of germinated conidia (means ± SE of $n = 5$). Different letters indicate significant differences among treatments ($P < 0.05$, Tukey *post hoc* test).

direct fungicidal effects can cause resistance phenotypes that are not dependent on the basal resistance in the respective plant.

The expression levels of resistance-related *PR*-genes *1, 2* and *4* indicate that a priming of the plant's own resistance response is likely to contribute significantly to the resistance that we observed in VOC-exposed plants. Already at 12 h after challenging, exposed plants of the normally susceptible cultivar NSL exhibited expression levels that were as least as high as those that we observed in the resistant cultivar (Fig. 4). Similarly, resistant cultivars of tomato respond faster than susceptible cultivars (Upadhyay *et al.* 2014). Studies of the incompatible interaction between bean and *C. lindemuthianum* showed the expression of *PR*-genes 60 h after inoculation (Oblessuc *et al.* 2012), which indicates that enhanced expression levels at 12 h post-inoculation can be absolutely sufficient to prevent infection.

However, our further results demonstrate that associational resistance represents a second, functionally independent

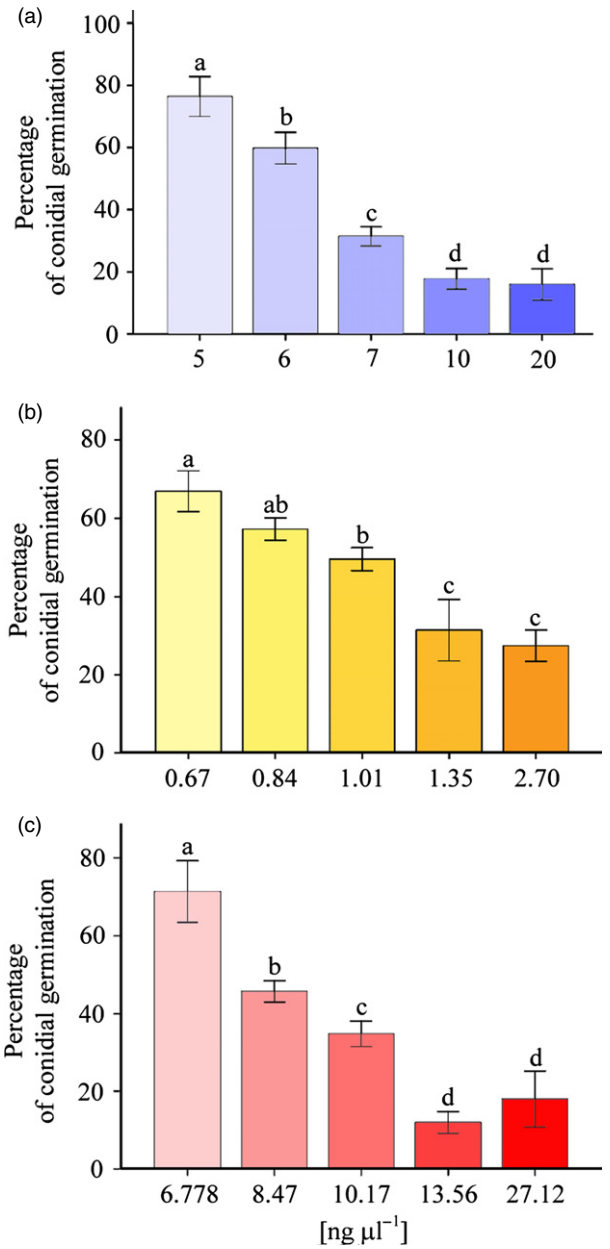

Fig. 8. Dosage-dependent effects of VOCs on conidial germination. We depict the percentage of germinated conidia (mean ± SE of $n = 5$) for conidia exposed to different concentrations of limonene (panel a), β-linalool (panel b) and methyl jasmonate (panel c). Different letters indicate significant differences among treatments ($P < 0.05$, Tukey *post hoc* test). VOCs, volatile organic compounds.

mechanism for VOC-mediated resistance. Single VOCs at the concentrations as found in the headspace of plants inhibited conidial germination *in vivo* (i.e. conidia applied to leaves) and the germination *in vitro* (i.e. conidia or living mycelia on Petri dishes; see Fig. 5). Resistance expression can be impaired in detached leaves (Jaber & Vidal 2010) and the conidia that had been applied to leaf surfaces showed a rapid (after 36 h) reduction in their germination rate (Fig. 5b). Moreover, given that conidial germination was also inhibited *in vitro* (Fig. 5c) and by the headspace of resistant plants (Fig. 7), we conclude that plant VOCs can also directly inhibit the development of the pathogen and that the effects observed in the receivers can be caused by a combination of induced resistance and associational resistance.

Our results demonstrate that associational resistance can contribute to the resistance phenotype of a plant exposed to VOCs. The direct inhibitory effects of purified VOCs (Fig. 5) and of the natural headspace of the plants (Fig. 7), together with the rapid and irreversible effects of individual VOCs on conidial germination (Fig. 6), indicate that plant-derived fungicidal VOCs at natural concentrations can directly and effectively inhibit the germination of conidia, a central part of the fungal reproductive cycle. Because VOCs in the emitting leaf are necessarily present at higher concentrations than in its headspace, we further conclude that the VOCs can also contribute to the direct resistance of the emitter. VOCs benefit the plant producing them by playing a direct protective role and, owing to their physico-chemical properties, inevitably leave the leaf (Peñuelas & Llusià 2004; Heil & Karban 2010). Resistance effects in neighbouring plants might, therefore, represent side effects that can be caused by 'eavesdropping' (defined as an active response of the receiver, that is a response mediated by changes in gene expression patterns), or by associational resistance (defined as a passive resistance that is mediated directly by VOC molecules that come from the emitter) or by a combination of both effects.

Which VOCs were involved in the observed effects? Limonene, β-linalool and methyl jasmonate caused a dose-dependent response (Fig. 8), and limonene and β-linalool were only found in the headspace of the resistant cultivars (Tables 1 and 2). However, the volatiles α-pinene and farnesene were also exclusive to the profile of PV plants (Table 2) and under our experimental conditions did not detectably inhibit spore germination. In several other studies, associations of certain VOCs with resistance phenotypes have been found. For example, terpenoid biosynthesis was induced in lettuce plants inoculated with *B. cinerea* (De Cremer *et al.* 2013) and resistance phenotypes in maize (*Zea mays*) correlated with the presence of certain VOCs in their headspaces (Zeringue *et al.* 1996; Erb *et al.* 2011). We found comparably few volatiles in our profiles, likely because plants expressing resistance to pathogens in general emit less rich bouquets than plants that have been damaged by herbivores (Yi *et al.* 2009). Still, it appears likely that additional analytical methods (e.g. using further adsorbents with different chemical properties) will reveal trace components that were not found by the methods chosen here.

More importantly, the resistance effect is unlikely to be caused by one single compound or one narrowly defined mechanism that occurs in or on the receiver plant. Multiple compounds with very different structures inhibited the germination of conidia (Fig. 5, Tables 1 and 2). Indeed, several VOCs have antimicrobial activity (Fernando *et al.* 2005; Arroyo *et al.* 2007; Neri *et al.* 2007). For example, nonanal inhibited the germination of fungi in leaves due to direct fungistatic effects (Zeringue *et al.* 1996) and induced resistance in lima bean to *Pseudomonas syringae* (Yi *et al.* 2009). Aldehydes emitted by resistant genotypes of maize have also been

suggested to contribute to the overall resistance to *Aspergillus niger* (Zeringue *et al.* 1996; Michereff *et al.* 2011). Furthermore, limonene has been reported to inhibit the growth of *Fusarium verticillioides* (Dambolena *et al.* 2008), methyl jasmonate inhibits the growth and aflotoxin production of *Aspergillus flavus* (Goodrich-Tanrikulu, Mahoney & Rodriguez 1995) and methyl salicylate has been shown to have antifungal activity against *Colletotrichum camelliae* (Zhang *et al.* 2006). Moreover, methyl salicylate represents the volatile form of salicylic acid, the central hormone in systemically induced plant resistance to biotrophs (Park *et al.* 2007). Therefore, methyl salicylate is likely to induce resistance to biotrophic pathogens in the majority of plant species. The volatile linalool (which was only present on the profiles of resistant plants) showed a negative effect in the conidial germination in our experiments and, interestingly, the same compound has been reported as inducer of resistance in rice to the bacterial pathogen, *Xanthomonas oryzae* (Taniguchi *et al.* 2014). Only the volatile *cis*-hexenyl acetate (the dominant component of the volatile profile of the susceptible cultivar) is more likely to be involved in herbivory-associated (Kost & Heil 2006; Heil, Lion & Boland 2008) rather than PR processes.

In summary, the headspace of bean plants enhanced the resistance in neighbouring plants to a hemibiotrophic fungal pathogen and this VOC-mediated resistance could be observed in a cultivar with very low basal resistance. The same air also inhibited the development of the fungal pathogen in the direct exposure experiments and single VOCs at natural concentrations inhibited the germination of the conidia both *in vivo* and *in vitro*. We conclude that the primary function of the production of VOCs is most likely to be the enhancement of the direct resistance of the emitting plant itself, and that the secondary effects observed in neighbouring plants can be caused by resistance induction as well as associational resistance.

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Data accessibility

All data are included in the article and the Supporting Information.

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Supporting Information

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

Figure S1. Direct effect of volatiles on conidial formation by live mycelium.