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Symptomless Endophytic Fungi Suppress Endogenous Levels of Salicylic Acid and Interact With the Jasmonate-Dependent Indirect Defense Traits of Their Host, Lima Bean (*Phaseolus lunatus*)

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Abstract Symptomless 'type II' fungal endophytes colonize their plant host horizontally and exert diverse effects on its resistance phenotype. Here, we used wild Lima bean (*Phaseolus lunatus*) plants that were experimentally colonized with one of three strains of natural endophytes (*Bartalinia pondoensis*, *Fusarium sp.,* or *Cochliobolus lunatus*) to investigate the effects of fungal colonization on the endogenous levels of salicylic acid (SA) and jasmonic acid (JA) and on two JA-dependent indirect defense traits. Colonization with *Fusarium sp.* enhanced JA levels in intact leaves, whereas *B. pondoensis* suppressed the induction of endogenous JA in mechanically damaged leaves. Endogenous SA levels in intact leaves were significantly decreased by all strains and *B. pondoensis* and *Fusarium sp.* decreased SA levels after mechanical damage. Colonization with *Fusarium sp.* or *C. lunatus* enhanced the number of detectable volatile organic compounds (VOCs) emitted from intact leaves, and all three strains enhanced the relative amount of several VOCs emitted from intact leaves as well as the number of detectable VOCs emitted from slightly damaged leaves. All three strains completely suppressed the induced secretion of extrafloral nectar (EFN) after the exogenous application of JA. Symptomless endophytes interact in complex and strain-specific ways with the endogenous levels of SA and JA and with the defense traits that are controlled by these hormones. These interactions can occur both upstream and downstream of the defense hormones.

Keywords Endophyte · Extrafloral nectar · Jasmonic acid · Plant-fungus interaction · Salicylic acid · Volatile organic compounds

Introduction

Symptomless endophytic fungi colonize their host plants without causing any symptoms of disease under the current conditions (Arnold et al. 2003; Saikkonen et al. 2004; Yuan et al. 2010). Whereas 'type I' endophytes in the family Clavicipitaceae systemically colonize grasses and are vertically transmitted, the so-called 'non-clavicipitaceous' or 'type II' endophytes (Yuan et al. 2010) have been found in seemingly all plant species that have been investigated for them so far, are transmitted horizontally, and are usually present in more than 80 % of the investigated samples (Arnold et al. 2000; Albrectsen et al. 2010; Gazis and Chaverri 2010; Partida-Martinez and Heil 2011; Yuan et al. 2010). These non-systemic endophytes colonize their hosts at lower densities than type I endophytes, but they can be extremely diverse: a hundred morphologically distinct groups comprising 33 taxa of endophytic fungi were found in leaves of European aspen (*Populus tremula*) (Albrectsen et al. 2010), 58 operational taxonomic units were detected in leaves of wild rubber trees (*Hevea brasiliensis*) in Peru (Gazis and Chaverri 2010), more than 100 morphospecies were found in a tropical palm (Fröhlich et al. 2000), and 418 fungal morphospecies were isolated from 83 leaves from different tropical tree species (Arnold et al. 2000).

Type I endophytes originally were discovered due to their negative effect on livestock: colonized tall fescue (*Festuca arundinacea*) causes severe livestock disorders, which are caused by alkaloids that are produced by the endophytic

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fungus (Clay 1990). Still, the role of type I endophytes as defensive mutualists of wild grasses has been questioned (Faeth 2002). The evidence for an enhanced anti-herbivore resistance in plants that are colonized by type II endophytes is even less convincing (Hartley and Gange 2009). For example, no direct relation between the frequency of fungi and the performance of herbivores was found in a study using mountain birch (*Betula bubescens*) (Ahlholm et al. 2002). Similarly, beetle larvae (*Chelymorpha alternans*) feeding on leaves of the tropical vine, *Merremia umbellata*, that were experimentally colonized with the endophyte, *Glomerella cingulata*, showed no differences in feeding behavior or larval growth (Van Bael et al. 2009). *Helicoverpa armigera* moths oviposited even more on leaves of tomato (*Lycopersicon esculentum*) plants that were inoculated with *Acremonium strictum,* as compared to endophyte-free plants (Jallow et al. 2008). By contrast, the larvae of *H. armigera* suffered from decreased growth when feeding on broad bean (*Vicia faba*) plants that were colonized with *A. strictum*, and they avoided these plants in choice assays (Jaber and Vidal 2010). Moreover, adults of *C. alternans* produced 80 % more offspring when they had fed as larvae on *M. umbellata* plants with low endophyte loads (Van Bael et al. 2009).

These seemingly contradictory observations might, in part, result from the high diversity of type II endophytes. Moreover, endophytes can interact with the resistance phenotype of their host via multiple mechanisms, which range from direct effects due to their role as entomopathogens (Ownley et al. 2010), or the production of toxic metabolites (Saikkonen et al. 2013) to indirect effects that are mediated via the manipulation of resistance-related hormones such as jasmonic acid (JA) and salicylic acid (SA) (Partida-Martinez and Heil 2011). In broad bean, the inoculation with endophytes enhanced the secretion of extrafloral nectar (EFN) (Jaber and Vidal 2009), which is an important indirect defense trait (Heil 2008). Likewise, the colonization with type II endophytes enhanced the emission of terpenoids from peppermint (*Mentha piperita*) (Mucciarelli et al. 2007). Both EFN and VOCs depend on octadecanoid signaling (Heil 2008), which indicates that the fungi interact with plant signaling cascades. Indeed, the exogenous application of SA or methyl jasmonate changed the abundance of several endophytic strains in oak, *Quercus serrata* (Kusumoto and Matsumura 2012), and JA played a role in the enhanced production of volatile essential oils in *Atractylodes lancea* plantlets that were colonized with the fungal endophyte, *Gilmaniella* sp. (Ren and Dai 2012).

In the present study, we investigated whether the colonization of Lima bean (*Phaseolus lunatus*) with selected strains of natural endophytic fungi alters the endogenous levels of the hormones, JA and SA, and the expression of the JA-dependent indirect defense traits, EFN secretion and VOC emission. Both traits play important roles in the defense of wild Lima bean against natural herbivores (Heil 2004; Heil and Silva Bueno 2007; Kost and Heil 2006, 2008). We isolated cultivable fungi from surface-sterilized leaves of wild plants, re-inoculated plants under sterile conditions with one of three selected strains, and followed the endogenous levels of SA and JA, the secretion of EFN, and the emission of VOCs. Our results show that the colonization of Lima bean with endophytes affects the endogenous levels of both hormones, and that it can inhibit the induction of EFN secretion, whereas VOC emission was positively affected. Moreover, the responses were strain-specific, which explains the lack of general patterns in the results of studies that use different combinations of endophytes and host plants.

Methods and Materials

Plants and Experimental Setup Seeds were collected from wild Lima bean (*Phaseolus lunatus*) plants in a natural population in the coastal area of southern Mexico (Pacific coast, 15°55′31.80”N and 97°094.68”′W) and were soaked in 70 % ethanol and in 10 % sodium hypochlorite for 5 min each to sterilize their surfaces. Then, plants were grown from these seeds in plastic pots (1.3 L) under greenhouse conditions. No endophytes can be cultivated from surface-sterilized seeds or plants grown from these seeds (data not shown). When plants had at least four secondary leaves, they were randomly assigned to three groups: intact, mechanical damage (MD), and application of exogenous JA.

The endophytes used (strains C015, U090, and U065) were isolated from healthy leaves of Lima bean plants growing in their natural environment (México, Pacific coast, 15°55′ 31.80”N and 97°094.68”′W) in July 2012. Leaves were rinsed under sterile water to remove spores from the surface, immersed in 70 % ethanol for 8 min, then in 10 % sodium hypochlorite for 5 min, and finally rinsed four times with sterile water. Pieces of leaves were placed in Petri dishes with potato dextrose agar (PDA) medium (Difco) supplemented with 10 U ml^{-1} penicillin G and 10 μg ml^{-1} streptomycin (Sigma, USA) to suppress the growth of bacteria. The emerging fungal colonies were repeatedly transferred to Petri dishes with PDA medium until we obtained axenic cultures. The fungi were preliminarily identified by the amplification of their internal transcribed spacer (ITS) region with primers ITS1F-KYO2 (5′TAGAGGAAGTAAAAGTCGTAA 3′) and ITS4R (5′ TCCTCCGCTTATTGATATGC 3′) (Gardes and Bruns 1993; Toju et al. 2012) and Sanger sequencing at LANGEBIO (CINVESTAV-Irapuato; www.langebio.cinvestav.mx) on an ABI 3,730-xl DNA Analyzer (Applied Biosystems; www.appliedbiosystems.com) as *Bartalinia pondoensis* (C015), *Fusarium sp.* (U090), and *Cochliobolus lunatus* (U065)(see supplementary Table 1 for sequences and annotation results). These fungi did not cause any symptoms

of disease in lima bean under our experimental conditions up to 22d post inoculation.

Inoculation Spore suspensions for inoculation were prepared by transferring an axenic mycelium to a Petri dish with PDA medium and incubating it at 28 °C in the dark, for 2 wk. The spores produced by the mycelium were suspended in distilled water with 0.1 % Tween (Sigma, USA), and their concentration was adjusted to 1×10^6 spores ml^{-1} by counting spores in aliquots in a Neubauer hemocytometer. Inoculation was performed by spraying 2 ml of the spore suspension on the four youngest secondary leaves of each plant. Water with 0.1 % Tween at equal amounts was sprayed on control plants. In order to quantify the level of colonization, leaves from all experimental plants were collected, surface-sterilized as described above, and 42 circles of 6 mm per plant were plated on PDA media and maintained for 7d. Colonization rates were expressed as the percentage of leaf discs from which fungi could be recovered.

Mechanical Damage and JA Application Preliminary experiments indicated that colonization rates are enhanced when the spore suspensions are inoculated on slightly damaged leaves (data not shown). Thus, the leaves of 30 % of the plants were damaged with a hole puncher (without perforating the leaf blade) immediately before inoculation. A second group of the plants was sprayed twice with 2 ml of an aqueous solution of 1 mM JA and allowed to dry, to apply the spore suspension (or, in the case of the last group of plants that remained intact as controls: water with 0.1 % Tween) 2 hr later.

VOC Collection and Analysis VOCs of 4-wk-old Lima bean plants were collected using a Stable Flex Solid Phase Micro-Extraction (SPME) collection system as described earlier (Ángeles-López et al. 2013). The VOCs were collected over the 22 hr after the inoculation with the endophytic fungus. Plants were enclosed in plastic bags (Toppits®, Minden, Germany) followed by the insertion of the fiber (50/30 μm, DVB/CAR/PDSM; Supelco, Bellefonte, PA, USA). Fibers were exposed for a period of 22 hr, and then desorbed for 60 sec directly into the gas chromatograph (GC) injector (180 °C). The VOCs were analyzed by GC-mass spectrometry (GC-MS) using an Agilent Technologies Gas Chromatograph 7890A with an HP5-MS column (30 min, 0.25 mm ID, 0.25 μm film thickness; Agilent Technologies) coupled to a MSD 5,973 detector. Following injection, the column temperature was maintained at 60 °C for 3 min, increased at 5 °C min^{-1} to 80 °C, and then at 80 °C min^{-1} to 210 °C, to be then held constant at 210 °C for 5 min. Mass spectra were compared with those of the NIST 2.0 library, and retention times and spectra were compared with authentic standards for the following compounds: β-ocimene, β-*trans*-ocimene, β-linalool, pinocarvone, methyl salicylate, β-caryophyllene, α-caryophyllene (Fluka Chemie, Steinheim, Germany; Sigma-Aldrich, St Louis, MO, USA), 4,8-dimethylnona-1,3,7-triene (DMNT) and 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) (kindly provided by W. Boland, MPI for Chemical Ecology, Jena, Germany). We estimated the relative quantities of individual VOCs by relating their peak area to the overall peak area of the respective chromatogram and controlled for putative differences among the fibers by using each individual fiber for each one VOC collection per treatment.

Quantification of EFN Wild Lima bean usually does not produce measurable amounts of EFN in the greenhouse (data not shown), for which reason the plants for the experiment on EFN secretion were grown in pots in a small greenhouse in the coastal area (see above) and then exposed to the natural environment, i.e., close to the naturally growing plants. Prior to inoculation, plants were sprayed with water to remove all accumulated EFN, grouped and treated as mentioned above. Plants were inoculated with one of the fungi and then protected with nets from flying nectar consumers and with Tangle Trap® from ants, over 24 hr. Twenty-four hours after the inoculation with the fungi, EFN was quantified as amounts of soluble solids by using microcapillaries to quantify the volume and a Atago® hand refractometer to quantify the concentration of soluble solids (Kost and Heil 2006).

Quantification of JA and SA Plants were cultivated for 4 wk and then subjected to the before-mentioned treatments (intact, mechanically damaged, and JA-application, and consecutive inoculation with one of the fungal strains). Leaf material was collected before and 24 hr after inoculation. Jasmonic acid was extracted with ethyl acetate (Pluskota et al. 2007), adding [9,10-H2] dihydrojasmonic acid as an internal standard, and derivatized by adding 10 μl pentafluorobenzyl bromide (Mueller and Brodschelm 1994). Salicylic acid was extracted with methanol, adding *ortho*-methoxybenzoic acid as an internal standard and derivatized with 80 μl N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) and 20 μl pyridine (Malamy et al. 1992). One micro-liter of each sample was injected in the splitless mode and analyzed by gas chromatography-single ion-mass spectrometry in an Agilent Technologies Gas Chromatograph 7890A using a DB-1MS column (60 m×0.25 mm×0.5 μm Agilent Technologies) coupled to a MSD 5,973 detector in SIM mode for 141, 181, 390, and 392 *m/z*, and 73,135, 267, and 282 for JA and SA, respectively. The GC-MS conditions were as described previously (Ramírez-Chávez et al. 2004).

Statistical Analyses The data fulfilled the requirements for parametric tests and, thus, were analyzed using ANOVA followed by least significant difference (LSD) *post hoc* tests. Different letters are used to indicate means that differ significantly ($P<0.05$). Analyses were performed using the program SPSS® (SPSS Inc., Chicago).

Results

Screening for the inoculated endophytes of leaf discs obtained from experimental plants revealed that control plants had been kept successfully free of these fungi (Table 1). Colonization rates in inoculated plants were highly variable, and depended on the experimental conditions as well as on the identity of the fungus. Highest colonization rates were obtained with *Fusarium sp.* and ranged from to 6 to almost 40 %, whereas lowest rates were obtained with *B. pondoensis* and ranged from 3 to 18 %. In general, the highest colonization levels were reached in the experiment on EFN secretion, i.e., under semi-field conditions. In the greenhouse (experiments on VOCs and plant hormones), mechanically damaged plants were colonized significantly more than intact plants, whereas JA-treatment had weak and inconsistent effects, but more frequently decreased rather than increased colonization rates (Table 1).

Inoculation of intact plants with *Fusarium sp.* or *Cochliobolus lunatus* significantly enhanced the number of detectable VOCs emitted from these plants from seven to, on average, 18 (*Fusarium sp.*) and 23 (*C. lunatus*) different compounds (Fig. 1a). In plants that were slightly damaged to enhance the degree of colonization (see Table 1), the number of detectable VOCs increased from an average of 10 compounds emitted from mock-inoculated plants to 19–24 when the plants were colonized with an endophyte. All three strains had a significant effect in this case (Fig. 1a). When considering relative amounts of VOCs rather than the number of detectable compounds, all three fungi significantly decreased the emission of (*Z*)-3-hexen-1-yl acetate from intact plants, whereas they enhanced the emission of DMNT (Table 2). *Fusarium sp.* or *C. lunatus* further induced the *de-novo* emission of several other VOCs from intact plants (Table 2). The release of DMNT also was significantly enhanced by all three fungi when we considered mechanically damaged plants, and all three strains elicited the *de novo* appearance of several mono- and diterpenes in the VOC pattern of mechanically damaged plants (i.e., these compounds were not detected as VOCs of endophyte-free damaged plants, see Table 2). At the level of individual compounds, most (34 out of 45) VOCs

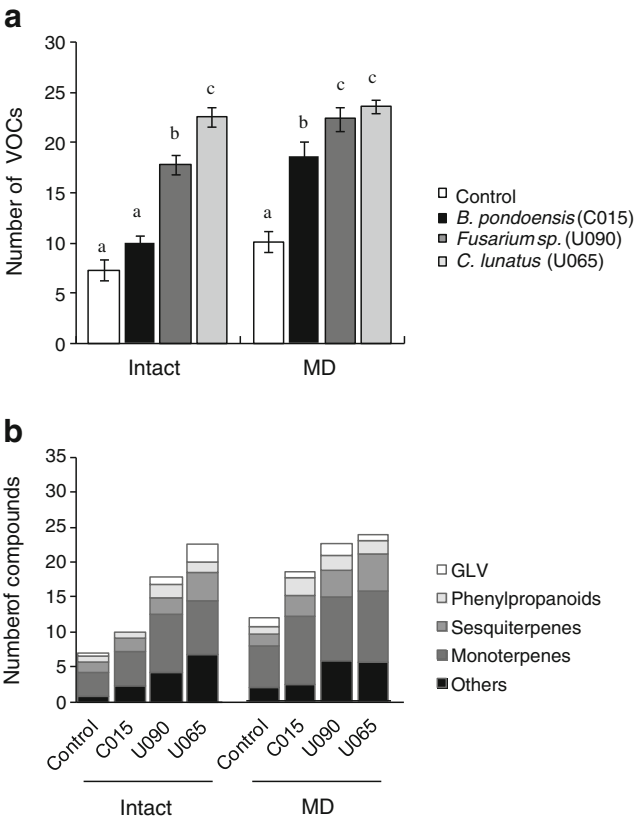

Fig. 1 Effects of the colonization by endophytes on volatile organic compound (VOC) emission. The VOCs emitted over the first 22 hr after inoculation are depicted separately for control plants without endophytes (*white bars*) and plants colonized with *Bartalinia pondoensis* (*black*), *Fusarium sp.* (*dark gray*), and *Cochliobolus lunatus* (*light gray*), and separately for intact plants or mechanically damaged (MD) plants. Panel **a** indicates the number of detectable compounds, panel **b** the effects of the colonization with endophytes on major classes of VOCs: green leaf volatiles (GLV), phenylpropanoids, sesquiterpenes, and monoterpenes

Table 1 Percentage of colonization in lima bean leaves by endophytic fungi (EF) in the different experiments

	VOCs		Extrafloral Nectar			Phytohormones		
	Intact	MD	Intact	MD	JA	Intact	MD	JA
Control	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
B. pondoensis	5.7±0.6^{a}	18.4±3.0^{b}	4.7±2.0^{a}	2.9±1.8^{a}	3.3±2.1^{a}	4.3±0.9^{ab}	7.1±1.1^{a}	2.9±1.2^{b}
Fusarium sp.	6.3±0.9^{a}	20.0±3.0^{b}	38.6±4.2^{b}	33.3±3.4^{b}	33.3±4.2^{b}	8.1±1.2^{a}	14.3±2.1^{b}	7.6±1.4^{a}
C. lunatus	12.4±3.2^{a}	32.4±5.5^{b}	30.0±6.1^{b}	31.9±6.1^{b}	30.2±1.6^{b}	3.3±0.9^{a}	10.0±1.2^{b}	4.8±0.8^{a}

Values represent the mean±SE of the % of colonization by the EF to each experimental set (VOCs, Extrafloral Nectar and Phytohormones) and correspond to the experimental set shown in Fig. 1, Fig. 2 and Fig. 3. Leaves were harvested and disinfected from 5 plants per treatment and 42 circles of 6 mm per plant were plated in PDA media and appearance of EF were quantified. Statistical analysis with *t*-test ($P<0.05$) was conducted in the first experimental set (VOCs) to determine differences between the Intact and MD groups. Significant differences between groups (intact, MD and JA-application) to EFN and Phytohormones experimental set was performed using ANOVA followed by LSD test. Different letters show significant differences, $P<0.05$

Table 2 Volatile organic compounds emitted from plants colonized by different endophytes

RT	NAME	Intact				Mechanical Damage				
		Control	*B. pondoensis*	*Fusarium sp*	*C. lunatus*	Control	*B. pondoensis*	*Fusarium sp.*	*C. lunatus*	Class
6.63	(*Z*)-3-Hexen-1-yl acetate *	14.12±9.47^{a}	0.00^{b}	2.12±0.78^{ab}	0.00^{b}	5.97±3.48^{a}	3.44±0.87^{ab}	2.44±0.96^{ab}	0.00^{b}	GLV
7.17	Geraniol	0.00	0.00	0.00	0.36±0.18	0.00	0.00	0.45±0.23	0.00	Monoterpene
7.31	(*E*)-β-Ocimene *	3.27±0.68^{a}	2.50±1.04^{ab}	1.53±0.64^{b}	3.14±0.15^{ab}	3.34±0.38	2.92±0.17	2.83±0.08	2.99±0.09	Monoterpene
7.58	β-Ocimene *	61.59±12.83^{a}	58.32±5.01^{ab}	54.71±5.13^{b}	61.99±6.75^{ab}	65.99±6.64^{a}	57.66±2.44^{ab}	49.41±4.88^{b}	68.46±2.34^{a}	Monoterpene
8.69	Unknown	0.00	0.00	1.14±0.17	0.00	0.00^{a}	0.46±0.14^{b}	0.63±0.26^{b}	0.00^{a}	
8.75	Linalool *	4.47±3.75	2.29±0.50	0.00	1.17±0.49	0.76±0.33	1.45±0.25	0.75±0.33	1.06±0.33	Monoterpene
9.12	4,8-Dimethylnona-1,3,7-triene (DMNT) *	0.00^{a}	18.44±3.82^{b}	29.23±3.09^{c}	17.70±4.97^{b}	14.41±2.69^{a}	24.60±1.42^{b}	29.71±3.05^{b}	16.65±2.46^{a}	Terpenoid
9.46	(*E,E*)-2,6-Dimethyl-1,3,5,7-octatetraene	1.02±0.33^{a}	0.00^{b}	1.19±0.05^{a}	1.18±0.09^{a}	1.12±0.15	1.06±0.15	1.38±0.16	1.30±0.12	Monoterpene
9.72	1,5,5-Trimethyl-6-methylene cyclohexene	0.00	0.00	0.48±0.18^{a}	0.90±0.14^{b}	0.26±0.14^{ab}	0.00^{a}	0.00^{a}	0.72±0.25^{b}	
10.06	Pinocarvone *	0.00	0.00	0.10±0.04	0.00	0.36±0.13^{a}	0.00^{b}	0.12±0.05^{b}	0.00^{b}	Monoterpene
10.14	Isopinocarveol *	0.00	0.00	0.65±0.13	0.00	0.00^{a}	0.46±0.19^{b}	0.54±0.15^{b}	0.59±0.18^{b}	Monoterpene
10.57	(*Z*)-Cinerone	0.00	0.00	0.00	0.42±0.15	0.00^{a}	0.38±0.14^{b}	0.45±0.12^{b}	0.40±0.07^{b}	Terpenoid
10.63	2,6-Dimethyl-3,7-octadiene-2,6-diol	0.00	0.00	0.39±0.10	0.00	0.00^{a}	0.89±0.20^{ab}	1.42±0.55^{b}	0.66±0.14^{ab}	Monoterpene
10.90	Methyl salicylate *	6.63±2.02	3.65±1.05	3.66±1.17	4.06±1.78	2.19±0.62^{ab}	1.45±0.31^{ab}	2.51±0.53^{a}	0.89±0.11^{b}	Aromatic comp.
11.41	cis-Verbenol	0.00	0.39±0.28	0.17±0.02	0.20±0.04	0.00^{a}	0.19±0.06^{b}	0.00^{a}	0.21±0.06^{b}	Monoterpene
11.60	3-Cyclopropyl-7-hydroxymethylbicyclo [4.1.0] heptane	0.00	0.00	0.00	0.26±0.11	0.00	0.00	0.00	0.00	Monoterpene
11.61	cis-3-Hexenyl isovalerate	0.40±0.13^{a}	0.49±0.14^{a}	0.00^{b}	0.00^{b}	0.39±0.19	0.50±0.13	1.06±0.56	0.39±0.21	Fatty acid ester
12.25	Unknown	0.00	0.00	0.00	0.18±0.08	0.00	0.00	0.00	0.00	
12.32	Unknown	0.00	1.52±0.87	0.00	0.00	0.00	0.00	0.00	0.00	
12.50	Unknown	0.00	0.00	0.56±0.30	0.00	0.00	0.00	1.04±0.32	0.00	
12.54	Unknown	0.00^{a}	1.24±0.52^{b}	0.00^{a}	0.56±0.20 ^{a}	0.00	0.00	0.00	0.76±0.23	
12.76	α-Limonene diepoxide	0.00^{a}	0.00^{a}	0.38±0.14^{b}	0.22±0.07^{a}	0.00^{a}	0.28±0.12^{ab}	0.59±0.19^{b}	0.32±0.04^{ab}	Monoterpene
12.86	Indole	0.00	0.00	0.00	0.00	0.00	0.29±0.12	0.00	0.00	Phenylpropanoid
13.09	Undecenal	0.00	0.00	0.00	0.38±0.16	0.00	0.00	0.00	0.00	GLV
13.77	2-Hexen-1-yl dodecanoate	0.00	0.00	0.00	0.00	0.00	0.00	0.08±0.03	0.00	Fatty acid ester
13.85	Unknown	0.00	0.00	0.10±0.03	0.00	0.00	0.00	0.00	0.00	
13.94	Unknown	0.00	0.00	0.00	0.00	0.00	0.00	0.13±0.05	0.00	
14.08	Eugenol	0.00^{a}	0.00^{a}	0.21±0.04^{b}	0.15±0.08^{b}	0.00^{a}	0.38±0.23^{ab}	0.63±0.22^{b}	0.26±0.10^{ab}	Phenylpropanoid
14.16	(*E*)-2-Undecenal	0.00	0.00	0.00	0.18±0.08	0.00	0.00	0.12±0.06	0.11±0.05	GLV
14.23	3-Methyl tridecane	0.00	0.00	0.00	0.05±0.02	0.00	0.00	0.00	0.00	Hydrocarbon
14.33	Unknown	0.00	0.00	0.00	0.20±0.14	0.00	0.00	0.00	0.00	
14.49	Copaene	0.00	0.00	0.00	0.00	0.52±0.48	0.22±0.15	0.07±0.03	0.16±0.04	Sesquiterpene
14.75	Tetradecane	0.75±0.27^{ab}	1.46±0.71^{a}	0.30±0.06^{b}	0.51±0.12^{ab}	1.01±0.41	0.37±0.11	0.48±0.12	0.54±0.17	Hydrocarbon

Table 2 (continued)

RT	NAME	Intact				Mechanical Damage				
		Control	*B. pondoensis*	*Fusarium sp*	*C. lunatus*	Control	*B. pondoensis*	*Fusarium sp.*	*C. lunatus*	Class
15.32	β-Caryophyllene *	7.60±1.44^{ab}	8.85±2.78^{a}	2.20±0.63^{b}	4.81±1.82^{a}	3.69±1.27	2.40±0.37	1.99±0.30	2.41±0.66	Sesquiterpene
15.73	Geranylacetone	0.00	0.00	0.00	0.00	0.00	0.24±0.10	0.00	0.09±0.04	Terpenoid
15.83	2,6,10-Trimethyltetradecane	0.00	0.00	0.00	0.10±0.05	0.00	0.00	0.00	0.10±0.04	Terpenoid
15.91	α-Caryophyllene *	0.16±0.06^{a}	0.00^{b}	0.09±0.04^{ab}	0.24±0.10^{a}	0.00^{a}	0.07±0.03^{b}	0.07±0.02^{b}	0.09±0.02^{b}	Sesquiterpene
16.06	Unknown	0.00	0.00	0.00	0.18±0.10	0.00	0.00	0.00	0.33±0.11	
16.12	Cedrene	0.00	0.00	0.00	0.00	0.00	0.00	0.07±0.05	0.00	Sesquiterpene
16.25	Cubenol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03±0.01	Sesquiterpene
16.35	Unknown	0.00	0.00	0.00	0.00	0.00	0.00	0.20±0.09	0.00	
16.68	Unknown	0.00	0.00	0.00	0.11±0.06	0.00	0.00	0.00	0.00	
16.89	γ-Muurolene	0.00	0.00	0.00	0.12±0.06	0.00	0.00	0.00	0.15±0.08	Sesquiterpene
17.02	δ-Cadinene	0.00	0.00	0.00	0.18±0.10	0.00	0.00	0.00	0.00	Sesquiterpene
17.80	4,8,12-Trimethyltrideca-1,3,7,11-tetraene (TMTT) *	0.00	0.86±0.45	0.50±0.32	0.44±0.18	0.00^{a}	0.31±0.15^{a}	0.83±0.23^{b}	0.31±0.11^{a}	Terpenoid

Compounds in the table are ordered according to their retention time (RT). Mean normalized data of 5 replicates are expressed as % of total peak area per gram of fresh weigh (% peak area gr^{-1} FW±SE) 24 hr after inoculation with the endophytes. Different letters represent statistically significant differences between treatments to each group (ANOVA-LSD posthoc test, $P<0.05$)

*Compounds were identified by comparison with a reference standard

were differentially emitted, that is, they were emitted only, or at significantly higher relative amounts, from plants colonized with endophytes (see Fig. 1b for general patterns).

The secretion of EFN by intact plants did not respond significantly to the inoculation with any of the fungal strains, although plants inoculated with *B. pondoensis* or *Fusarium sp.* exhibited tendencies, i.e., an increase of the average secretion rates by 33 % and 75 %, respectively (Fig. 2). Similarly, slightly damaged plants exhibited no significantly different EFN secretion rates depending on their colonization with endophytes, although *Fusarium* sp. tended to enhance EFN secretion. By contrast, colonization with each of the three strains significantly and completely inhibited the induced EFN secretion that could be observed in endophyte-free plants after exogenous application of JA (Fig. 2).

Endogenous JA levels in intact plants were significantly enhanced after colonization by *Fusarium* sp. but not by one of the other two strains, whereas *B. pondoensis* significantly inhibited the increase in endogenous JA levels after slight damage (Fig. 3a). A (non-significant) tendency towards reduced levels of JA after exogenous application of the hormone (to 60 % of control levels) was observed in plants colonized with *C. lunatus*.

Endogenous SA levels in intact plants were significantly reduced after colonization with each of the three strains (Fig. 3b). Similarly, colonization by *B. pondoensis* and *Fusarium* sp. inhibited the increase in endogenous SA levels that was observed in the damaged plants. By contrast, neither *B. pondoensis* nor *Fusarium* sp. had significant effects on the decreased levels of endogenous SA in plants that had been treated with exogenous JA before inoculation, whereas

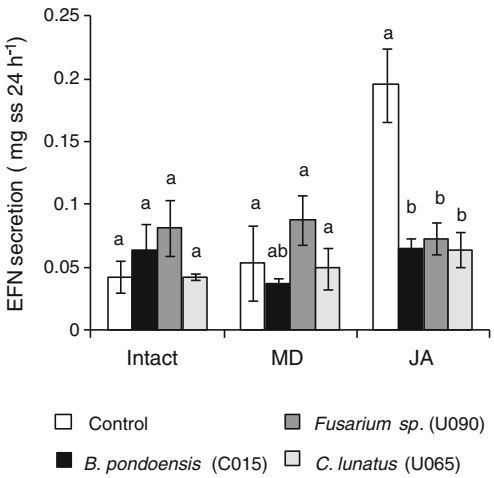

Fig. 2 Effects of the colonization by endophytes on extrafloral nectar (EFN) secretion. We present means±SE (N=5) of the soluble solids that were secreted over the first 24 hr after inoculation. Different letters indicate significant ($P< 0.05$) effects of endophytes among intact, mechanically damaged (MD), or JA-treated plants, according to LSD tests. White bars: control plants without endophytes; black bars: plants with *Bartalinia pondoensis;* dark gray bars: plants with *Fusarium sp.* and light gray bars: plants with *Cochliobolus lunatus*

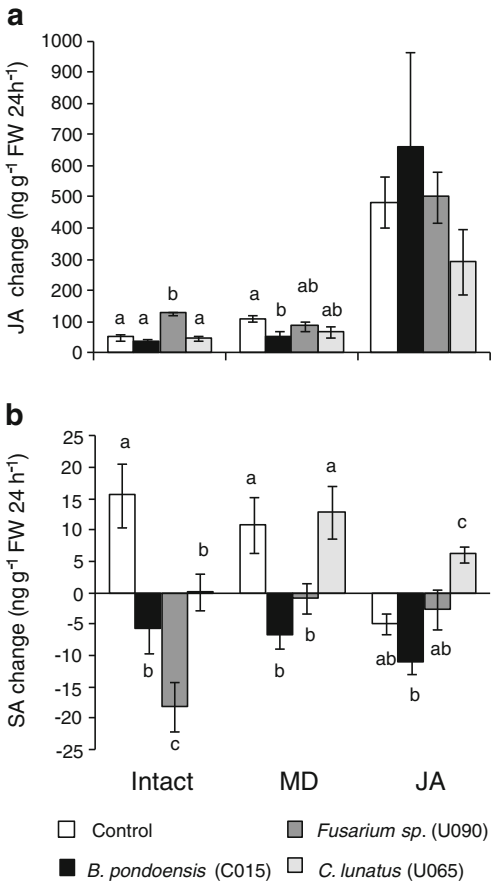

Fig. 3 Changes in the endogenous levels of jasmonic acid (JA) and salicylic acid (SA) over the first 24 hr after inoculation. Intact, mechanically damaged (MD), or JA-treated plants were mock-inoculated (*white bars*) or inoculated with *Bartalinia pondoensis* (*black bars*), *Fusarium sp.* (*dark gray bars*), or *Cochliobolus lunatus* (*light gray bars*). Concentrations [ng per gram leaf fresh mass] of JA (panel **a**) or SA (panel **b**) were quantified before and 24 hr after inoculation to depict absolute changes in the concentration of each hormone. Bars represent means obtained from $N= 5$ plants per treatment, lower case letters above columns indicate significant ($P< 0.05$) differences among treatments according to *post-hoc* analysis (LSD)

colonization by *C. lunatus* significantly enhanced the endogenous levels of SA in these plants (Fig. 3b).

Discussion

Endophytic fungi colonize the extracellular space of living plant tissue and, thus, can potentially interact with the defensive signaling pathways of their host, as has been described for mycorrhiza and rhizobia (Pozo et al. 2005; Pozo and Azcón-Aguilar 2007). Most of the 'type II' endophytes are taxonomically related to necrotrophs, rather than biotrophs, and several of them can switch to a necrotrophic lifestyle under certain conditions (Delaye et al. 2013). Therefore, JA-dependent signaling might be involved in keeping the fungus in an asymptomatic stage, and colonization with endophytes should

enhance the endogenous JA levels. Indeed, the inoculation of *Atractylodes lancea* plants with the 'type II' endophyte *Gilmaniella* sp. enhanced endogenous JA levels in this plant, although this effect became obvious not earlier than 8d after inoculation (Ren and Dai 2012). In our study, we found strain–specific effects of the colonization with endophytes on endogenous JA levels, at least over the first 24 hr after inoculation. One strain (*Fusarium* sp.) significantly induced endogenous JA levels in intact leaves, whereas *B. pondoensis* significantly decreased JA levels in slightly damaged leaves. The other strains had no significant effects under our experimental conditions.

Nevertheless, two of three strains (*Fusarium* sp. and *C. lunatus*) enhanced the emission of VOCs from intact plants, and all three strains enhanced the number of detectable VOCs emitted from slightly damaged leaves, over the first 22 hr after inoculation. Concordantly, endogenous levels of SA in intact plants were significantly decreased by all of the strains. These changes indicate the up-regulation of at least parts of the classical JA-dependent defenses. Similarly, peppermint plants responded to the inoculation with an endophyte with the enhanced emission of VOCs such as menthone and neomenthol (Mucciarelli et al. 2007), although these changes were observed starting on day 14 after inoculation. In broad bean, colonization with *Acremonium strictum* transiently enhanced EFN secretion (Jaber and Vidal 2009). All these findings are in line with the hypothesis that the endophytes, at least transiently, induce JA-dependent responses in the plants, and that these responses negatively affect endogenous levels of SA, likely due to SA-JA trade-offs (Thaler et al. 2012).

Future studies will have to disentangle whether these VOCs represent induced compounds that were emitted from the plant itself or whether some of them were produced by the fungi. We will also require further studies to characterize the ecological effects of these changes in the VOC profiles of colonized Lima bean plants. Since in our study more VOCs were emitted from the colonized plants, it appears safe to assume that these changes should generally tend to contribute to the indirect defence of Lima bean.

No major differences in the patterns of emitted VOCs were found between endophyte-free and colonized tall fescue (*Festuca arundinacea*) plants (Yue et al. 2001), whereas Li et al. (2014) found that the colonization with type I endophytes reduced the constitutive emission of VOCs from intact grasses but enhanced their induced emission after herbivore feeding (see also Saikkonen et al. 2013). Similarly, the emission of VOCs from *Lolium perenne* plants that were colonized with the type I endophyte *Neotyphodium lolii* changed significantly when the host was then infected with a pathogenic fungus (*Fusarium poae*) (Pańka et al. 2013). Thus, the colonization by some endophytes might prime plants for enhanced VOC emission once they are attacked, rather than directly inducing the emission from the intact plant, as we observed in our study. All these effects are highly likely to be strain-specific, as, for example, the exogenous application of methyl jasmonate or SA to *Quercus serrata* increased the density of some strains of natural endophytes and decreased the density of others (Kusumoto and Matsumura 2012). Similarly, the colonization of cursed thistle (*Cirsium arvense*) with *Chaetomium cochliodes* reduced the growth of cabbage moth (*Mamestra brassicae*) but increased feeding by the thistle tortoise beetle (*Cassida rubiginosa*), whereas another fungus, *Cladosporium cladosporioides*, had no effect on *M. brassicae* (Gange et al. 2012). We conclude that interactions among endophytes, the endogenous hormone levels, and the SA- or JA-responsive defense traits depend on the specific strain or, most likely, the 'strain X host plant' combination, and that the action of further organisms (herbivores or pathogens) adds an additional layer of possible outcomes.

The most interesting result of the present study is the blocked response of EFN secretion to exogenous JA that was caused by all three strains, at least during the early phase of colonization. In another study, the application of ibuprofen and nordihydroguaiaretic acid inhibited the synthesis of JA and of the JA-dependent essential oils in *Atractylodes lancea* plants, but not the induction of the essential oils by the endophyte, *Glimaniella* sp. (Ren and Dai 2012). Thus, endophytes can interfere with octadecanoid signaling downstream of JA. The observed inhibition of EFN is highly counterintuitive, because we used strains that naturally colonize Lima bean and wild plants exhibit JA-induced EFN secretion (Heil 2004; Kost and Heil 2005). Unfortunately, the signaling that controls EFN secretion downstream of JA is unknown, for which reason we cannot even speculate on a putative mechanism that underlies this inhibitory effect.

It appears possible that this inhibition of EFN secretion represents a transient effect that occurs only during the early phase of the colonization process. Alternatively, the presence of one single instead of several fungal strains in the plant, or unnaturally high inoculum densities, might have caused this effect. In nature, a single leaf usually carries several strains of endophytes. For example, a collection of 83 leaves from several tropical tree species revealed 1,472 fungal isolates that represented 418 fungal morphospecies (Arnold et al. 2000). More than 1,000 fungal isolates were obtained from 175 leaves of aspen (*Populous tremula*) (Albrectsen et al. 2010) and in *Hevea* spp., on average six fungal species co-occurred in leaf discs of ca 3 cm^2 (P. Chaverri, pers. comm.). Type II endophytes comprise mutualists, latent pathogens, and commensalistic species such as dormant saprophytes (Arnold and Lutzoni 2007; Sieber 2007), and they form diverse communities within a single host, or even in a single leaf. Finally, the outcome of plant-endophyte interactions is highly context-dependent and depends, among others, on the nutritional

status of the plant and other abiotic factors as well as on the presence and identity of plant enemies (Arnold 2007; Partida-Martinez and Heil 2011). Thus, the field suffers from the common 'relevance *versus* reproducibility' dilemma (Heil 2014): the commonly applied experimental scheme, in which plants are colonized by one single strain of endophyte and then investigated under controlled conditions, is not likely to reveal the true physiological and ecological effects of type II endophytes (Estrada et al. 2013; Mejía et al., 2008; Van Bael et al. 2012). Studies using natural colonization (Van Bael et al. 2012) might reveal ecologically more relevant results, although they necessarily suffer from a lower degree of reproducibility.

In summary, symptomless endophytes interact in multiple and complex ways with the endogenous levels of the plant defense hormones, SA and JA. Thereby, the endophytes can affect the capacity of their host to express indirect defense traits such as the emission of VOCs and the secretion of EFN. The interactions can occur both upstream and downstream of the hormones and are strain-dependent. We conclude that we have to consider the identity (and species-specific biology) of the fungi when we aim at predicting effects of the colonization by endophytes on the resistance phenotype of their host. Many responses will depend on the developmental phase of both, host and endophyte, on interactions among different endophytes, and on the densities that certain endophytes reach within the host. Future studies aimed to obtain ecologically relevant results should mimic the natural situation more carefully (i.e., inoculate plants with multiple strains of endophytes at natural densities) to more closely resemble the effects of type II endophytes on their host plants in nature, whereas studies aimed to disentangle putatively general physiological processes that could underlie these effects should try to standardize for factors such as inoculum density and time after colonization, to obtain data that can be compared among different study systems.

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