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PLANT-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Induced plant defense via volatile production is dependent on rhizobial symbiosis

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Abstract Nitrogen-fixing rhizobia can substantially influence plant–herbivore interactions by altering plant chemical composition and food quality. However, the effects of rhizobia on plant volatiles, which serve as indirect and direct defenses against arthropod herbivores and as signals in defense-associated plant–plant and within-plant signaling, are still unstudied. We measured the release of jasmonic acid (JA)-induced volatiles of rhizobia-colonized and rhizobia-free lima bean plants (Fabaceae: *Phaseolus lunatus* L.) and tested effects of their respective bouquets of volatile organic compounds (VOCs) on a specialist insect herbivore (Mexican bean beetle; Coccinellidae: *Epilachna varivestis* Mulsant) in olfactometer choice trials. In a further experiment, we showed that VOC induction by JA reflects the plant responses to mechanical wounding and insect herbivory. Following induction with JA, rhizobia-colonized plants released significantly higher amounts of the shikimic acid-derived compounds, whereas the emission of compounds produced via the octadecanoid, mevalonate and non-mevalonate pathways was reduced. These changes affected the choice behavior of beetles as the preference of non-induced plants was much more pronounced for plants that were colonized by rhizobia. We showed that indole likely represents the causing agent for the observed repellent effects of jasmonic acid-induced VOCs of rhizobia-colonized lima bean plants. Our study demonstrates a rhizobia-triggered efficacy of induced plant defense via volatiles. Due to these findings, we interpret rhizobia as an integral part of legume defenses against herbivores.

Keywords Above–belowground interactions · Legumes · Nitrogen fixation · *Phaseolus lunatus* · Volatile organic compounds

Introduction

Mutualistic interactions between plants and nitrogen (N_2)-fixing bacteria are widespread in the plant kingdom. More than 15,000 plant species from at least 12 families have the ability to form associations with these bacteria (Corby 1981; Sprent 2001). N_2-fixing bacteria play a key role for global and local N cycles and crucially determine the productivity of natural and agricultural ecosystems (Sprent and Sprent 1990). One of the economically and ecologically most important plant families is the Fabaceae (legumes), which form stable associations with N_2-fixing rhizobia (Brockwell et al. 1995; Wardle 2002).

Recent research indicates that associations between plants and mutualistic soil microbiota not only influence plant growth, but also centrally affect the outcome of interactions between plants and organisms of higher

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trophic levels such as herbivores and pathogens (Van der Putten et al. 2001; Bonte et al. 2010; Pineda et al. 2010). While many ecological studies focus on bottom-up effects of mycorrhizal fungi (see Koricheva et al. 2009 for a review), the ecological effects of root-colonizing N_2-fixing bacteria are comparably little studied (but see Dean et al. 2009; Kempel et al. 2009; Katayama et al. 2010, 2011; Thamer et al. 2011).

The provision of additional N to plants by rhizobia can be expected to profoundly affect plant interactions with herbivores. Plants show plastic responses to N availability by altering their N and carbon (C) metabolism, which can have substantial effects on the C/N ratio of plant tissue (Lou and Baldwin 2004; Scheible et al. 2004). N deficiency, for example, may lead to the accumulation of carbohydrates in leaves, and subsequently can cause negative feedback loops on photosynthesis (Hermans et al. 2006). Variation in plant N content may directly affect herbivore food selection and development (Fischer and Fiedler 2000; Coley et al. 2006) as N-containing amino acids and proteins represent nutritive compounds of major importance for all plant consumers. In addition to primary plant compounds, the concentration and/or compositions of secondary metabolites crucially determines the outcome of plant–herbivore interactions. It has been suggested that the balance between C and N in plant tissue determines the relative investment of plants in C- or N-based defense compounds (carbon/nutrient-balance hypothesis; see Bazzaz et al. 1987; Bryant et al. 1983). During the last decade, this simple view has been criticized as being not logically or philosophically justifiable (e.g., Hamilton et al. 2001; Koricheva 2002). Nevertheless, plant available N has repeatedly been reported to affect the synthesis of constitutive and induced N-based defensive secondary compounds such as alkaloids and cyanogenic glycosides (e.g., Cipollini et al. 2002; Chen et al. 2008; Simon et al. 2010).

While the above-mentioned compounds accumulate in plant cells, plants generally also produce a wide range of volatile organic compounds (VOCs), which comprise mainly fatty acid derivatives, terpenoids, phenyl propanoids, and benzenoids (Winter and Rostás 2010; Ballhorn et al. 2011a). Many of these volatiles are induced by herbivores, that is, they are synthesized de novo in response to feeding damage, are released to the air, and can trigger multiple functions in plant–animal and plant–plant interaction (Fukushima et al. 2002; Baldwin 2010). One of the best-studied effects of VOCs is their function as an indirect plant defense. VOCs provide olfactory cues that attract carnivores, mainly parasitoid wasps, to the damaged VOC-emitting plant (e.g., Rostás and Turlings 2008). The parasitoids lay their eggs into herbivore bodies (in most cases lepidopteran, coleopteran larval stages or aphids), the wasps' larvae feed on inner tissues, finally kill the herbivore and thus reduce herbivore pressure on the plant (e.g., Digilio et al. 2010). Furthermore, VOCs can serve as signals in defense-associated plant–plant signaling by priming or inducing herbivore defenses in neighboring plants of the same and also other species (Heil and Silva Bueno 2007).

In addition to providing indirect defenses and playing a role in plant–plant and within-plant communication, herbivore-induced VOCs can act as a direct defense by repelling herbivores (Heil 2004). Therefore, any influences of belowground symbionts on volatile production may translate into changes of aboveground interactions in many different ways. Evidence for a positive influence of belowground mutualists on the inducibility of defense mechanisms in plants again comes from mycorrhizal fungi (Gange et al. 2003; Pozo and Azcon-Aguilar 2007), which may trigger induced resistance of plants to herbivores (Kempel et al. 2010). Recent studies further indicate that belowground mutualists do not only directly affect herbivores. For instance, Guerrieri et al. (2004) showed that parasitoid wasps were attracted to mycorrhizal tomato plants. Leitner et al. (2010) demonstrated that mycorrhization of *Medicago truncatula* changes volatile production even though this effect was additionally mediated by plant genotype. These findings indicate that root symbionts importantly govern plant secondary chemistry with consequences for trophic interactions and aboveground food webs. In the light of shared signaling pathways used to establish the symbiosis of plants with both mycorrhiza and rhizobia (Marx 2004), similar effects of rhizobia can be hypothesized. However, the importance of rhizobia for induced plant responses have not been investigated until now.

Currently, we are just beginning to understand the importance of rhizobia in plant–herbivore interactions (Ballhorn et al. 2009a). First studies demonstrated that the performance of aboveground herbivores is affected by rhizobia and that these effects can be related to non-volatile defense compound production in leaves (Kempel et al. 2009; Thamer et al. 2011). For a complete picture of the effects in food webs, however, information on the importance for defense-associated volatile compounds are needed and would critically contribute to our knowledge on the role of rhizobia in ecosystems. In the present study, we used rhizobia-free and rhizobia-colonized wildtype lima bean plants (Fabaceae: *Phaseolus lunatus* L.) to study effects of these symbionts on jasmonic acid (JA)-induced VOC emission. In olfactometer experiments with the specialist Mexican bean beetle (Coccinellidae: *Epilachna varivestis* Muls.), we analyzed whether rhizobia-mediated shifts in compositions of JA-induced VOCs affect plant selection by the insects.

Materials and methods

Plants

Lima bean plants (Fabaceae: *Phaseolus lunatus* L.) used in this study were grown from seeds collected in a natural population in southern Mexico (15°55′N, 097°09′W, elevation 15 m). In nature, lima bean forms a close association with N_2-fixing soil bacteria of the family Rhizobiaceae. Screenings of wild lima bean plants at natural sites revealed nodulation of all plants investigated. However, there was substantial quantitative variability in number of nodules (D. J. Ballhorn; unpublished data). The nodules are visible (0.5–5 mm in diameter), ball-like structures formed on the roots containing the rhizobia in a structurally modified form (bacteroids) (Van Brussel 1977). Plants were cultivated in a climatic chamber (Conviron BDW 160-R walk-in CE chamber; Conviron, Winnipeg, Canada) adjusted to resemble conditions at natural sites in Mexico as recorded for September to October 2007. Light in the chamber was provided by a combination (1:1) of HQI-BT 400W (Osram) and RNP-T/LR 400W (Radium) lamps with a light regime of 13:11-h light:dark under a photon flux density of 450–500 μmol photons m^{-2} s^{-1} at table height. The temperature was set at 30 °C during the light period and at 23 °C during the dark period. Relative air humidity was adjusted to 70–80 %. Plants were cultivated in plant containers of 10 × 10 × 11 cm (width, length, height; one plant per pot) in a 1:1 ratio of potting soil (Fox Farms, Arcata, CA) and sand (grain size 0.5–2.0 mm). The substrate was autoclaved at 121 °C for 35 min at a pressure of 1,260 mbar. All plants were fertilized with 50 ml of a 0.1 % aqueous solution of Flory-3 [N–phosphorus (P)–potassium (K) plus magnesium (%); 15, 10, 15 +2-Fertilizer; EUFLOR, Munich, Germany] once a week and watered daily. We applied this low amount of fertilizer to avoid a strongly reduced growth of control plants, which might affect other parameters than tested with potential effects on leaf palatability to herbivores (leaf toughness, leaf tissue hydration). To avoid contamination, control plants were placed in plastic trays (50 × 70 × 8 cm). Position of trays in the climatic chamber was changed every 3 days to exclude any position effects. Experiments and chemical analyses of leaf material were conducted after a cultivation period of 5 weeks.

Rhizobia: cultivation and inoculation of plants

The rhizobial strain used in our study was isolated from lima bean roots derived from natural sites in Mexico as described in Eilmus (2009) and 16S ribosomal ribonucleic acid (rRNA) sequence data had previously been deposited in GenBank (EU842040). In nature, lima bean is associated with *Bradyrhizobium* (Thies et al. 1991; Ormeño-Orillo et al. 2006) but forms associations with a broad range of other bacteria from the genus *Rhizobia*, including *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* (Triplett et al. 1981; Ormeño et al. 2007). In our study, bacteria were cultivated in liquid medium (pH 7.0) containing 1 g yeast extract (AppliChem, Darmstadt, Germany), 10 g mannite (Roth, Karlsruhe, Germany), 800 ml deionized water, and 200 ml soil extract. The soil extract was prepared from 160 g dry, non-fertilized loamy soil (taken from a fallow grass-covered area) that was suspended in 400 ml deionized water under addition of 0.4 g sodium carbonate (Roth) and autoclaved at 121 °C for 30 min at a pressure of 1,260 mbar. Three days prior to plant inoculation, rhizobia were cultivated at 28 °C and 180 r.p.m. on a laboratory shaker (Eppendorf, Westbury, NY). The bacteria solution was then diluted with tap water in a ratio of 1:10 and plants were watered with 100 ml of this solution. Media solutions applied to the control plants contained no bacteria while all other parameters remained unchanged. Establishment of rhizobia (i.e., nodulation; forming of visible root nodules) as well as the rhizobia-free status of controls were evaluated at regular time intervals by carefully removing plant containers from the root system and recording the occurrence of nodules at its periphery.

Phylogenetic characterization of rhizobial strain

To classify the rhizobial strain in greater detail, we downloaded the closest BLAST hits from GenBank as well as for other representatives from the family Rhizobiaceae and generated a molecular phylogeny. An overview of included sequences is given in Table S1. Sequences were aligned using the Ribosomal Database Project (RDP) (Cole et al. 2009). For phylogenetic analyses, we used a Bayesian approach and a maximum likelihood (ML) analysis as described previously (Kautz et al. 2009). The Bayesian–Markov chain Monte Carlo (B/MCMC) analyses were performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). Posterior probabilities were approximated by sampling the trees using a MCMC method. The sequences were tested for the most appropriate model of DNA substitution analyses by the program MrModeltest version 2.3 (Nylander et al. 2004). Using Akaike's information criterion, general time reversible with gamma distributed rate heterogeneity and a proportion of invariant sites (GTR+I+Γ) was determined as the most appropriate ML model of evolution for our dataset. MrBayes estimated the proportion of invariant sites, the gamma distribution shape parameter, base frequencies, and the substitution rates. No molecular clock was assumed. A run with 10,000,000 generations starting with a random tree and employing four simultaneous chains was executed. Every

100th tree was saved into a file. The first 2,500,000 generations (i.e., the first 25,000 trees) were deleted as the "burn-in" of the chain. We plotted the log-likelihood scores of sample points against generation time using TRACER v1.5 (http://tree.bio.ed.ac.uk/software/tracer/) to ensure that stationarity was reached after the burn-in by checking whether the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck and Ronquist 2001). Of the remaining 150,000 trees (75,000 from each of the parallel runs) a majority rule consensus tree with average branch length was calculated using the "sumt" option of MrBayes. Posterior probabilities were obtained for each clade.

The ML analysis was performed with GARLI version 0.951 (Zwickl 2006) using default settings. Bootstrap support was based on 1,000 replications.

Insects

The Mexican bean beetle (Coccinellidae: *E. varivestis* Muls.) is a natural herbivore of wild lima bean plants with adults and larvae feeding on foliage. This beetle is native to southern Mexico and represents a serious pest of cultivated lima beans in some areas of the United States. It commonly causes total defoliation in years of high infestation. The insect has a narrow host range of fabaceous food plants but with distinct preference for lima bean plants at natural sites (D. J. Ballhorn, personal observation). The insects were kept under ambient conditions as outlined for plant cultivation.

JA treatment of plants

The emission of plant VOCs was induced by spraying foliage with JA (1.0 mmol l^{-1}) 90 min after the start of the light period. JA is a natural plant hormone centrally involved in the release of herbivore-induced plant volatiles and is frequently used for experimental induction of these volatiles (e.g., Mithöfer et al. 2005a, b; Heil and Silva Bueno 2007; Ballhorn et al. 2008). The concentration of 1.0 mmol JA l^{-1} we used in this study is consistent with previous studies on the induction of plant volatiles (e.g., Heil 2004; Kost and Heil 2006; Ballhorn et al. 2011a). Plants used for VOC collection and insect choice experiments were treated identically and were randomly selected for the respective experiment. Control plants were treated the same way, but were sprayed with water instead of JA solution. Leaves were sprayed with JA solution until completely moistened and allowed to dry (10 min) before being sprayed a second time. Plants were allowed to dry again, and then were placed into polyethylene terephthalate (PET) bags (Bratenschlauch; Toppits, Minden, Germany, a PET foil that does not emit detectable amounts of volatiles even under exposure to temperatures of up to 150 °C) (Ballhorn et al. 2008). Bagged plants were placed in the growth chamber at unchanged conditions. The temperature in the bags was controlled and did not exceed 32 °C.

Comparison of volatile blends released in response to JA treatment, herbivore damage, and mechanical damage

The application of JA represents an elegant and quantitatively reproducible way to induce plants for the release of volatiles. However, the JA-induced volatile spectra might differ from those released in response to other treatments such as mechanical or herbivore damage. To test for the transferability of data obtained from JA-induced plants to plants induced by more natural means, in a comparative approach we applied JA treatment, mechanical, and herbivore damage to rhizobia-free lima bean plants ($n = 7$ plants per group) and measured the subsequent release of volatiles. Leaves were mechanically damaged by punching holes into the leaf blade with a metal brush (approximately 17 holes cm^{-2}, diameter of individual holes 0.2 mm). Each leaf of each plant was damaged up to a total leaf area damage of about 25 %. Herbivore damage was achieved by placing 20 larvae (second larval stage) of the Mexican bean beetle on the plants (from three to four larvae per leaf). Larvae were removed when plants had been damaged to a comparable extent as in experiments with mechanical wounding (approximately 25 % of total leaf area removed after 6 h).

Gas chromatography–mass spectrometry

Volatiles were collected continuously over 24 h on charcoal filters (1.5 mg charcoal; CLSA-Filters, Le Ruissaeu de Montbrun, France) using air circulation in closed loop stripping as described in Donath and Boland (1995). After 24 h, volatiles were eluted from the carbon filter with dichloromethane (40 µl) containing 1-bromodecane (200 ng μl^{-1}) as internal standard. Samples were analyzed on a gas chromatography trace mass spectrometer (Trace GC Ultra DSQ; Thermo Electron, Austin, TX). The program for separation [RTX5-MS column (Restek, Philadelphia, PA), 15 m × 0.25 mm; 0.25-µm coating] was 40 °C initial temperature (2 min), 10 °C min^{-1} to 200 °C, then 30 °C min^{-1} to 280 °C with helium (constant flow 1.5 ml min^{-1}) as carrier gas. Compounds were identified by comparison to standard substances (Fluka, Seelze, Germany) and with the Nist 05 library (Xcalibur 1.4 software; Thermo Electron). Individual compounds (peak areas) were quantified with respect to the peak area of the internal standard (1-bromodecane). Only compounds for which reference substances were available were included in quantitative analysis. Quantitative data on released VOCs were corrected for gram plant fresh weight.

Olfactometer experiments

Tests on beetles' choice behavior in response to VOCs released from JA-treated lima bean plants with and without rhizobia were conducted in a Y-olfactometer, which was set up in the growth chamber to avoid any reactions of plants or insects due to changed ambient conditions. The glass olfactometer consisted of a stem (length 16 cm) and two arms (length 13 cm, inside angle 60°). Glass tubes had an inner diameter of 4 cm. Inflowing air was cleaned by charcoal filters (1.5 mg of charcoal; CLSA-Filters) and then passed the plants placed in PET foil bags. Plants used for insect choice tests were connected 1 h prior to the experiment to the running olfactometer setup to avoid bias of insect behavior due to accumulation of volatiles in the bags exceeding naturally occurring levels. Constant air flow (ca. 750 ml min^{-1}) was provided by means of an inverted ventilator at the end of the olfactometer. In the olfactometer, individual beetles were given a choice one after the other, and altogether eight replicates were conducted per beetle. Only beetles entering one of the arms within 3 min were counted as having made a decision. Within one experiment, the arms of the olfactometer were exchanged between the rounds.

Each experiment consisted of a choice situation between two different odor sources. In the olfactometer experiments, we tested combinations of four different odor sources [plants induced with JA (JA+), non-induced plants (JA−), plants colonized with rhizobia (R+), rhizobia-free plants (R−)] on insect behavior. Since indole turned out to be the dominant compound released by induced rhizobial plants (see "Results"), we further tested the effect of indole added to induced non-rhizobial plants on beetle behavior. We experimentally enhanced levels of indole within otherwise natural VOC blends rather than testing indole as an individual, isolated compound, because components of the host blend may not be recognized when perceived by plant consumers outside the context of that blend (Bruce and Pickett 2011). Altogether, we applied the following five independent experimental setups: JA+ R+ versus JA+ R− (treatment 1), JA− R+ versus JA− R− (treatment 2), JA+ R+ versus JA− R+ (treatment 3), JA+ R− versus JA− R− (treatment 4), indole+ JA+ R− versus JA+ R− (treatment 5). In addition, we applied two control treatments with similar odor qualities: JA+ R+ versus JA+ R+ (control 1) and JA+ R− versus JA+ R− (control 2).

To prevent bias due to possible unforeseen asymmetries in the experimental set-up, after every ten trials, the odor source was changed and the odorant and control arms were reversed. Before changing the odor source, both volatile chambers and the glass tubes were washed with water and detergent, and then wiped with hexane and acetone. With the exception of choice experiments with artificially added indole ($n = 10$ beetles) $n = 26$ beetles were used in each experiment including the controls.

Olfactometer experiments with indole-enriched volatile blends

Plants used for olfactometer experiments were induced for volatile production by JA treatment as described above. Indole was experimentally added to these natural VOC blends by applying lanolin paste (both Sigma Aldrich), in which indole was dissolved in a concentration of 0.1 $\mu\text{g}\ \mu\text{l}^{-1}$. Lanolin paste (0.25 g paste per plant) was applied on stripes of Bratenschlauch foil which were attached to the plants in order to avoid direct contact of the paste with the plants. The plants were then packed in PET foil bags and used for olfactometer experiments as described above. Amounts of VOCs released from the plants together with indole released from the lanolin paste into the plants' headspace were monitored in a parallel experiment using a closed-loop stripping system as described previously. The concentration of indole in the lanolin paste and the amount of lanolin paste applied to the experimental plants resulted in amounts of volatile indole which quantitatively corresponded to the amount of indole released by rhizobial plants.

Data analyses

All data were checked for the normal distribution of residuals and homogeneity of variances. The effects of JA and rhizobia on the total amount of emitted volatile compounds were assessed by a two-way ANOVA. The effects of these factors on the single compounds were simultaneously tested by a multivariate ANOVA (MANOVA). We further calculated standardized canonical coefficients to assess the contribution of each compound in the context of all other compounds in the model. This analysis was followed by separate (protected) two-way ANOVAs for each compound. The effects of different induction treatments on volatile production were investigated in a one-way ANOVA (factor induction with four levels: no induction, JA, mechanical wounding and herbivory) followed by a Tukey's post hoc test. Calculations were done in SAS 9.2 (procedure ANOVA).

For preferences of beetles in the olfactometer trials, a binary response model was used to test whether the probability of the response is 0.5. For this, we used a logistic intercept-only regression model to test the null hypothesis that the intercept is 0. We considered the intercept as random to account for the probability of an increased variability of the intercept estimate due to a variability of responses across individuals. Calculations were done using the GLIMMIX procedure in SAS 9.2 with ML estimation using adaptive Gaussian quadrature (Lange 1999).

Results

Phylogenetic classification of rhizobial strain

Based on 16S rRNA sequence data (GenBank accession no. EU842040), the bacterial isolate was classified as order Rhizobiales, family Bradyrhizobiaceae, genus *Bradyrhizobium* according to RDP (Cole et al. 2009). To generate a molecular phylogeny of Rhizobiales a total of 48 sequences plus two outgroup taxa were used. A matrix with 1,382 unambiguously aligned nucleotide position characters was produced for analysis. The alignment is available in TreeBASE (http://www.treebase.org/treebase; accession no. 13681).

Our results show the placement of the used strain within the genus *Bradyrhizobium*, and were most closely related to *Bradyrhizobium elkanii* and *Bradyrhizobium pachyrhizi* (Fig. 1). The bacterial strain used for inoculation of lima bean plants formed a single unresolved clade with its closest GenBank relatives due to high sequence similarity. The downloaded sequences had a similarity of 99.0 % in the case of accession FJ192679 and 98.8 % in case of all other close relatives (Fig. 1; Table S1). Most of these close relatives were isolated from root nodules of different Fabaceae including *Glycine max*, *Pachyrhizus erosus* and *Maackia amurensis*.

Effects of induction treatments on production of VOCs

Lima bean plants treated with different methods (JA, mechanical damage and herbivory) for induction of VOCs produced significantly higher amounts of total VOCs than control plants (ANOVA: $F_{3,24} = 32.7$, $P < 0.001$, Tukey's test for differences with all other treatments $P < 0.05$) but yielded no significant differences between the different elicitors JA, mechanical wounding and herbivory (Tukey's-test: $P >> 0.1$; Fig. 2a). The same pattern could be observed for the single compounds (Fig. 2b–l). Only for methyl salicylate (MeSA) (Fig. 2h) and *cis*-jasmone (Fig. 2l) the reduced emission by control plants was not statistically significant. However, there were no indications for specific effects of JA application on VOC emission compared to mechanical wounding or herbivory.

Effects of rhizobia on induction of VOCs

In total, JA-induced plants used for the olfactometer trials produced more VOCs than non-induced plants ($F_{1,44} = 196.0$, $P < 0.001$; Fig. 3). The symbiosis with rhizobia marginally decreased total VOC production across the JA treatments ($F_{1,44} = 3.55$, $P = 0.07$) and alleviated the JA-induced increase (interaction JA × rhizobia: $F_{1,44} = 3.64$, $P = 0.06$). Using a MANOVA, we found highly significant effects of JA, rhizobia and the interaction between both factors. Inspection of the standardized canonical coefficients revealed that *cis*-3-hexenyl butyrate, (*E*)-β-caryophyllene and methyl jasmonate contributed most to the effect of JA, whereas the effects of rhizobia and of the rhizobia × JA interaction were mainly expressed via changes in the emission of (*E*)-β-caryophyllene, indole and *cis*-3-hexenyl isovalerate (Table 1).

Separate ANOVAs revealed that the emission of all VOCs was clearly dependent on the induction by JA whereas non-induced plants showed only minimal emissions (Fig. 3a; Table 1). In contrast, the rhizobial symbiosis inconsistently affected the emission of individual VOCs. Whilst some VOCs were not affected by rhizobia (2-ethylhexan-1-ol, *cis*-β-ocimene, linalool, *cis*-jasmone; Table 1), most VOCs reflected the pattern of total VOCs with a significant [methyl jasmonate, *cis*-3-hexenyl butyrate, (*E*)-β-caryophyllene; Fig. 3b, e, f] or marginally significant (*cis*-3-hexenyl acetate, *cis*-3-hexenyl isovalerate; Fig. 3c, d) negative effect of rhizobia in general but especially on the JA-induced emission of these VOCs (Table 1). In contrast, the emission of indole (Fig. 3g) and MeSA (Fig. 3h) was significantly increased in rhizobia-colonized plants (Table 1). As a consequence, the proportional composition of VOCs changed according to treatment. For instance, whilst in non-rhizobial plants the JA-induced VOCs were dominated by *cis*-3-hexenyl isovalerate, indole was the major VOC in rhizobial plants.

Olfactometer trials

After induction with JA, Mexican bean beetles significantly avoided rhizobial compared to non-rhizobial plants (Fig. 4a). However, no significant preference could be observed for non-induced plants (Fig. 4b). Furthermore, the avoidance of induced plants could be observed for both rhizobial (Fig. 4c) and for non-rhizobial plants (Fig. 4d). Thus, the negative effect of VOCs on beetles was independent from the rhizobial symbiosis, whereas the negative effect of rhizobia on the preference by the beetles was only observed after induction. Control olfactometer assays offering two different JA-induced (JA+) plants with the same colonization status [with rhizobia (R+) or without rhizobia (R−)] indicated that the experimental setup per se did not affect beetle behavior (mean number of decisions per beetle: JA+ R+ vs. JA+ R+ = 4.00 vs. 4.00; JA+ R− vs. JA+ R− = 3.84 vs. 4.16, $P << 0.05$).

In a further olfactometer trial, we added indole to the natural VOC blend of induced non-rhizobial plants (see

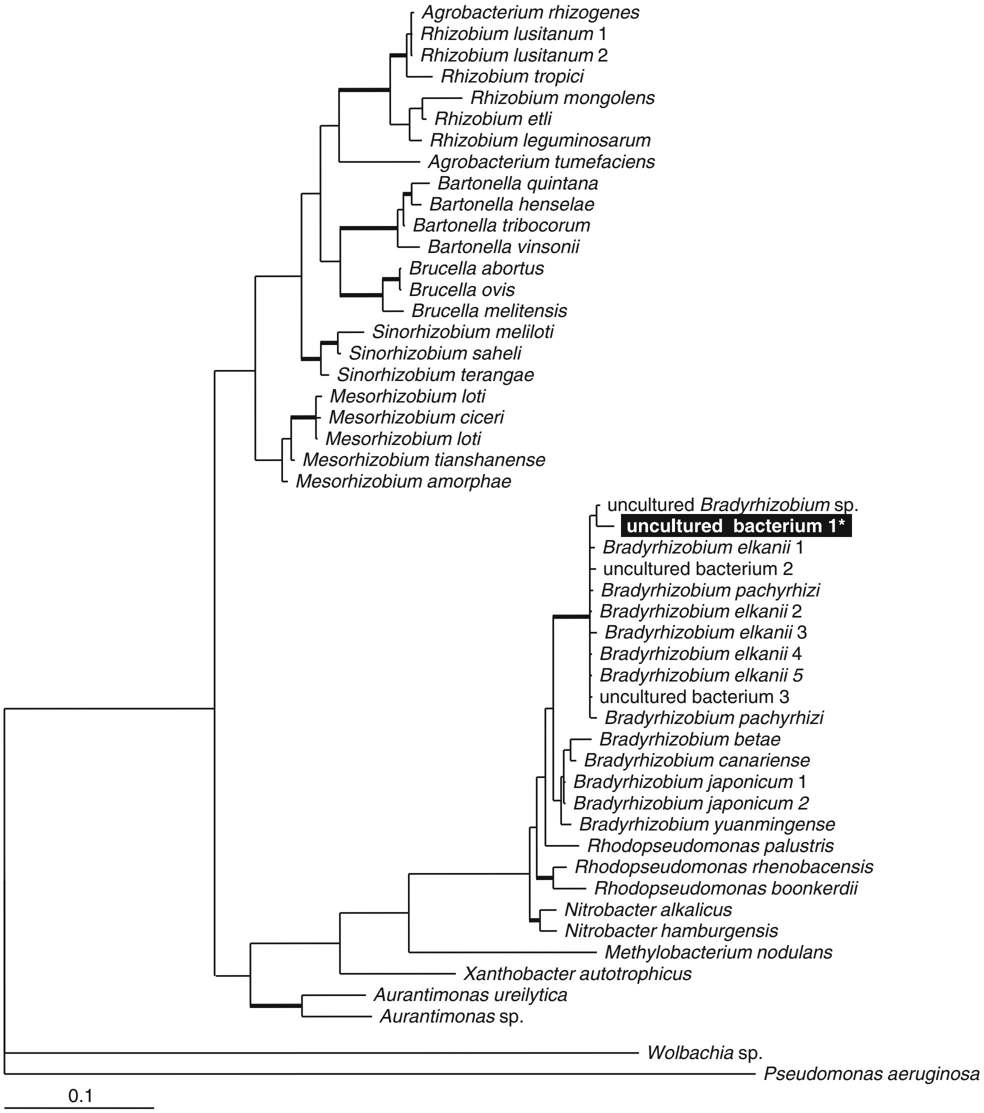

Fig. 1 Phylogenetic placement of the rhizobial strain used for inoculation of lima bean plants as inferred from the 16S ribosomal ribonucleic acid region. Since the topologies of the maximum likelihood (ML) and Bayesian–Markov chain Monte Carlo (B/MCMC) analyses did not show any strongly supported conflicts, only the 50 % majority-rule consensus tree of Bayesian tree sampling is shown. Those nodes that received strong support (i.e., posterior probability ≥0.95 in B/MCMC analysis as well as ML bootstrap ≥70 %) in both the ML and Bayesian were considered significant and are highlighted in *bold*. The bacterial strain used for inoculation of lima bean plants is indicated by *white font* on black background and highlighted by an *asterisk*

"Materials and methods"), to test whether the strong release of indole from induced rhizobial plants could explain the avoidance by beetles. In fact, beetles exposed to VOC blends experimentally manipulated by the addition of indole significantly avoided these VOC blends as compared to controls (Fig. 5).

Discussion

In this study, we demonstrated that rhizobia change the composition of the JA-induced volatile blend of lima bean. As a consequence, a specialist insect herbivore showed a much stronger olfactory preference for non-induced plants when they grew in symbiosis with rhizobia. Thus, the efficacy of this defense mechanism clearly depends on the association with belowground mutualistic biota. We suggest that the mediation of indirect and direct defense mechanisms is a common but largely overlooked function of belowground mutualists for trophic aboveground interactions. We further showed that the production of volatile compounds following treatment of plants with JA did not differ from effects of mechanical wounding and insect herbivory. Thus, JA application avoids some of the

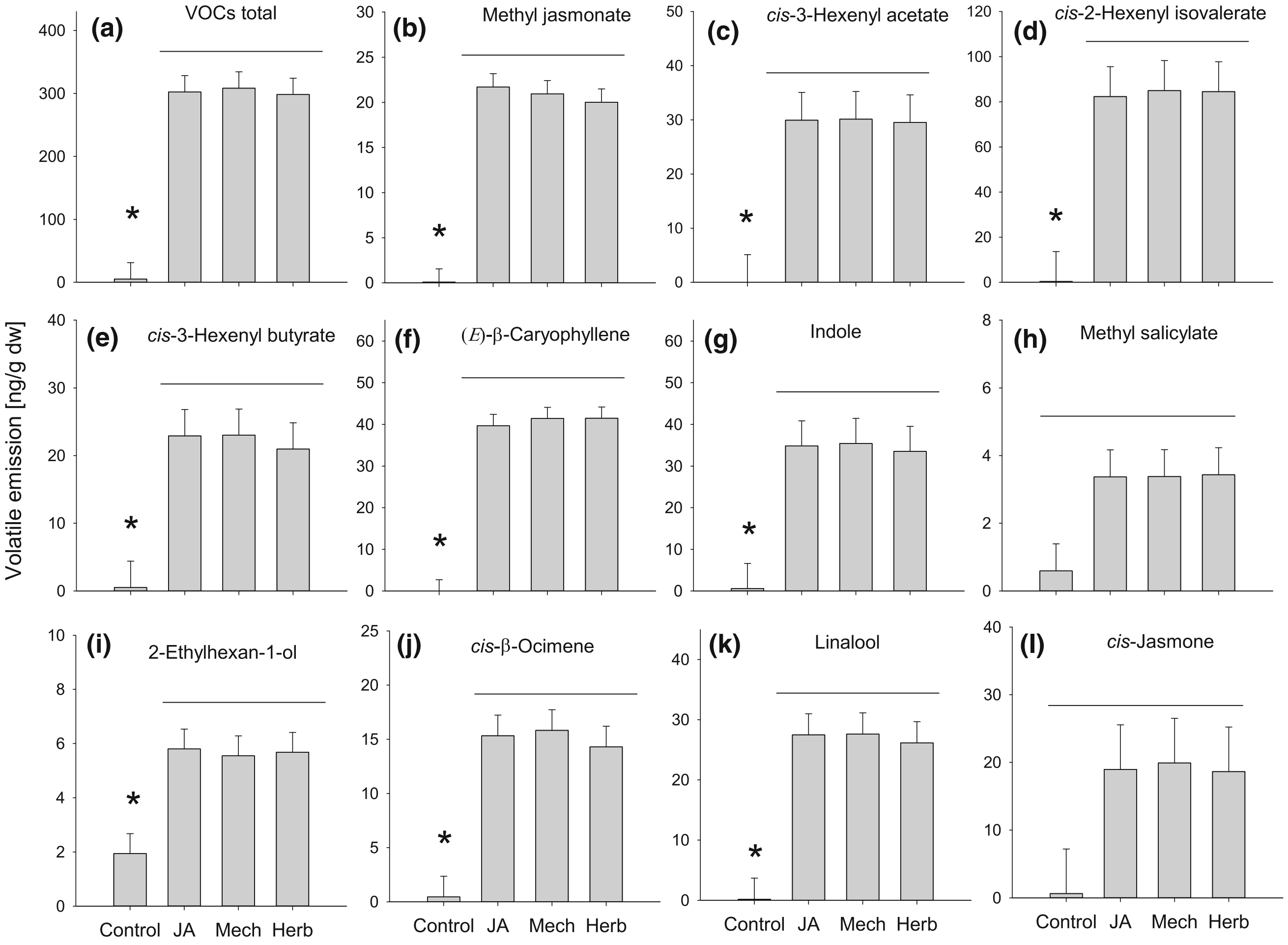

Fig. 2 Volatile emission [volatile organic compounds (*VOCs*); mean ± SE] of lima bean in response to different treatments. *Lines* indicate non-significant differences, *asterisks* indicate significant deviation from all other values (Tukey's test, $P < 0.05$). Sample size is $n = 7$ for each treatment. *Control* Untreated plants, *JA* jasmonic acid-treated, *Mech* mechanical wounding, *Herb* insect herbivory

problems associated with other methods (e.g., differences in herbivore preference and feeding activity, confounding effects of removed plant tissue) and is therefore a suitable tool for the investigation of herbivore-induced plant responses while allowing standardization in our system.

N_2 fixation by legume-associated rhizobia is known to increase the N content of host plant tissue (Sprent and Sprent 1990), which represents a crucial parameter determining food plant quality to insect herbivores (Schädler et al. 2007; Chen et al. 2008). Moreover, these effects may be overridden by influences on the secondary chemistry of plants with consequences for aboveground trophic interactions involving herbivores (Kempel et al. 2009; Katayama et al. 2010). Accordingly, rhizobial colonization can increase the production of defense compounds in legumes like alkaloids (Johnson and Bentley 1991) and cyanogenic compounds (Thamer et al. 2011). The specific outcome of rhizobia on aboveground herbivores may therefore depend on the relative strength of effects on nutritional quality versus production of defense compounds. To complicate things, plant chemotype (Kempel et al. 2009) and rhizobial strain (Dean et al. 2009) further mediate these effects and therefore despite increasing knowledge of the ecology of plant–rhizobia interactions, the complex effects of this mutualistic association from a multitrophic perspective still remain widely cryptic.

Rhizobia-mediated changes of induced VOCs we report here add a further dimension of complexity to the ecological effects of these mutualists. In the present study, we showed that rhizobia colonization of lima bean plants resulted in the alteration of JA-induced volatile blends. Within these VOC blends, individual compounds were differently affected. While the concentrations of indole (the only N-containing volatile compound detected) and MeSA were significantly increased in induced rhizobia-colonized lima bean plants (by more than 100 % compared to rhizobia-free controls), the majority of exclusively C-based volatiles remained unaffected (2-ethylhexan-1-ol,

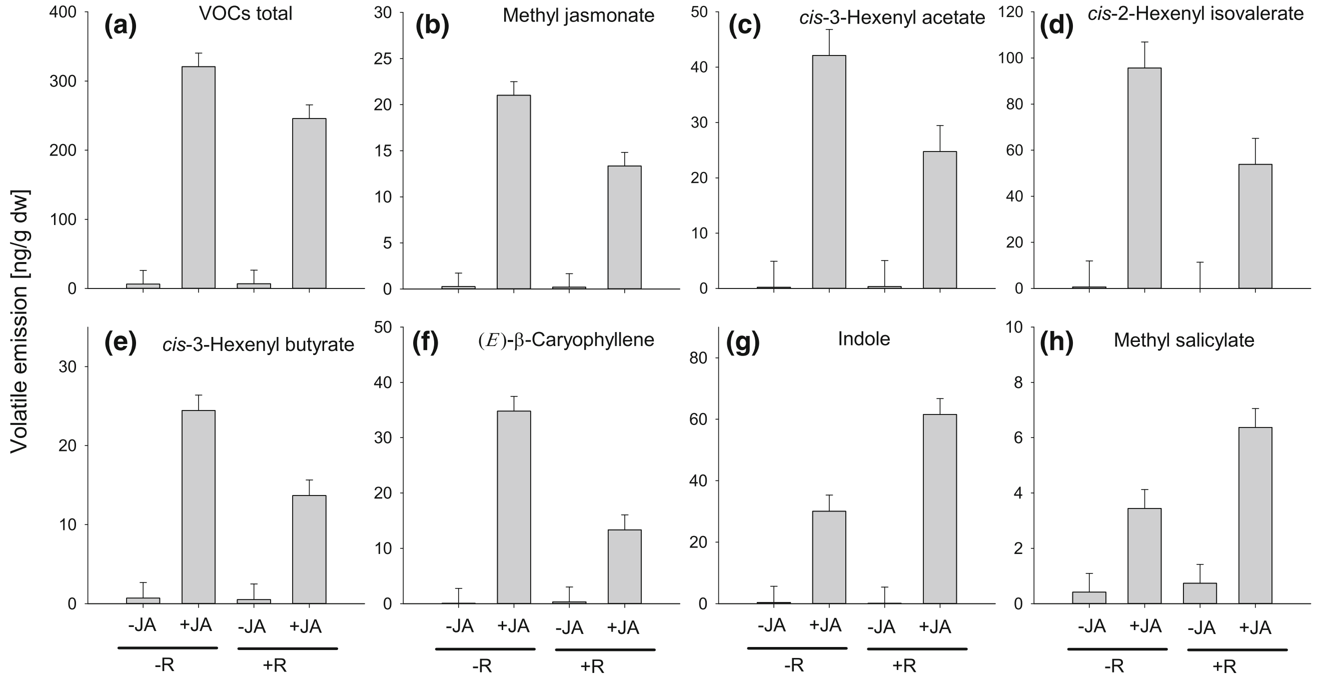

Fig. 3 Volatile emission (mean ± SE) of lima bean in response to rhizobial symbiosis [without (−*R*), with (+*R*)] and induction by JA application [without (−*JA*), with (+*JA*)]. Sample size is $n = 12$ for each mean

Table 1 *F*-values from separate ANOVAs and standardized canonical coefficients (*SCC*) from the multivariate ANOVA on the effects of rhizobia and jasmonic acid on the emission of volatile compounds

	Rhizobia		Jasmonic acid		Interaction	
	F-values	SCC	*F*-values	SCC	*F*-values	SCC
cis-3-Hexenyl acetate	3.37†	0.41	49.74***	0.03	3.47†	0.57
2-Ethylhexan-1-ol	0.01	−0.20	21.90***	−0.01	0.02	−0.25
cis-β-Ocimene	0.03	−0.73	113.12***	0.42	0.31	−0.68
Linalool	0.11	−0.92	85.70***	−0.35	0.26	−0.88
cis-3-Hexenyl butyrate	7.62**	1.02	86.76***	1.12	7.10*	0.81
Methyl salicylate	5.66*	−0.66	39.96***	−0.14	3.64†	−0.56
cis-3-Hexenyl isovalerate	3.52†	1.36	43.50***	0.22	3.35†	1.31
Indole	8.84**	−1.47	75.47***	0.37	9.18**	−1.54
cis-Jasmone	0.85	0.70	15.20***	0.75	0.87	0.66
(*E*)-β-Caryophyllene	15.51***	2.32	78.70***	0.91	16.28***	2.42
Methyl jasmonate	6.86*	0.30	132.30***	0.92	6.67*	0.29

df for the *F*-values are 1, 44

† $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

cis-β-ocimene, linalool, *cis*-jasmone) or were even decreased by 59 % (*cis*-3-hexenyl acetate), 56 % (*cis*-3-hexenyl butyrate and *cis*-3-hexenyl isovalerate), and 38 % [(*E*)-β-caryophyllene]. As a consequence, the total volatile emission after induction was reduced for rhizobial plants and the volatile blend was clearly dominated by indole.

The increase of indole emission points to an important role of symbiotically fixed N for volatile production. Effects of plant nutrient availability on the induced release of VOCs have been demonstrated for a range of different plant species (Gouinguené and Turlings 2002). Maize seedlings, for example, show a lowered emission of induced plant VOCs when N–P–K fertilization is reduced (Gouinguené and Turlings 2002) and manipulating N availability alone enhanced levels of induced VOCs in maize (Schmelz et al. 2003) and cotton (Chen et al. 2008). Due to the rhizobial symbiosis, legumes generally are less dependent on soil N. Nevertheless, N deficiency may occur in patches where appropriate symbionts are lacking or whenever plants and bacteria fail to establish extensive root nodulation (e.g., Zilli et al. 2009) contributing to spatial variability in N supply to legumes. Such variability in plant–rhizobia interaction and resulting VOC emission may explain our observation that lima bean plants in nature

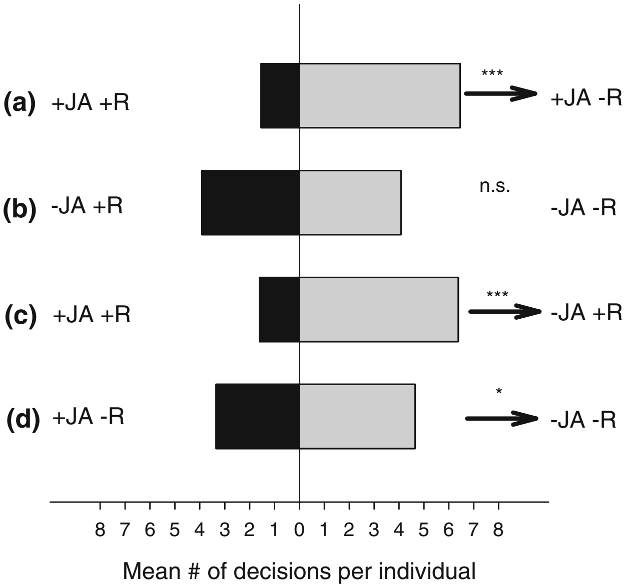

Fig. 4 Mean number of decisions per individual of *Epilachna varivestis* in the olfactometer experiments for plants −R or +R and/or −JA or +JA application. *Arrows* indicate significant preferences. For each comparison 26 beetle individuals and eight decisions per individual were used. *n.s.* (Not significant) $P > 0.05$, $^{*}P < 0.05$, $^{***}P < 0.001$. For other, abbreviations, see Fig. 3

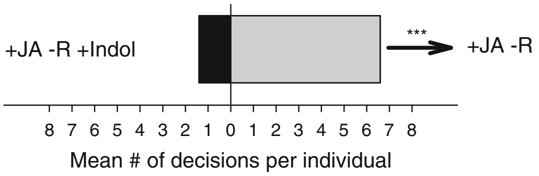

Fig. 5 Effect of indole application to JA-induced non-rhizobial lima bean plants on the mean number of decisions per individual of *E. varivestis* in the olfactometer experiment. The *arrow* indicates a significant preference for plant without additional indole. Sample size is ten beetles with eight decisions per individual. $^{***}P < 0.001$. For abbreviations, see Fig. 3

commonly have varying degrees of nodulation and also vary in VOC profiles (D. J. Ballhorn, personal observation). Distinct effects of N availability on induced VOC emission in soybean (*G. max*) suggest that N is a key factor determining this trait (Winter and Rostás 2010). Thus, also for lima bean it can be speculated that differences in the N supply by symbiotic bacteria contribute to the observed variation in VOC blends.

With focus on individual compounds, it is remarkable that particularly the release of N-containing indole is enhanced by high N availability. Furthermore, the fact that several exclusively C-based VOCs were significantly reduced in rhizobia-colonized plants may also suggest resource allocation competition between rhizobia and the plants' synthetic machinery as up to 25 % of net photosynthates may be required for N_2 fixation by rhizobia (Oono and Denison 2010 and references therein). It may further be argued that the C/nutrient balance within plants may not only have implications for direct plant defenses, but also for the composition of volatile blends. However, in addition to indole, also non-N-containing MeSA was significantly increased (by 118 %) in induced rhizobia-colonized plants. Indole and MeSA are the only compounds we have analyzed that are synthesized via the shikimate pathway (Paré and Tumlinson 1997). It is noteworthy that the increase in indole is in line with the statement by Lerdau and Coley (2002) that products of the shikimate pathway follow the predictions of the C/nutrient-balance hypothesis. In contrast, the increased production of the purely C-based MeSA contradicts these predictions. Thus, even within specific pathways changes in metabolite production cannot be predicted from changes in the supply of C or N alone. However, our findings suggest that rhizobia colonization may enhance specific biosynthetic pathways while others such as the octadecanoid pathway that is involved in the production of *cis*-jasmone, methyl jasmonate, *cis*-3-hexenyl acetate, 2-ethylhexan-1-ol, *cis*-3-hexenyl butyrate, *cis*-3-hexenyl isovalerate (von Dahl and Baldwin 2004; Matsui 2006; D'Auria et al. 2007; Frost et al. 2007, 2008), the methyl-D-erythriol 4-phosphate pathway that leads to the monoterpenes *cis*-β-ocimene and linalool (Dudareva et al. 2005; Hampel et al. 2005; Mithöfer et al. 2005a, b), or the mevalonate pathway producing sesquiterpenes such as (*E*)-β-caryophyllene (Paetzold et al. 2010; Walters 2011) were little or negatively affected.

To our knowledge there are no other studies on the effects of rhizobia on plant VOCs. However, Leitner et al. (2008) observed induction of volatiles, mainly sesquiterpenes, in response to specific nodulation factors (oligosaccharides) in bioassays with *M. truncatula*. As at least one of the oligosaccharidic elicitors tested was able also to induce nodulation, an involvement of rhizobial Nod factors in triggering the release of plant volatiles seems possible. While information on the effects of living rhizobia on plant volatiles is still lacking, for mycorrhizal fungi, which represent another major group of microbial plant mutualists, Leitner et al. (2010) found slight variations of induced VOCs between mycorrhizal *M. truncatula* plants and non-inoculated controls. Altogether, for *M. truncatula* the effect of plant cultivar on VOC emission was stronger than effects of mycorrhiza. In lima bean, however, the observed rhizobia-mediated effects on the emission of prominent VOCs were distinct and likely independent from plant genotype as they were constantly observed among plants that were grown from seeds collected from different mother plants in a natural lima bean population in southern Mexico (Ballhorn et al. 2009b). This is further supported by one of our previous studies, in which plants grown from the same batch of seeds showed substantial variability of chemical traits (cyanogenesis, total phenolics). Such variability of traits could be largely eliminated by producing clones indicating genetic variability as a major source of variation (Ballhorn et al. 2011b).

The findings on variation of induced plant VOCs depending on colonization with rhizobia that we report here potentially have far reaching implications for multiple ecological interactions as many of the compounds affected are centrally involved in plant–insect, plant–pathogen, and also plant–plant and within-plant communication. Some of the compounds significantly down-regulated in rhizobia-colonized plants, such as methyl jasmonate and *cis*-jasmone, are key hormones for induced plant defenses against herbivores and priming of plant defenses (Rodriguez-Saona and Thaler 2005; Karban 2011). Thus, rhizobia colonization may quantitatively impact the outcome of plant–herbivore interactions. On the other hand, MeSA—one of the two up-regulated compounds—is involved in activating disease resistance of plants against pathogens. MeSA triggers the expression of defense-related genes in healthy tissue of infected plants as well as in neighboring plants (Shulaev et al. 1997; Ozawa et al. 2000), and like jasmonates has priming effects on plant defenses (Turlings and Ton 2006). Therefore, the enhanced emission of MeSA in rhizobia-colonized plants could benefit the plant through protecting it at herbivory-derived wounds against infectious pathogens (Brown et al. 1995; Shiojiri et al. 2006).

In contrast to jasmonates and MeSA, the role of indole is much less understood. Indole, a product of the shikimate pathway, is formed from indole-3-glycerol-P either as an intermediate in tryptophan (Trp) biosynthesis or by a Trp-independent pathway leading to a family of N-containing defense compounds (e.g., 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; Frey et al. 1997). In maize seedlings, indole is the major shikimic acid-derived VOC released in response to attack of *Spodoptera* moths (Turlings et al. 1998). It has been shown to attract females of different parasitoid species, *Cotesia marginiventris* (Hymenoptera: Braconidae), *Cotesia kariyai* (Hymenoptera: Braconidae) and *Microplitis rufiventris* (Hymenoptera: Braconidae) (D'Alessandro et al. 2006, 2009). However, other studies also have recorded repellent effects of indole to carnivores. For example, naive *M. rufiventris* parasitoids preferred VOC blends of maize lacking the induced compound indole (D'Alessandro et al. 2006).

In contrast to effects on parasitoid and predatory arthropods, for which variation in attraction of carnivorous arthropods could partly be attributed to the presence and relative abundance of specific compounds within the VOC blend (D'Alessandro et al. 2009), the effects of inducible plant volatiles on arthropod herbivores have been studied to a much lesser extent (Bruce and Pickett 2011). Here, we could show for the first time that the rhizobia-mediated variation of induced lima bean VOCs can constitute a signal with altered information that modifies the host-finding behavior of a specialist insect herbivore, the Mexican been beetle (*E. varivestis*). Whether relative changes in volatile compositions or the up-regulation of individual compounds such as indole caused the observed effects requires further research. However, our olfactometer experiments with artificially enhanced indole levels in otherwise natural VOC blends strongly suggest a key function of this compound in herbivore deterrence. In contrast to the existing data on the effects of indole on parasitoids, until now there is no information available on repellent effects of this compound on insect herbivores. This present study is the first in which a direct defensive function of indole could be demonstrated. Remarkably, this function is strongly dependent on the symbiosis with rhizobia.

In this study, the release of VOCs was induced by experimental application of JA at a concentration of 1.0 mmol l^{-1}. This concentration represents the standard JA treatment applied in many studies on the induction of VOCs and extrafloral nectar of lima bean (Heil 2004; Kost and Heil 2006). However, the questions arises whether the effects observed in response to an externally applied plant hormone correspond to natural situations such as plant damage, for example by an herbivore. Our control experiments with mechanically damaged and herbivore damaged rhizobia-free lima bean plants showed no significant differences of VOC release in response to these treatments and induction with JA indicating a transferability of our data to the situation in nature.

Altogether, we demonstrated that rhizobia colonization has a substantial impact on the emission of inducible plant VOCs and host plant selection by an insect herbivore. Moreover, for plants grown in symbiosis with rhizobia we could show a clearly more efficient function of volatiles as repellent against the herbivore. As these volatile compounds have multiple effects in interaction with various trophic levels, our study indicates a complexity of ecological functions of plant–microbe interactions, which is beyond our current understanding of these ubiquitous associations. More studies are needed to unravel the physiological and genetic basis of these effects. In particular, other plant species and the effects of multiple N_2-fixing bacteria should be analyzed to obtain more general insights into the functional ecology of these bacteria–plant interactions and the bottom-up effects on higher trophic levels. With focus on the rhizobia-lima system, different strains should be tested as the effects we observed for the specific strain used in the present study might not be identical with other strains as rhizobia frequently show distinct variability in N_2-fixing efficiency, that is the quality as a mutualistic partner (Oono and Denison 2010).

Nevertheless, our study shows that belowground symbionts may be regarded as important players in the evolution of plant defense with implications for concepts of the organization of ecological communities.

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