RESEARCH LETTER

Order of arrival shifts endophyte–pathogen interactions in bean from resistance induction to disease facilitation

Rosa-María Adame-Álvarez, Jaime Mendiola-Soto & Martin Heil

Departamento de Ingeniería Genética, Centro de Investigación y de Estudios Avanzados (CINVESTAV), Irapuato, Guanajuato, México

Correspondence: Martin Heil, Departamento de Ingeniería Genética, CINVESTAV-Irapuato, km 9.6 Libramiento Norte, 36421 Irapuato, GTO, México.
Tel.: +52 (462) 623 9657;
fax: +52 (462) 623 9650;
e-mail: mheil@ira.cinvestav.mx

Received 28 April 2014; accepted 29 April 2014. Final version published online 3 June 2014.

DOI: 10.1111/1574-6968.12454

Editor: Bernard Glick

Keywords
biocontrol; *Colletotrichum*; fungal endophytes; induced resistance; *Phaseolus lunatus*; *Pseudomonas syringae*.

Abstract

Endophytic fungi colonize plants without causing symptoms of disease and can enhance the resistance of their host to pathogens. We cultivated 53 fungal strains from wild lima bean (*Phaseolus lunatus*) and investigated their effects on pathogens using *in vitro* assays and experiments *in planta*. Most strains were annotated as *Rhizopus*, *Fusarium*, *Penicillium*, *Cochliobolus,* and *Artomyces* spp. by the sequence of their 18S rRNA gene. *In vitro* confrontation assays between endophytes and three pathogens (the bacteria *Pseudomonas syringae* pv. *syringae* and *Enterobacter* sp. strain FCB1, and the fungus *Colletotrichum lindemuthianum*) revealed strong and mainly symmetric reciprocal effects: endophyte and pathogen either mutually inhibited (mainly *Enterobacter* FCB1 and *Colletotrichum*) or facilitated (*P. syringae*) the growth of each other. *In planta*, the endophytes had a strong inhibitory effect on *P. syringae* when they colonized the plant before the bacterium, whereas infection was facilitated when *P. syringae* colonized the plant before the endophyte. Infection with *Enterobacter* FCB1 was facilitated when the bacterium colonized the plant before or on the same day with the endophyte, but not when the endophyte was present before the bacterium. The order of arrival determines whether fungal endophytes enhance plant resistance to bacterial pathogens or facilitate disease.

Introduction

Plants are colonized by microorganisms even when they are phenotypically healthy (Wilson, 1995; Arnold *et al.*, 2000; Yuan *et al.*, 2010; Partida-Martinez & Heil, 2011; Porras-Alfaro & Bayman, 2011). Fungal endophytes colonize plant tissues without causing disease under the current environmental conditions and fall into two major groups, which are characterized by different taxonomic and ecological properties. Fungal endophytes were first discovered in cultivated tall fescue (*Lolium arundinaceum*) plants that caused livestock disorders due to fungal alkaloids (Clay, 1990). Whereas these 'type I' endophytes in the family *Clavicipitaceae* systemically colonize grasses and are vertically transmitted, the so-called type II endophytes (nonsystemic, horizontally transmitted species that colonize *de novo* every leaf) are less studied but much more diverse and ubiquitous (Yuan *et al.*, 2010). Type II endophytes have been isolated from all plant species investigated to date and are usually present in between 80% and 100% of the investigated samples (Arnold *et al.*, 2000; Arnold & Herre, 2003; Albrectsen *et al.*, 2010; Gazis & Chaverri, 2010).

Interestingly, the colonization of cacao (*Theobroma cacao*) by natural horizontally transmitted endophytes significantly decreases both necrosis and mortality of leaves challenged with a pathogenic *Phytophthora* sp. or other pathogens (Arnold *et al.*, 2003; Mejia *et al.*, 2008). Indeed, an enhanced resistance to pathogens might be a common outcome of the colonization of plants by type II endophytes (Gao *et al.*, 2010; Ownley *et al.*, 2010). Therefore, these endophytes are increasingly being discussed as promising biocontrol agents (Gao *et al.*, 2010; Porras-Alfaro & Bayman, 2011; O'Hanlon *et al.*, 2012). However, endophytes can enhance the resistance in their host either locally (Raghavendra & Newcombe, 2013) or systemically (Waller *et al.*, 2005). Furthermore, producing endophyte-free plants and then colonizing them one by one with isolated strains is time-consuming. In one study, 217 strains of endophytic bacteria and 17 fungi from coffee tissues

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were evaluated for their potential to control coffee leaf rust (*Hemileia vastatrix*), and only nine of the bacterial strains and none of the fungi reduced disease severity (Silva *et al.*, 2012). Fast and cost-efficient screening methods are therefore required. Furthermore, endophytes and pathogens interact in multiple ways, ranging from direct competition and antibiosis to manipulation of the endogenous levels of resistance-related plant hormones such as jasmonic acid and salicylic acid (Conn *et al.*, 2008; Brock *et al.*, 2013; Garcia-Guzmán & Heil, 2014). Therefore, the main focus of our study was to test the hypothesis that the order of arrival affects the outcome of the interaction *in planta*.

We focused on fungal endophytes in lima bean (*Phaseolus lunatus* L.) and asked the following questions. (1) Which fungi can be isolated from symptomless leaves of wild lima bean plants? We focused on cultivable strains so as to be able to examine their function *in vitro* and *in planta*. (2) Which fungi inhibit the growth of pathogenic microorganisms in Petri dishes? For these assays, we used the biotrophic *Enterobacteria Pseudomonas syringae* pv. *syringae* and *Enterobacter* sp. strain FCB1 (Córdova-Campos *et al.*, 2012), and the hemibiotrophic fungus *Colletotrichum lindemuthianum*. All three pathogens cause disease in lima bean (Yi *et al.*, 2009; Heil & Adame-Álvarez, 2010; Córdova-Campos *et al.*, 2012). (3) Does the order of arrival affect the outcome of the endophyte–pathogen interaction *in planta*? Facilitation occurs when one organism enhances the development of another one owing to its presence. For example, facilitation affected the assembly of endophyte communities in maize (*Zea mays*) (Pan & May, 2009). Therefore, we hypothesized that the order of arrival might be crucial in determining whether the endophyte has a negative (i.e., resistance-enhancing), neutral, or positive (facilitation) effect on the development of the bacterial pathogens.

Material and methods

Biologic material and growth conditions

We collected five phenotypically healthy leaves from each of ten lima bean plants growing in their natural habitat in Oaxaca, South Mexico (Pacific coast; *c.* 15°55′N and *c.* 097°09′W). Leaves were washed with distilled, sterilized water and then surface-sterilized by immersion in 70% ethanol for 1 min, followed by immersion in 5% sodium hypochloride for 5 min, and then washed with distilled, sterilized water. Consecutively, the leaves were cut into pieces (0.7 × 0.7 cm), placed on potato dextrose agar (Difco), and maintained at 28 °C for 3 days. Developing fungal mycelia were repeatedly transferred to new medium until we obtained axenic cultures. Lima bean seeds were collected from the same site and surface-sterilized. Preliminary experiments have demonstrated that plants cultivated from these seeds are free of endophytes (n = 50, R.M. Adame-Álvarez, J. Mendiola-Soto, unpublished data). The bacterial pathogen *Pseudomonas syringae* pv. tomato was kindly provided by Dr Choong-Min Ryu (KRIBB, Daejeon, South Korea) and causes disease in lima bean under both greenhouse and field conditions (Yi *et al.*, 2009; Heil & Adame-Álvarez, 2010). *Enterobacter* sp. strain FCB1 was isolated from diseased common bean (*Phaseolus vulgaris*) leaves (Córdova-Campos *et al.*, 2012). The fungus, *Colletotrichum lindemuthianum* strain 1088, was kindly provided by Dr June Simpson (CINVESTAV Irapuato).

Characterization of endophytic fungi

Axenic cultures of endophytic fungi were assigned to morphotypes, that is, mycelia that showed stable, homogenous, and optically distinguishable phenotypes on the culturing media were assigned to morphotypes based on these phenotypes (Arnold *et al.*, 2000; Albrectsen *et al.*, 2010). We then extracted DNA from the 53 most common morphotypes (Nicholson *et al.*, 2001). The 18S rRNA gene was amplified with primers MR1 and FR1 (Vainio & Hantula, 2000) and submitted to Sanger sequencing at LANGEBIO (CINVESTAV-Irapuato; www.langebio.cinvestav.mx) on an ABI 3730-xl DNA Analyzer (Applied Biosystems; www.appliedbiosystems.com). Next, the fungal strains were tested for their capacity to sporulate on potato dextrose agar medium and to recolonize lima bean plants under greenhouse conditions. To obtain a spore suspension, sporulating colonies were immersed in distilled water and the spore suspension was adjusted to a concentration of 1×10^6 conidia mL^{-1} by counting spores in a hemacytometer set (Hausser Scientific, Horsham, PA). This suspension was sprayed onto plants. For consecutive experiments, we considered only strains that did not cause symptoms of disease and that yielded a reproducible colonization of the leaves, demonstrated via staining with lactophenol blue (Merck, Darmstadt, Germany) and by amplifying the fungal 18S rRNA gene from the experimentally colonized plants to confirm the identity of the colonizing fungus.

Confrontation assays in Petri dishes

For the confrontation assays, the bacteria were cultivated in liquid media (*P. syringae* in 100 μg mL^{-1} King's broth medium at 28 °C and *Enterobacter* in yeast extract–dextrose–$CaCO_3$ medium at 37 °C) and shaken (GYRATORY SHAKER-MODEL G2) at 125 r.p.m. Fungi (see Fig. 1 for

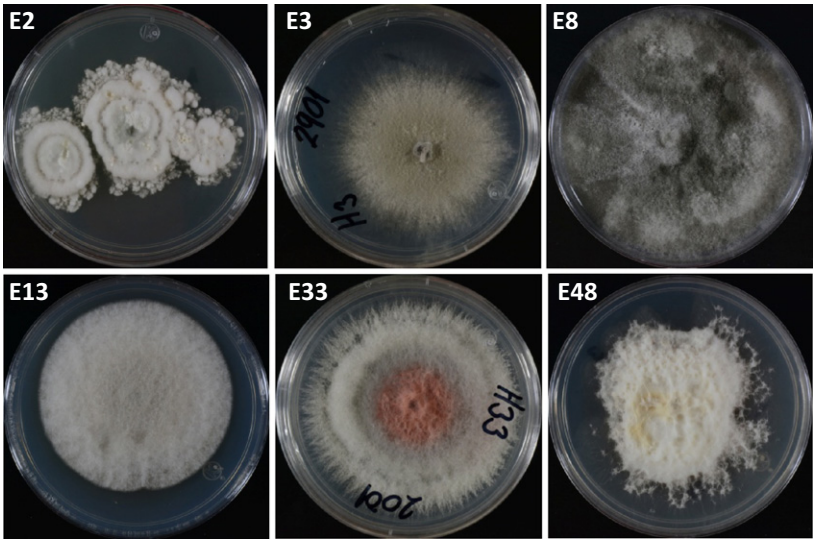

Fig. 1. Phenotypes of endophytic fungi growing on potato dextrose agar that were used in confrontation and colonization assays. E2: *Cochliobolus cynodontis*, E3: *Hyphozyma variabilis*; E8: *Cochliobolus australiensis*, E13: *Rhizopus oryzae*, E33: *Keissleriella genistae;* E48: *Fusarium* sp. 14201. See Table S1 for annotations and sequences.

morphotypes) were cultivated on potato dextrose agar at 37 °C. After 24 h, 20 μL of bacterial culture and a piece of 0.5 × 0.5 cm of fungal mycelium were placed 3 cm apart on a fresh potato dextrose agar plate. Bacteria or fungi cultivated alone served as controls. In two different experiments, plates were kept at 37 °C or at 20–28 °C (20 °C at night and 28 °C during the day, to mimic greenhouse conditions). Microbial growth was determined by measuring the diameter of the colonies twice a day over the next 5 days and relating the colony sizes as found in the confrontation situation to the sizes of the controls.

In planta assays

Phaseolus lunatus plants were cultivated from surface-sterilized seeds in a greenhouse under natural light conditions (temperature *c.* 20 °C at night and max. 30 °C during the day) until they had five trifoliate leaves. Bacteria (*P. syringae*) were cultivated in liquid medium as described above and then adjusted to suspensions with a density of 1×10^7 mL^{-1} (optical density at 600 nm = 0.065; determined in a GENESYS™ 20 spectrophotometer; Thermo Scientific; www.thermoscientific.com). The fungi were cultivated, and spore suspensions were obtained as described above. Two *in planta* assay experiments were performed with *P. syringae*. In the first experiment, either E2: *Cochliobolus cynodontis* or E33: *Keissleriella genistae* were applied 4 days or 2 days after the bacterial pathogen, simultaneously with the pathogen, or 4 days before the pathogen. In the second experiment, one of five endophytes (E2: *Cochliobolus cynodontis*, E3: *Hyphozyma variabilis*; E8: *Cochliobolus australiensis*, E33: *K. genistae;* E48: *Fusarium* sp. 14201; see Fig. 1) was applied 4 days before or after the bacterial pathogen, or simultaneously with the pathogen. Plants were challenged by spraying all leaves with the bacterial or fungal suspensions until the whole surface was wet and received water spraying on days on which bacterial or fungal suspensions were applied to some plants, according to the above-mentioned scheme. The plants were kept at 100% r.h. for 24 h before being transferred back to the greenhouse. Eight days after challenging the plants with *P. syringae*, three randomly selected leaves from each plant were removed, weighed (fresh weight), homogenized in distilled water, diluted 1 : 10, 1 : 100 and 1 : 1000 (three replicates at each concentration), and plated onto solid King's broth medium to count colony-forming units (CFUs).

Similar *in planta* assays were performed using *Enterobacter* sp. FCB1 as the pathogen. *Phaseolus lunatus* plants were cultivated until they had five trifoliate leaves, bacteria were cultivated in liquid medium, the fungi were cultivated, and spore suspensions were obtained as described above. Then, one of five endophytes (E2, E3, E8, E33, and E48) was applied 4 days before or after the bacterial pathogen, or simultaneously with the pathogen, and infection levels were determined as describe above.

Results

Endophytes from lima bean

We isolated 346 fungal strains from which 53 morphologically distinct axenic mycelia were cultured (see Supporting Information, Table S1). Based on the sequences of their 18S rRNA gene, most of these strains were

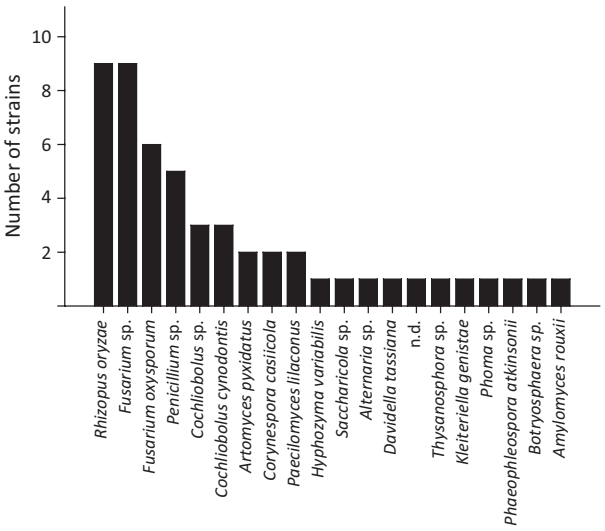

Fig. 2. Number of endophyte taxa represented by the morphologically different strains isolated from healthy leaves of wild lima bean (*Phaseolus lunatus*). Morphologically different strains were preliminarily annotated by sequencing their 18S rRNA gene and by BLAST searches in GenBank. n.d., not determined.

annotated as *Rhizopus oryzae* (8 strains), *Fusarium* spp. (8), *Fusarium oxysporum* (6), *Cochliobolus* sp. (6), *Penicillium* spp. (4), and *Artomyces* spp. (3) (Fig. 2).

Confrontation assays in Petri dishes

In direct confrontations in Petri dishes, most of the pairing combinations of endophyte and pathogen resulted in a symmetric growth pattern: either both showed reduced or increased growth in the confrontation situation as compared to growing alone. Thus, the average colony growth rates of endophytes and pathogens were positively correlated with each other (Spearman rank correlation: $P < 0.01$) over all endophyte–pathogen combinations and in both experiments (Fig. 3). At 37 °C, all six endophyte strains significantly inhibited the growth of colonies of *Enterobacter* FCB1. In five of six cases, this inhibition was a mutual one (i.e., the endophytic fungus also exhibited reduced colony growth; Fig. 3a). Similarly, five of six strains inhibited the growth of the pathogenic fungus *Colletotrichum lindemuthianum*. In three cases, the inhibition was mutual, in one case the growth of the endophyte was not affected and in one case the endophyte showed slightly increased growth, whereas the pathogen was inhibited (Fig. 3a). By contrast, four of the endophytes significantly enhanced the colony growth rate of *P. syringae*, three of which also showed significantly increased growth in co-culture. None of the endophytes significantly inhibited the growth of *P. syringae* (Fig. 3a).

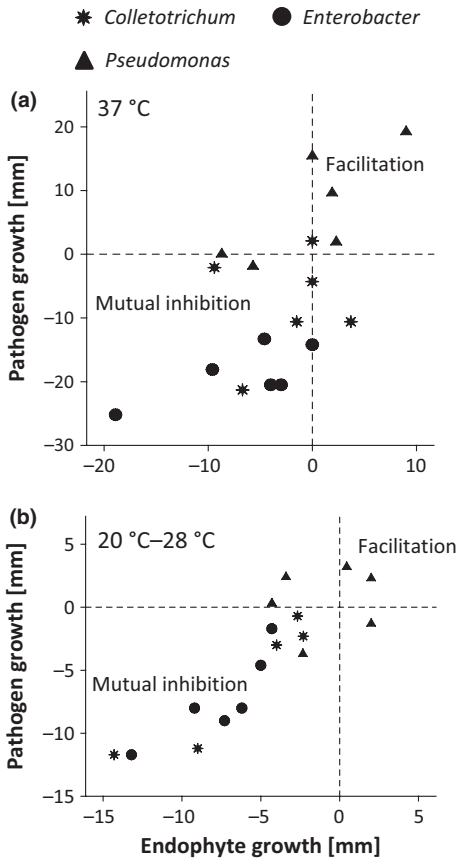

Fig. 3. Growth effects in confrontation assays in Petri dishes. Six fungal strains (E2: *Cochliobolus cynodontis*, E3: *Hyphozyma variabilis*; E8: *Cochliobolus australiensis*, E13: *Rhizopus oryzae*; E33: *Keissleriella genistae;* E48: *Fusarium* sp. 14201) were co-cultivated with *Colletotrichum lindemuthianum* (asterisks), *Enterobacter* strain FCB1 (black circles), or *Pseudomonas syringae* (black triangles) or were cultivated alone. The position of the symbols indicates the growth rate in the confrontation assays as compared with the growth rate when cultivated alone, with values of the endophyte defining the position on the *x*-axis and values of the pathogen defining the position on the *y*-axis. Both values were positively correlated at $P < 0.01$ (Spearman rank correlation, $n = 5$ for each pathogen–endophyte combination). The experiment was performed once at 37 °C (a) and one at 20 °C at night and 28 °C during the day (b).

Similar patterns at generally lower absolute growth rates were found in confrontations assays that were performed at 20–28 °C (Fig. 3b).

Induced resistance and facilitation *in planta*

In both *in planta* experiments using fungal endophytes and the bacterium *P. syringae* (Fig. 4), the species of endophyte and the temporal order in which the fungus and pathogen were applied significantly affected the numbers of bacterial CFU 8 days after challenging [results of

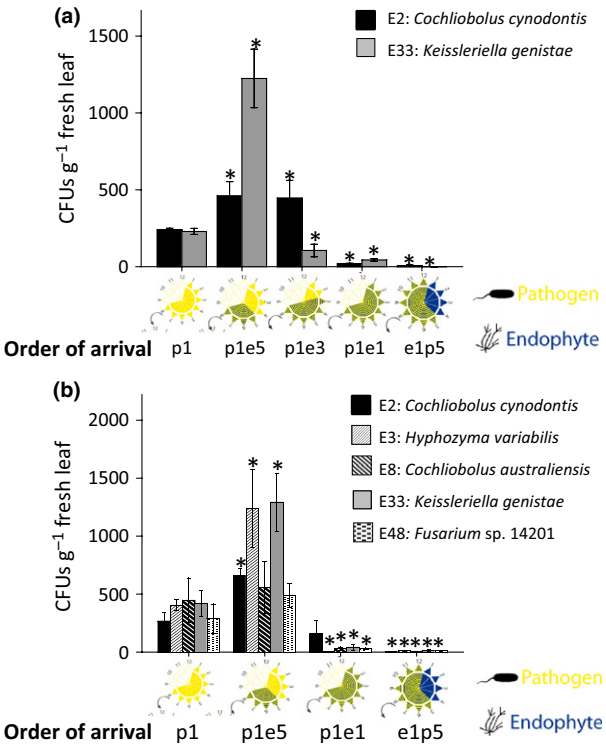

Fig. 4. Order of arrival shifts endophyte–*Pseudomonas* interactions from resistance to disease facilitation. The concentration of CFUs of *Pseudomonas syringae* is indicated for plants that were challenged with the bacterium before, at the same time or after being colonized with an endophyte. In the first experiment (a), one of two endophytes (E2: *Cochliobolus cynodontis* or E33: *Keissleriella genistae*) were applied 4 days (p1e5) or 2 days (p1e3) after the pathogen, simultaneously with the pathogen (p1e1), or 4 days before the pathogen (e1p5). In the second experiment (b), one of five endophytes (E2: *Cochliobolus cynodontis*, E3: *Hyphozyma variabilis*; E8: *Cochliobolus australiensis*, E33: *Keissleriella genistae;* E48: *Fusarium* sp. 14201) was applied 4 days before the pathogen (e1p5), simultaneously (p1e1), or 4 days after the pathogen (p1e5). The order of arrival is also indicated graphically: day in yellow: only *P. syringae*; days in blue: only endophyte; days in yellow/blue stripes: both microorganisms present on the plant. Bars represent means ± SE ($n = 3$), and asterisks over the bars indicate significantly ($P < 0.05$, *t*-test) higher or lower CFU numbers than in the controls (p1: endophyte-free plants challenged only with *P. syringae*).

univariate ANOVA: Experiment 1 (Fig. 4a): SS = 56 506, d.f. = 1, $P < 0.001$ for factor 'fungus'; SS = 2 737 381, d.f. = 4, $P < 0.001$ for factor 'temporal order', and SS = 997 002, d.f. = 4, $P < 0.001$ for the interaction. Experiment 2 (Fig. 4b): SS = 519 830, d.f. = 4, $P = 0.46$ for factor 'fungus', SS = 6 743 794, d.f. = 3, $P < 0.001$ for factor 'temporal order', and SS = 1 410 090, d.f. = 12, $P = 0.018$ for the interaction; Fig. 4]. When the plants were inoculated with *P. syringae* 4 days before the endophyte, the numbers of bacterial CFU were significantly higher than those found on the control plants (challenged only with *P. syringae*). By contrast, when *P. syringae* and the endophyte were inoculated on the same day, the bacterial titers were significantly lower than for the controls, and they were further reduced when the bacteria were applied after the fungal endophyte (Fig. 4).

In the *in planta* experiments using *Enterobacter* FCB1 (Fig. 5), the temporal order in which the fungus and pathogen were applied, but not the species of endophyte, significantly affected the numbers of bacterial CFU 8 days after challenging (results of univariate ANOVA: SS = 67 170, d.f. = 1, $P < 0.001$ for factor 'temporal order'; $P > 0.05$ for the factor 'fungus' and the interaction). When the plants were inoculated with *Enterobacter* FCB1 4 days before the endophyte or on the same day, the numbers of bacterial CFU were significantly higher than those found on the control plants (challenged only with *Enterobacter* FCB1; Fig. 5). By contrast, no effect of the endophyte on bacterial infection levels was seen when the bacteria were applied after the fungal endophyte (Fig. 5).

Discussion

We isolated 53 morphologically different axenic cultivable endophytes from 50 phenotypically healthy leaves of wild lima bean. Most of these strains were annotated as *Rhizopus oryzae*, *Fusarium oxysporum* and other *Fusarium* spp.,

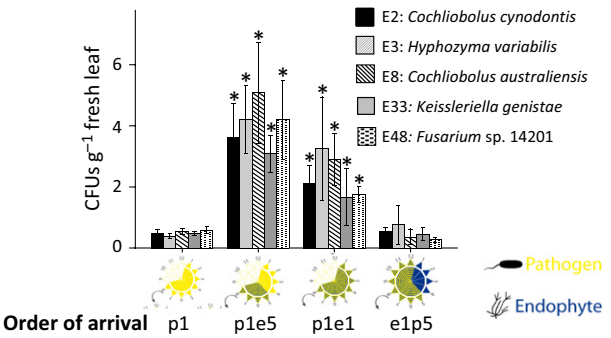

Fig. 5. Order of arrival affects facilitation of *Enterobacter* by endophytes. The concentration of CFUs of *Enterobacter* FCB1 is indicated for plants that were challenged with the bacterium before, at the same time, or after being colonized with an endophyte. One of five endophytes (E2: *Cochliobolus cynodontis*, E3: *Hyphozyma variabilis*; E8: *Cochliobolus australiensis*, E33: *Keissleriella genistae;* E48: *Fusarium* sp. 14201) was applied 4 days before the pathogen (e1p5), simultaneously (p1e1), or 4 days after the pathogen (p1e5). See legend to Fig. 4 for symbology. Bars represent means ± SE ($n = 4$), and asterisks over the bars indicate significantly ($P < 0.05$, *t*-test) higher or lower CFU numbers than in the controls (p1: endophyte-free plants challenged only with *Enterobacter* FCB1).

Cochliobolus cynodontis and other *Cochliobolus* spp., *Penicillium* spp., and *Artomyces* sp., based on the sequence of their 18S rRNA gene. Further genes will need to be sequenced and the resulting annotations combined with morphological traits to obtain reliable identifications. However, these annotations indicate that most of the fungi belong to taxa that have frequently been reported as endophytes in other plants species. Although we are unlikely to have sampled the entire diversity of endophytes in lima bean at our field site, the number of strains discovered falls within the lower range of what has been reported from other plants. For example, 100 morphologically distinct groups comprising 33 taxa of endophytic fungi have been discovered in leaves of European aspen (*Populus tremula*) (Albrectsen *et al.*, 2010), 58 operational taxonomic units have been isolated from leaves of wild rubber trees (*Hevea brasiliensis*) in Peru (Gazis & Chaverri, 2010), more than 100 morphospecies of endophytes have been found to be associated with tropical palms (Frohlich *et al.*, 2000), and 418 fungal morphospecies have been isolated from 83 leaves from different tropical tree species (Arnold *et al.*, 2000).

The high diversity of endophytes makes efficient screening methods highly important when attempting to identify suitable strains for biocontrol. The most common experimental scheme relies on confrontation assays in Petri dishes followed by *in planta* assays in which plants that are already colonized by the endophyte are challenged with a target pathogen or pest insect (Maciá-Vicente *et al.*, 2008; Mejia *et al.*, 2008; Gurulingappa *et al.*, 2010; Silva *et al.*, 2012; Raghavendra & Newcombe, 2013). Our study demonstrates that the results of this standard procedure are unlikely to reliably predict all the effects that an endophyte can have on the host plant. In the confrontation assays in Petri dishes, most of the endophytes inhibited the growth of *Enterobacter* FBC1 and *Colletotrichum lindemuthianum*. By contrast, the endophytes had the opposite effect on *Enterobacter* FCB1 *in planta* and facilitated infection when the fungus was applied after the bacterium or on the same day. The other way round, most endophytes facilitated the growth of *P. syringae* in the Petri dishes, whereas three of five endophytes significantly inhibited the growth of this bacterium *in planta* when the endophyte colonized the plant before or at the same time as the bacterium. Endophytes only facilitated bacterial growth of *P. syringae* when arriving after the bacterium.

These differences between *in planta* assays and assays in Petri dishes were also observed when both types of experiment were performed at the same temperature. Thus, what we see here are no temperature effects, but rather a consequence of the different conditions that the microorganisms face in plant tissue as compared to artificial culturing media. We conclude that the 'order of arrival' is a crucial predictor of the effect of an endophyte on bacterial infection. Plants that received a synchronous application of both the bacteria and endophyte best resembled the situation in Petri dishes (both pathogen and endophyte start growth at the same time) but revealed opposite results for both bacterial strains tested: facilitation of *P. syringae* in the Petri dish vs. inhibition *in planta*, and inhibition of *Enterobacter* FCB1 in the Petri dish vs. facilitation *in planta*.

To date, few studies have compared different orders of microorganism arrival on the plant leaf. When endophytic *Fusarium verticillioides* strains were inoculated onto maize seedlings before, simultaneously, or after inoculation with the pathogen *Ustilago maydis*, smut disease severity was significantly decreased only when *F. verticillioides* was simultaneously inoculated with *U. maydis* (Lee *et al.*, 2009). When tomato (*Lycopersicon esculentum*) plants were inoculated with the nonpathogenic *Verticillium dahliae* strain Dvd-E6 before or together with the pathogenic strain Vd1, the pathogen was almost completely excluded from the root. However, when Vd1 was inoculated first, Dvd-E6 competed on an equal basis (Shittu *et al.*, 2009). Nine bacterial strains have been shown to control coffee leaf rust (*Hemileia vastatrix*) severity when applied 72 or 24 h before being challenged with the pathogen, but a further six strains significantly reduced rust severity only when applied 72 h, but not 24 h, before challenging with the pathogen (Silva *et al.*, 2012).

Three of six endophytes showed increased growth when they were co-cultivated with *P. syringae* in the Petri dish, as did the bacterium, which indicates that direct interactions are likely to contribute to the facilitation effect. However, molecules that are exchanged in a mutualistic manner cannot explain the induced resistance to *P. syringae* in plants that were already colonized by the fungus. Whereas competition for space might contribute to the lower infection levels by *P. syringae* in plants that were already colonized by endophytes, it is tempting to speculate that costs of resistance or a cross talk between jasmonic acid- and salicylic acid-dependent signaling pathways (Pieterse *et al.*, 2009; Thaler *et al.*, 2012) contributed to the facilitation effects. Biotrophic pathogens such as *P. syringae* are controlled via salicylic acid-dependent pathways (Cameron *et al.*, 1999; Cui *et al.*, 2005) and these usually inhibit jasmonic acid signaling, which controls infection by necrotrophic pathogens and infestation by herbivores (Cui *et al.*, 2005; Pieterse *et al.*, 2009). Endophytic fungi can adopt a necrotrophic lifestyle under certain circumstances and are taxonomically related to necrotrophs rather than to biotrophs (Delaye *et al.*, 2013). Therefore, jasmonic acid signaling is likely to

maintain endophytes in the nonsymptomatic stage (Garcia-Guzmán & Heil, 2014), and salicylic acid–jasmonic acid cross talk could enhance the susceptibility to biotrophs in plants that express resistance to necrotrophs and symptomless endophytes.

However, surprisingly, the facilitation effect on *P. syringae* was only visible when the fungus arrived after the bacterium, and *Enterobacter* FBCB1 was facilitated when the fungus arrived on the same day or after the bacterium, a pattern that is not consistent with the typical phenomenon of induced susceptibility due to hormonal cross talk. Still, plants might have responded to the colonization by endophytes with enhanced JA-dependent signaling to control the new invader and thereby suppressed SA-dependent resistance against the biotrophic bacteria. Indeed, lima bean responds to the inoculation with endophytes with a transient decrease in endogenous SA levels (M. Heil & A. Navarro, unpublished data). An alternative, nonexclusive explanation could be that resistance expression is costly (Bergelson *et al.*, 1996; Heil & Baldwin, 2002). Therefore, we hypothesize that the plants could not continue to suppress the bacterium when they then also had to control the fungus. Future studies are required to understand the mechanisms of this facilitation effect.

Most importantly, Petri dish assays did not allow for a reliable prediction of the effects that endophytes have on the level of resistance in their host plant to infection by two pathogenic bacteria, *P. syringae* or *Enterobacter* FCB1. *In planta* assays testing different orders of arrival were required to observe the full spectrum of possible effects that endophytes can have on bacterial infections in the host, and order of arrival determined whether the endophytes affected the bacteria positively (facilitation), negatively (enhanced resistance), or had no effect. From an applied perspective, these results are highly relevant. In case this effect also occurs in other endophyte–pathogen combinations, farmers would need to make sure that the plant is not already infected by bacterial pathogens when they apply endophytes for biocontrol purposes.

Acknowledgements

This investigation was supported by SAGARPA-CONACyT grant 2009-109621 to M.H. We gratefully acknowledge valuable comments on earlier versions of this manuscript made by two anonymous referees and by Caroline Woods. The authors report no financial or other conflict of interests relevant to the subject of this study.

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Supporting Information

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

Table S1. Annotation results for endophytes isolated from Phaseolus lunatus and sequences of their 18S rRNA genes.