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The use of fungal entomopathogens as endophytes in biological control: a review

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ABSTRACT

Fungal entomopathogens have been proposed as environmentally friendly alternatives to chemical control. Unfortunately, their effectiveness continues to be limited by their susceptibility to ultraviolet (UV) light and low moisture. A relatively recent development, the use of fungal entomopathogens as endophytes, might overcome the traditional obstacles impeding the widespread adoption of fungal entomopathogens and also provide a novel alternative for management of insect pests and plant pathogens. In addition, some fungal entomopathogens could also function as biofertilizers. Eighty-five papers covering 109 individual fungal entomopathogen studies involving 12 species in six genera are reviewed. Thirty-eight plant species in 19 families were studied, with maize, common bean, and tomato being the most investigated. Of the 85 papers, 39 (46%) examined the effects of fungal entomopathogen endophytism on 33 insect species in 17 families and eight orders. Thirty-four (40%) examined plant response to endophytism, corresponding to 20 plant species. Various inoculation techniques (e.g., foliar sprays, soil drenching, seed soaking, injections, etc.) are effective in introducing fungal entomopathogens as endophytes, but colonization appears to be localized and ephemeral. The field of insect pathology will not substantially profit from dozens of additional studies attempting to introduce fungal entomopathogens into a wider array of plants, without attempting to understand the mechanisms underlying endophytism, the responses of the plant to such endophytism, and the consequent responses of insect pests and plant pathogens. This review presents several areas that should receive focused attention to increase the probability of success for making this technology an effective alternative to chemical control.

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Beauveria; endophytic; entomopathogenic fungi; *Metarhizium*

INTRODUCTION

Biological control of insect pests involves the use of living organisms to reduce pest populations (Eilenberg et al. 2001). These living organisms include other insects that act as parasitoids or as predators, and entomopathogenic microbial agents such as fungi, bacteria, viruses, and protozoa (Vega and Kaya 2012; Heimpel and Mills 2017). Although fungal entomopathogens have been studied for more than 100 years, their field efficacy and commercial adoption are limited by susceptibility to ultraviolet (UV) light and low moisture, and problems with field application, reaching the target pest, and an absence of cost/benefit analyses. From this experience, based on using entomopathogens in a manner analogous to chemical insecticides (i.e., as sprays), there is interest in using fungal entomopathogens as fungal endophytes to determine their effects on insect pests and/or plant pathogens, as well as on the plant. Fungal endophytes (hereafter referred to as endophytes) are fungi that at some point in their life cycle inhabit the internal tissues of a plant without causing any adverse symptoms (Carroll 1986; Wilson 1995; Hyde and Soytong 2008). Most endophytic associations are generally recognized within the Ascomycota and involve two fundamentally different kinds of relationships between the host plant and colonizing fungus: clavicipitalean and nonclavicipitalean (Petrini 1986; Carroll 1988; Rodriguez et al. 2009). A vast body of literature shows mutualistic associations between clavicipitalean endophytes (e.g., *Neotyphodium*) and grasses (e.g., *Festuca, Lolium*; White et al. 2003; Cheplick and Faeth 2009 and references therein) and, most recently, a symbiotic association between *Periglandula* spp. and morning glories (Convolvulaceae) (Beaulieu et al. 2015). Although the broader category of nonclavicipitalean endo-

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phytes mostly involves members of subphylum Pezizomycotina in the Ascomycota, endophytic Basidiomycota, Glomeromycota, and Zygomycota are also reported (Rodriguez et al. 2009; Huang et al. 2015; Shi et al. 2016). Atsatt and Whiteside (2014) reported on endophytic fungi that produce a protoplast phase inside plant cells, a life stage for which Atsatt (2003) coined the term "mycosome." If the "mycosome hypothesis" (Atsatt 2003; Atsatt and Whiteside 2014) is correct and their presence is widespread, then this could explain how fungi came to be so prevalent in plants.

Colonization by various fungal entomopathogens can be established using different inoculation techniques, such as foliar sprays, soil drenching, seed soaking, and injections (TABLE 1). Unfortunately, most interest seems to focus on demonstrating endophytism by recovering the fungal entomopathogen after inoculation and limited attention is paid to possible effects on insects and plant pathogens. The field of insect pathology could substantially profit if dozens of additional experiments that attempted to introduce fungal entomopathogens into a wider array of plants had attempted to understand and optimize mechanisms underlying endophytism, the responses of the plant to such endophytism, and the consequent responses of insect pests and plant pathogens. This review considers 85 studies of endophytic fungal entomopathogens and suggests areas that should receive increased attention.

SCOPE OF THIS REVIEW

Eighty-five peer-reviewed papers were published from 1990 to 2017 describing inoculation of entomopathogens into plants using foliar sprays, soil sprays, radicle inoculations, root dipping, granular applications, soil drenching, seed treatments (e.g., soaking, coating, dressing), or injections (TABLE 1). The 85 papers encompass 109 individual fungal entomopathogen studies (20 papers presenting results for more than one fungal entomopathogen), and 93% of the studies involve just two genera: *Beauveria* (73 studies, i.e., 67%) and *Metarhizium* (28 studies, 26%). Thirty-eight plant species in 19 families were investigated, with maize (14 papers), common bean (12), and tomato (11) predominating, totaling 105 individual plant assessments (TABLES 1, 2). The vast majority of papers focus on annual or perennial crops of agronomic importance, one plant used by the pharmaceutical industry (opium poppy), and only four papers on woody perennials, i.e., coffee, cacao, date palm, and Monterey pine (TABLE 1, SUPPLEMENTARY TABLE 1).

There has been a marked increase in the number of papers published since 2010 compared with the two previous decades: 1990–1999 (5 papers); 2000–2009 (20 papers); 2010–present (60 papers) (TABLE 1). The work reported was conducted in 23 countries, distributed as follows: USA (18 papers), Spain (12), India (7), Germany (6), Canada and Kenya (5 each), Jordan and Uganda (4 each), Colombia and Mexico (3 each), Argentina, Benin, Egypt, New Zealand, and Switzerland (2 each), and Australia, China, Greece, Korea, Pakistan, Palestine, Poland, and Thailand (1 each).

POSSIBLE MECHANISMS FOR NEGATIVE EFFECTS OF ENDOPHYTISM ON INSECTS

Of the 85 papers, 39 (46%) examined the effects of fungal entomopathogen endophytism on 33 insect species in 17 families and eight orders (TABLE 1, SUPPLEMENTARY TABLE 2). Some species were studied more than once, and some papers present results on more than one insect species. Negative effects of endophytism on insects were reported in 38 studies; 2 reporting a negative effect on the third trophic level (i.e., parasitoids; Quesada-Moraga et al. 2009; Akutse et al. 2014). Three studies reported no effect on insects (Lefort et al. 2016; Ramírez-Rodríguez and Sánchez-Peña 2016a), including a parasitoid species (Jaber and Araj 2018). Possible modes of action of endophytic fungal entomopathogens were discussed by Vega (2008), Vega et al. (2008a), and McKinnon et al. (2017).

Ascribing a mechanism to explain negative effects of introduced fungal entomopathogens on insects is difficult. The only way to convincingly do this is by using endophyte-free plants, which would be difficult to obtain in the laboratory and impossible in the field, because endophytes are considered to be ubiquitous in plants from contiguous vegetation and would rapidly infest newly introduced plants (Arnold et al. 2000; Arnold and Herre 2003; Arnold 2005). At least one paper reports the absence of endophytes in field-collected plants (*Phragmites australis* subsp. *australis*) in northeastern USA (Lambert and Casagrande 2006), but subsequent work in Michigan revealed the presence of endophytes in the plants (Fischer and Rodriguez 2013).

One technique used to produce "endophyte-free" leaves is to grow plants in closed environments while preventing moisture on the leaves, followed by leaf sampling to confirm that endophytes are not present (Wilson 1993; Herre et al. 2007; Mejía et al. 2008). It would be prudent to generate endophyte-free seedlings of agricultural crops using this technique for experimental use, although the presence of seed-endophytic fungi might thwart the effort. If successful, it would

Table 1. Summary of the 85 published peer-reviewed papers on inoculation of fungal entomopathogens into plants.

Fungal entomopathogen[a]	Plant	Inoculation method	Endophytic establishment[b]	Substrate	Effect on insect assessed?[c]	Effect on plant assessed?[d]	Reference (country where work was conducted)[e]
Beauveria bassiana	*Zea mays* (Poaceae)	Foliar spray (also isolated as natural endophyte)	+	Field-grown plants	Yes (–) (*Ostrinia nubilalis*, Lepidoptera: Pyralidae)	Yes	Vakili 1990★ (USA)
B. bassiana	*Z. mays*	Granular application to the whorl; injection into the base of plant	+	Field-grown plants	Yes (–) (*O. nubilalis*)	No	Bing and Lewis 1991★ (USA)
B. bassiana	*Z. mays*	Granular application to the whorl; injection into the base of plant	+	Field-grown plants	Yes (–) (*O. nubilalis*)	No	Bing and Lewis 1992a★ (USA)
B. bassiana	*Z. mays*	Injection below primary ear	+	Field-grown plants	Yes (–) (*O. nubilalis*)	No	Bing and Lewis 1992b (USA)
B. bassiana	*Z. mays*	Pseudo-stem injection; topical application	+	Sterile soil for injection experiment; topical application for seedlings on sterile soil; transplanted to the field after treatment	Yes (–) (*Sesamia calamistis*, Lepidoptera: Noctuidae)	No	Cherry et al. 1999 (Benin)
B. bassiana	*Z. mays*	Hand brushed onto leaves; foliar spray	+	Not mentioned	No	Yes	Wagner and Lewis 2000★ (USA)
B. bassiana	*Solanum lycopersicum*[f] (Solanaceae)	Seed coating	+	Sterile vermiculite	No	No	Leckie 2002; Ownley et al. 2004 (USA)
B. bassiana	*Z. mays*	Granular formulation on foliage; seed soaking	+	Field-grown plants for granular formulations and seed soaking; sterile vermiculite for seed soaking greenhouse experiment	No	Yes	Lewis et al. 2001★ (USA)
B. bassiana	*Z. mays*	Seed dressing, topical applications into leaf axils; stem injection	n.d.	Field-grown plants	Yes (–) (*S. calamistis*)	No	Cherry et al. 2004 (Benin)
B. bassiana	*Theobroma cacao* (Malvaceae)	Radicle	+	Sterile water agar	No	No	Posada and Vega 2005 (USA)
B. bassiana, *Lecanicillium* sp.[g]	*Phoenix dactylifera* (Arecaceae)	Conidial suspension pipetted into wounded petiole	+	Field-grown plants	No	Yes	Gómez-Vidal et al. 2006 (Spain)
B. bassiana	*Coffea arabica* (Rubiaceae)	Radicle	+	Sterile water agar	No	No	Posada and Vega 2006 (USA)
B. bassiana	*Papaver somniferum* (Papaveraceae)	Foliar spray; seed dressing	+*	Lab: plants in pots (substrate not mentioned) or in Petri dishes	No	No	Quesada-Moraga et al. 2006 (Spain)
B. bassiana	*Musa* sp. (Musaceae)	Root and rhizome dip; rhizome injection; solid substrate (*B. bassiana* in rice mixed with sterile soil)	+	Tissue culture plants in sterile soil	No	Yes (+)	Akello et al. 2007 (Uganda)
Metarhizium anisopliae	*Z. mays*	Seed coating	n.d.	Field-grown plants	Yes (–) (*Agriotes obscurus*, Coleoptera: Elateridae)	Yes (+)	Kabaluk and Ericsson 2007 (Canada)
B. bassiana	*C. arabica*	Foliar sprays; stem injection; soil drench	+	Commercial seedlings transplanted to sterile potting media	No	No	Posada et al. 2007 (USA)
B. bassiana	*Musa* sp.	Root and rhizome dip	+	Tissue culture plants in sterile soil	Yes (–) (*Cosmopolites sordidus*, Coleoptera: Curculionidae) Mycosis reported	Yes (+)	Akello et al. 2008a (Uganda)

(*Continued*)

Table 1. (Continued).

Fungal entomopathogen[a]	Plant	Inoculation method	Endophytic establishment[b]	Substrate	Effect on insect assessed?[c]	Effect on plant assessed?[d]	Reference (country where work was conducted)[e]
B. bassiana	*Musa* sp.	Root and rhizome dip	+	Tissue culture plants in sterile soil	Yes (–) (*C. sordidus*) Mycosis reported.	Yes (+)	Akello et al. 2008b (Uganda)
B. bassiana	*Gossypium hirsutum* (Malvaceae), *Phaseolus vulgaris* (Leguminosae), *S. lycopersicum*	Seed dressing	+*	Gnotobiotic system (details not included in paper)	No (Paper focuses on effects on various plant pathogens)	No	Ownley et al. 2008 (USA)
B. bassiana	*Musa* sp.	Root and rhizome dip	+	Tissue culture plants in sterile soil	No	Yes	Akello et al. 2009 (Uganda)
B. bassiana, *Lecanicillium* sp.[h]	*P. dactylifera*	Injection into petioles; topical application into wounded petiole	n.d.	Field-grown plants; tissue culture plants	No	No	Gómez-Vidal et al. 2009 (Spain)
B. bassiana	*S. lycopersicum*	Seed coating	+	Seeds germinated in sterile vermiculite and transferred to sterile potting medium	Yes (–) (*Helicoverpa zea*, Lepidoptera: Noctuidae) Mycosis reported	No	Powell et al. 2009 (USA)
B. bassiana	*P. somniferum*	Seed dressing; foliar and soil sprays	+	Field-grown plants	Yes (–) (*Iraella luteipes*, Hymenoptera: Cynipidae)	Yes	Quesada-Moraga et al. 2009 (Spain)
B. bassiana	*Sorghum bicolor* (Poaceae)	Conidiated rice culture placed on whorl; foliar sprays	+*	Field-grown plants	Yes (–) (*Chilo partellus*, Lepidoptera: Pyralidae) Negative effect also reported on three nontarget insect pests	Yes (+)	Reddy et al. 2009 (India)
B. bassiana	*S. bicolor*	Seed dressing; foliar sprays; soil inoculation	+ (lowest colonization in nonsterile soil)	Sterile or nonsterile potting soil; vermiculite	No	Yes	Tefera and Vidal 2009 (Germany)
B. bassiana, *Lecanicillium lecanii*[i]	*G. hirsutum*, *Triticum aestivum* (Poaceae), *P. vulgaris*, *Z. mays*, *S. lycopersicum*, *Cucurbita maxima* (Cucurbitaceae)	Foliar sprays; solid substrate soil inoculation (cotton and wheat)	+ for foliar sprays; variable for soil inoculations	Sterile potting mix (black clay soil, sand, peat)	Yes (–) (*Aphis gossypii*, Hemiptera: Aphididae; *Chortoicetes terminifera*, Orthoptera: Acrididae)	Yes (+)	Gurulingappa et al. 2010 (Australia)
M. anisopliae	*S. lycopersicum*	Soil inoculation	+	Sterile vermiculite	No	Yes (+)	García et al. 2011 (Argentina)
B. bassiana, *M. anisopliae*	*Vicia faba* (Leguminosae)	Seed soaking followed by reinoculation of seedling rhizosphere with endophyte	+	Sterile soil/sand	Yes (–) (*Acyrthosiphon pisum* and *Aphis fabae*, Hemiptera: Aphididae)	No	Akello and Sikora 2012 (Germany)
B. bassiana	*P. dactylifera*	Injection	+	Nonsterile sandy loam soil	Yes (–) (*Rhynchophorus ferrugineus*, Coleoptera: Curculionidae) Negative effect on plant pathogen (*Rhizoctonia solani*)	No	Arab and El-Deeb 2012★ (Egypt)
Metarhizium robertsii	*Panicum virgatum* (Poaceae), *P. vulgaris*	Mycosed *Galleria mellonella* placed in soil	n.d. (endophyte colonization assumed but not tested)	Sterile soil	No	No	Behie et al. 2012 (Canada)

(*Continued*)

Table 1. (Continued).

Fungal entomopathogen[a]	Plant	Inoculation method	Endophytic establishment[b]	Substrate	Effect on insect assessed?[c]	Effect on plant assessed?[d]	Reference (country where work was conducted)[e]
B. bassiana	*Corchorus olitorius* (Malvaceae)	Seed soaking	+*	Sterile soil	No	No	Biswas et al. 2012 (India)
B. bassiana	*Pinus radiata* (Pinaceae)	Seed coating; root dip coatings	+ (very low recovery)	Nonsterile compost	No	No	Brownbridge et al. 2012 (New Zealand)
B. bassiana	*S. lycopersicum*	Foliar sprays; injection	+ for injection	Information not provided	Yes (–) (*Bemisia tabaci*, Hemiptera: Aleyrodidae)	Yes	El-Deeb et al. 2012★ (Egypt)
M. anisopliae	*Glycine max* (Leguminosae)	Mycelium added to soil (details not provided)	n.d.	Sterile soil	No	Yes (+)	Khan et al. 2012 (Korea)
M. robertsii	*P. virgatum, P. vulgaris*	Seed soaking; fungal plugs placed in soil	– for seed soaking; + for plugs in soil and *P. vulgaris*	Sterile moistened filter paper; sterile and nonsterile potting mixture	No	Yes (+)	Sasan and Bidochka 2012 (Canada)
B. bassiana, M. anisopliae	*V. faba, P. vulgaris*	Seed soaking	+ for *B. bassiana*; – for *M. anisopliae*	Sterile soil:manure mixture	Yes (–) (*Liriomyza huidobrensis*, Diptera: Agromyzidae)	No	Akutse et al. 2013 (Kenya)
M. anisopliae	*Brassica napus* (Brassicaceae)	Foliar sprays	+	Sterile peat moss and sand	Yes (–) (*Plutella xylostella*, Lepidoptera: Plutellidae)	No	Batta 2013 (Palestine)
B. bassiana	*Corchorus capsularis* (Malvaceae)	Seed soaking	+*	Sterile soil	Yes (–) (*Apion corchori*, Coleoptera: Apionidae)	No	Biswas et al. 2013 (India)
B. bassiana	*Fragaria* x *ananassa* (Rosaceae)	Mixing conidia into vermiculite; dipping roots; drenching	+	Vermiculite (not stated whether it was sterile)	No	No	Dara et al. 2013 (USA)
B. bassiana	*Oryza sativa* (Poaceae)	Foliar sprays	+*	Nonsterile paddy soil	No	No	Jia et al. 2013 (China)
B. bassiana	*P. somniferum*	Foliar sprays	+*	Sterile clay loam and peat mixture	No	No	Landa et al. 2013 (Spain)
B. bassiana	*Vigna unguiculata* (Leguminosae)	Spraying of leaves and stems	+	Not mentioned	No	No	Maketon et al. 2013 (Thailand)
B. bassiana	*P. vulgaris*	Foliar spray; soil drench	+	Sterile soil and sand mixture	No	No	Parsa et al. 2013 (Colombia)
M. robertsii	*P. vulgaris*	Fungal plugs placed in soil	n.d. (endophyte colonization assumed but not tested)	Sterile potting mixture	No (Paper focuses on antagonism against plant pathogen: *Fusarium solani* f. sp. *phaseoli*)	Yes (+)	Sasan and Bidochka 2013 (Canada)
B. bassiana	*V. faba*	Seed soaking	+	Sterile soil:manure mixture	Yes (–) (*Phaedrotoma scabriventris*, Hymenoptera: Braconidae; *Diglypus isaea*, Hymenoptera: Eulophidae)	No	Akutse et al. 2014 (Kenya)
B. bassiana, Purpureocillium lilacinum	*G. hirsutum*	Seed soaking	+* for greenhouse study; n.d. in field study	Greenhouse study: soaked seeds germinated in unsterilized potting medium Field study: soaked seeds directly planted in the field	Yes (–) (*A. gossypii*)	No	Castillo Lopez et al. 2014★ (USA)
M. acridum, M. robertsii	*Cucumis sativus* (Cucurbitaceae), *V. unguiculata*	Seed soaking	+	Sterile moistened filter paper	No	Yes	Golo et al. 2014 (USA)

(*Continued*)

Table 1. (Continued).

Fungal entomopathogen[a]	Plant	Inoculation method	Endophytic establishment[b]	Substrate	Effect on insect assessed?[c]	Effect on plant assessed?[d]	Reference (country where work was conducted)[e]
B. bassiana	*Echinacea purpurea* (Asteraceae)	Seed coating	+	Commercial substrate (Turface)	No	Yes (+)	Gualandi et al. 2014 (USA)
B. bassiana	*Cynara scolymus* (Compositae)	Foliar sprays	+	Not mentioned	No	No	Guesmi-Jouini et al. 2014 (Spain)
B. bassiana	*Cucurbita pepo* (Cucurbitaceae)	Foliar spray	+	Sterile sandy loam:peat mixture	No (Paper focuses on effects on plant pathogen: zucchini yellow mosaic virus)	No	Jaber and Salem 2014 (Jordan)
B. bassiana, M. anisopliae, M. robertsii	*S. bicolor*	Foliar sprays	+	Nonsterile sand:peat mixture	Yes (–) (*Sesamia nonagrioides*, Lepidoptera: Noctuidae)	Yes	Mantzoukas et al. 2015 (Greece)
Clonostachys rosea	*Allium cepa* (sin)	Seed soaking or soaking of roots from uprooted plants followed by replanting	Yes	Sterilized mixture of red soil and livestock manure (5:1)	Yes (–) (*Thrips tabaci*, Thysanoptera; Thripidae)	No	Muvea et al. 2014 (Kenya)
B. bassiana	*P. somniferum*	Seed soaking	+*	Sterile clay loam:peat mixture	No	No	Quesada-Moraga et al. 2014 (Spain)
B. bassiana, M. anisopliae, Metarhizium brunneum	*Brassica oleracea* (Brassicaceae)	Pipetting into soil in seedling stage	+	Seeds germinated in commercial potting medium, transplanted to pots containing sterile or nonsterile field soil	No	Yes (+)	Razinger et al. 2014 (Switzerland)
B. bassiana, M. robertsii	*P. vulgaris*	Fungal plug placed in soil	+	Sterile soil potting mixture	No	No	Behie et al. 2015 (Canada)
B. bassiana	*C. olitorius*	Foliar sprays	+*	Field-grown plants	No	No	Biswas et al. 2015 (India)
B. bassiana, P. lilacinum	*G. hirsutum*	Seed soaking	n.d.	Nonsterile potting medium	Yes (–) (*H. zea*)	Yes (+)	Castillo Lopez and Sword 2015 (USA)
B. bassiana	*Vitis vinifera* (Vitaceae)	Foliar spray	+*	Nonsterile soil	No (Paper focuses on effects on plant pathogen: *Plasmopara viticola*)	No	Jaber 2015 (Jordan)
B. bassiana	*B. napus*	Various formulations: beads placed in roots; seed film coatings; liquid formulations for topical application	+*	Sterile or nonsterile soil: sand mixture	No	No	Lohse et al. 2015 (Germany)
Metarhizium pingshaense	*Z. mays*	Seed soaking	+	Not mentioned	Yes (–) (*Anomala cincta*; Coleoptera: Scarabaeidae)	No	Peña-Peña et al. 2015 (Mexico)
B. bassiana	*S. lycopersicum*	Root dip; stem injection; solid substrate (*B. bassiana* in rice mixed with sterile soil); foliar spray	+	Not mentioned	Yes (–) (*Helicoverpa armigera*: Lepidoptera: Noctuidae)	Yes (–)	Qayyum et al. 2015 (Pakistan)
B. bassiana	*Z. mays, T. aestivum, G. max, Nicotiana tabacum* (Solanaceae)	Seed soaking; foliar spray; root immersion	+	Sterile mixture of perlite, vermiculite, soil	No	Yes	Russo et al. 2015 (Argentina)
B. bassiana	*S. lycopersicum, T. aestivum*	Seed soaking	+	Sterile simulated calcareous substrates	No	Yes (+)	Sánchez-Rodríguez et al. 2015 (Spain)

(*Continued*)

Table 1. (Continued).

Fungal entomopathogen[a]	Plant	Inoculation method	Endophytic establishment[b]	Substrate	Effect on insect assessed?[c]	Effect on plant assessed?[d]	Reference (country where work was conducted)[e]
B. bassiana	*S. lycopersicum*	Seed dressing	+*	Nonsterile calcined montmorillonite clay	Yes (–) (*Spodoptera exigua*; Lepidoptera: Noctuidae)	No	Shrivastava et al. 2015 (USA)
B. bassiana	*B. napus, V. faba*	Foliar spray	+	Not mentioned	Yes (–) (*H. armigera* - tested on *V. faba*) Mycosis reported.	No	Vidal and Jaber 2015 (Germany)
B. bassiana	*P. vulgaris*	Seed soaking	+	Field experiment	Yes (–) (*L. huidobrensis*, L. *sativae, L. trifolii*)	Yes (+)	Gathage et al. 2016 (Kenya)
B. bassiana	*B. oleracea*	Foliar spray	+*	Sterile mixture of soil; vermicompost, peat	Yes (–) (*P. aphidiustella*)	No	Gautam et al. 2016 (India)
B. bassiana, M. anisopliae	*Manihot esculenta* (Euphorbiaceae)	Soil drench	+	Sterile soil	No	Yes (+)	Greenfield et al. 2016 (Colombia)
B. bassiana, M. brunneum	*V. faba*	Seed soaking	+	Sterile soil; sand; peat	No	Yes (+)	Jaber and Enkerli 2016 (Jordan)
M. anisopliae	*Camellia sinensis* (Theaceae)	Foliar spray; soil drench	+	Field-grown plants	No	No	Kaushik and Dutta 2016 (India)
B. bassiana	*S. lycopersicum*	Foliar spray	+	Not mentioned	Yes (–) (*Tuta absoluta*, Lepidoptera: Gelechiidae) Mycosis reported	Yes	Klieber and Reineke 2016 (Germany)
C. rosea, Isaria fumosorosea, M. anisopliae	*Quercus robur* (Fagaceae)	Soil drench	+ (*C. rosea* only) –	Sterile sandy forest soil	No	Yes (+, –)	Kwaśna and Szewczyk 2016 (Poland)
B. bassiana	*P. radiata*	Natural occurrence in seeds and 35-y-old trees	+ (1/30 seedlings)	Nonsterile potting mix	Yes (–) (*Costelytra zealandica*, Coleoptera: Scarabaeidae, belowground insect pest) No effect (*H. armigera*)	No	Lefort et al. 2016 (New Zealand)
B. bassiana, M. anisopliae	*P. vulgaris*	Seed soaking	+	Sterile mixture of manure and soil	Yes (–) (*Ophiomyia phaseoli*, Diptera: Agromyzidae)	No	Mutune et al. 2016 (Kenya)
B. bassiana	*Z. mays*	Foliar spray	+*	Sterile soil	No	No	Renuka et al. 2016 (India)
B. bassiana	*Z. mays*	Seed coating; foliar and stem spray	+	Peat moss	Yes (no effect) (*Spodoptera frugiperda*, Lepidoptera: Noctuidae)	No	Ramírez-Rodríguez and Sánchez-Peña 2016a (Mexico)
B. bassiana	*P. vulgaris*	Seed coating	+	Sterile peat moss	No	No	Ramírez-Rodríguez and Sánchez-Peña 2016b (Mexico)
B. bassiana, M. brunneum	*Cucumis melo* (Cucurbitaceae), *Medicago sativa* (Leguminosae), *S. lycopersicum*	Foliar spray	+	Sterile commercial substrate	Yes (–) (*Spodoptera littoralis*, Lepidoptera: Noctuidae)	No	Resquín-Romero et al. 2016 (Spain)
M. brunneum	*Solanum tuberosum* (Solanaceae)	Foliar spray	+	Sterile commercial substrate	No	No	Ríos-Moreno et al. 2016 (Spain)
B. bassiana, M. brunneum	*C. melo*	Foliar spray	+	Sterile substrate based on washed sand	Yes (–) (*Bemisia tabaci*, Hemiptera: Aleyrodidae)	No	Garrido-Jurado et al. 2017 (Spain)

(*Continued*)

Table 1. (Continued).

Fungal entomopathogen[a]	Plant	Inoculation method	Endophytic establishment[b]	Substrate	Effect on insect assessed?[c]	Effect on plant assessed?[d]	Reference (country where work was conducted)[e]
B. bassiana, *M. brunneum*	*Capsicum annuum* (Solanaceae)	Soil drench	+	Sterile soil:sand:peat (1:1:1)	Yes (–) for *Myzus persicae* (Homoptera: Aphididae) No effect on *Aphidius colemani* (Hymenoptera: Braconidae)	Yes (+)	Jaber and Araj 2017 (Jordan)
B. bassiana, B. brongniartii, M. brunneum	*V. faba*	Foliar spray	+	Nonsterile compost	No	Yes (+)	Jaber and Enkerli 2017 (Switzerland)
B. bassiana, M. anisopliae	*P. vulgaris*	Seed soaking	+	Sterile sand:peat; sterile vermiculite; nonsterile field-collected soils	No	No	Parsa et al. 2018 (Colombia)
B. bassiana	*V. vinifera*	Foliar spray (greenhouse and field)	+	Clay/white peat substrate	Yes (–) (*Planococcus ficus*, Homoptera: Pseudococcidae)	No	Rondot and Reineke 2018 (Germany)
B. bassiana	*T. aestivum, T. durum*	Soil treatment; seed soaking; foliar spray	+	Sterile sandy soil	Yes (–) (*S. littoralis*)	Yes (+)	Sánchez-Rodríguez et al. 2018 (Spain)

Phylum Ascomycota, Order Hypocreales, families Bionectriaceae (*Clonostachys*), Clavicipitaceae (*Metarhizium*), Cordycipitaceae (*Beauveria, Isaria, Lecanicillium* [current name: *Akanthomyces*]), and Ophiocordycipitaceae (*Purpureocillium*).
b"+" = fungal entomopathogen was detected in the inoculated plants. "–" = fungal entomopathogen was not detected in the inoculated plants.
"*" = molecular methods were used for detection. "n.d." = not determined.
c"(–)" = at least one negative effect on the insect was reported.
d"(+)" = at least one positive effect on the plant was reported. "Yes" without "+" sign indicates no differences between treated and control plants. (–) = at least one negative effect on the plant was reported.
e"★" denotes a paper not reporting the use of a sterilization technique prior to isolating endophytes.
*f*The preferred scientific name for tomatoes is *Solanum lycopersicum* (Spooner et al. 2005), instead of *Lycopersicon esculentum*.
*g*Current name: *Akanthomyces.*
*h*Current name: *Akanthomyces.*
*i*Current name: *Akanthomyces lecanii*.

allow researchers to clearly assess the effects of endophytic fungal entomopathogens when no other endophytes are present. Naturally occurring endophytes within one crop species can exhibit wide variation in species composition and infection frequencies (Vega et al. 2010; Parsa et al. 2016). The term "inconstant microbiota," used to define the variation in the internal gut microbiota in insects (Wong et al. 2013), also could be used to reflect the endophyte situation in plants, i.e., the inconstant endophyte community (inconstant microbiota), which is unpredictable in time and space. This inconstant microbiota is a confounding variable that cannot be controlled in the field. Several scenarios exist, all based on the presence of an inconstant microbiota: (i) plant inoculation with the fungal entomopathogen (e.g., foliar spray using 10^8 conidia mL^{-1}) results in colonization, with either an induction of plant defenses or no induction, neither affecting insects; (ii) colonization induces plant defenses (e.g., salicylic and/or jasmonic acid pathways), with negative effects on insects. In this scenario, the effect is indirect, i.e., mediated by the fungal entomopathogen but not caused by it; in such a case, a nonentomopathogenic fungus might also induce the same effect; (iii) the introduced fungal entomopathogen could be "sensed" by other endophytic fungi (competitors), which might respond in various ways (e.g., metabolite production) that induce plant defenses; this would be another example of indirect effects; (iv) colonization results in metabolite production by members of the unknown inconstant microbiota, which has a direct negative effect on insects (e.g., antibiosis, feeding deterrence), yet another indirect effect; and (v) colonization results in production of fungal metabolites by the introduced entomopathogen, causing a direct negative effect on the insect. None of these scenarios depend on insect infection by the fungal entomopathogen.

Feeding on hyphae.—Wagner and Lewis (2000) reported on the presence of *B. bassiana* hyphae in the vascular tissue of corn plants, and several papers reported negative effects of *B. bassiana* endophytism on the Lepidopteran *Ostrinia nubilalis* (TABLE 1), but

Table 2. Reports of detection of fungal entomopathogens naturally infecting plants in the field.

Fungal species	Plant (common name) (family)	Reference (country where samples originated)
Beauveria sp.	*Coffea arabica* (coffee) (Rubiaceae)	Vega et al. 2010 (Colombia)
	Theobroma cacao (cacao) (Malvaceae)	Amin et al. 2014 (Indonesia)
	Zea mays (maize) (Poaceae)	Pimentel et al. 2016 (Brazil)
Beauveria bassiana	*Atractylodes lancea* (Compositae)	Lü et al. 2014 (China)
	Carpinus caroliniana (ironwood) (Betulaceae)	Bills and Polishook 1991 (USA)
	C. arabica	Posada et al. 2007 (USA); Vega et al. 2008a (Colombia); Vega et al. 2010 (Colombia, USA)
	Dactylis glomerata (orchard grass) (Poaceae)	Sánchez Márquez et al. 2007 (Spain)
	Datura stramonium (jimsonweed) (Solanaceae)	Jones 1994 (USA)
	Gossypium hirsutum (cotton) (Malvaceae)	Jones 1994 (USA)
	Pinus monticola (western white pine) (Pinaceae)	Ganley and Newcombe 2006 (USA)
	Pinus radiata (Monterey pine) (Pinaceae)	Reay et al. 2010 (New Zealand); Lefort et al. 2016 (New Zealand)
	Theobroma gileri (Malvaceae)	Evans et al. 2003 (Ecuador)
	Z. mays	Vakili 1990; Jones 1994; Pingel and Lewis 1996; Arnold and Lewis 2005 (USA)
Beauveria brongniartii	*C. arabica*	Vega et al. 2010 (USA)
Clonostachys rosea	*C. arabica*	Vega et al. 2008a (Colombia)
	Quercus robur (English oak) (Fagaceae)	Kwaśna et al. 2016 (Poland)
Cordyceps sobolifera (current name: *Ophiocordyceps sobolifera*)	*T. cacao*	Rubini et al. 2005 (Brazil)
Isaria farinosa (current name: *Cordyceps farinosa*)	*Fagus sylvatica*	Unterseher and Schnittler 2010 (Germany)
	Q. robur	Kwaśna et al. 2016 (Poland)
Isaria fumosorosea (current name: *Cordyceps fumosorosea*)	*Q. robur*	Kwaśna et al. 2016 (Poland)
Lecanicillium lecanii (current name: *Akanthomyces lecanii*)	*D. glomerata*	Sánchez Márquez et al. 2007 (Spain)
Metarhizium anisopliae	*Glycine max* (soybean) (Fabaceae)	Khan et al. 2012 (Korea)
	Q. robur	Kwaśna et al. 2016 (Poland)
	Taxus chinensis (Chinese yew) (Taxaceae)	Liu et al. 2009*a* (China)
Paecilomycesb sp.	*C. arabica*	Posada et al. 2007 (USA); Vega et al. 2008a (Mexico, USA); Vega et al. 2010 (Colombia, Mexico, Puerto Rico)
	Musa acuminata (banana) (Musaceae)	Cao et al. 2002 (China)
	Oryza sativa (rice) (Poaceae)	Tian et al. 2004 (China)
	G. max	Pimentel et al. 2006 (Brazil)
	Z. mays	Pimentel et al. 2016 (Brazil)
P. farinosus (current name: *Cordyceps farinosa*)	*C. caroliniana*	Bills and Polishook 1991 (USA)
P. cf. *fumosoroseus* (= *Cordyceps* cf. *fumosorosea*)	*C. arabica*	Vega et al. 2008a (Puerto Rico)
Paecilomyces cf. *javanicus* (= *Cordyceps* cf. *javanica*)	*C. arabica*	Vega et al. 2008a (Colombia)
Verticillium lecanii (current name: *Akanthomyces lecanii*)	Araceae	Petrini 1981 (Switzerland)
	Arctostaphylos uva-ursi (Ericaceae)	Widler and Müller 1984 (Switzerland)
	C. caroliniana	Bills and Polishook 1991 (USA)

[a]Endophytic *Metarhizium anisopliae* produced high levels of the drug taxol (paclitaxel). See also Gond et al. (2014).
[b]Molecular analysis resulted in assigning samples to *Paecilomyces*. Entomopathogenic *Paecilomyces* species have been transferred to *Isaria* (Luangsa-ard et al. 2005).

none experimentally attempted to elucidate the mechanism. To determine the effects of hyphal consumption by corn earworm (*Helicoverpa zea*), Leckie (2002) and Leckie et al. (2008; see also Ownley et al. 2004) grew *B. bassiana* or *M. anisopliae* in liquid culture, harvested mycelia, dried and ground it to a fine powder, then incorporated the mycelium at different concentrations into an artificial diet used to rear neonate larvae. Negative effects depended upon hyphal powder concentration and included high mortality, delayed development, and lower larval and pupal weights. These negative effects were ascribed to the possible presence of metabolites in hyphae or to deterred feeding. Purification of the

metabolites and testing of pure metabolites would corroborate these observations. Interestingly, low hyphal concentrations resulted in higher larval and pupal weights, and shorter time to pupation, perhaps a consequence of increase in nutritional components being provided by the hyphae.

Mycosis.—Most endophyte-related studies have not examined vascular tissues for sporulation or mycelial colonization following endophytic establishment. Sporulation, growth, and distribution of mycelium could be assessed by examining histological preparations, which might or might not help provide a conclusive taxonomic identification for the fungus, unless it is proven that control plants are absolutely devoid of spores or hyphae. No studies are published explaining why endophyte sporulation or mycelial colonization might be inhibited in planta, although some theoretical possibilities could be proposed: (i) production of spores inside vascular tissue serves no practical purpose to the fungus (i.e., sporulation as a dead end), and consequently, energy expenditure would not be warranted; and (ii) factors needed to trigger sporulation are absent, perhaps including specific nutritional components, appropriate pH and photoperiod, etc. Fungal sporulation is vastly studied, and the literature might provide insights on possible reasons impeding sporulation in planta, if that is actually the case.

Two papers dealing with fungal entomopathogens report the presence of conidia in planta. Figure 1F in Wagner and Lewis (2000) presents a "conidium inside epidermal cell" in maize. The presence of a purported *B. bassiana* conidium in vascular tissues is perplexing. How could it gain access to the interior of an epidermal cell 12–48 h after leaves were inoculated? This would require complete in toto penetration of the conidium through the epidermal layer. Furthermore, how do we know that it is a *B. bassiana* conidium? Having additional information on how often such occurrences were recorded would have been invaluable. Similarly, Maketon et al. (2013) included a photograph of what is identified as *B. bassiana* conidia in parenchymal cells and vascular tissue. The paper lacks information on how its identity was determined or on how common the occurrence was. Finally, Kaushik and Dutta (2016) included photographs of purported *M. anisopliae* intravascular growth, including conidia and conidiophores, but the evidence is insufficient to definitively prove fungal identity.

If fungal entomopathogens do not sporulate in planta, then the infective propagule (spores) would be unable to infect insects and consequently an insect infection could not proceed in the usual sense of a spore landing on the insect cuticle, forming a germ tube, penetrating the cuticle, and reaching the hemocoel, where it propagates and eventually causes mycosis (Vega et al. 2012). Consequently, it is interesting to find six papers reporting insect mycosis following feeding on plants with fungal entomopathogen endophytism, and all cases involve *B. bassiana* (Powell et al. 2007, 2009; Akello et al. 2008a, 2008b; Vidal and Jaber 2015; Klieber and Reineke 2016) and chewing insects, i.e., *Cosmopolites sordidus* (Coleoptera) and *Helicoverpa zea, H. armigera*, and *Tuta absoluta* (Lepidoptera). The mechanism leading to mycosis remains unknown. Powell et al. (2009) speculated, "It is plausible that if hyphae are consumed intact and in sufficient quantity, successful mycosis might result." Oral infection in insects is reported (Gabriel 1959; Broome et al. 1976) but not conclusively proven with endophytic fungal entomopathogens and might be unlikely. For this to occur, endophytism would have to be systemic (i.e., throughout the entire plant or, at least, throughout the entire plant tissues insects are feeding on), which has not been reported. Nevertheless, Powell et al.'s (2009) hypothesis could be experimentally tested with plant material known to have ample endophytic growth (via staining or green fluorescent protein [GFP]-transformed entomopathogen or fluorescent in situ hybridization [FISH]) throughout the plant tissues consumed by a chewing insect. One possible mechanism leading to mycosis could be saprophytic growth by endophytic *B. bassiana* in plant tissues damaged by an insect, followed by sporulation, cuticular infection and penetration, and subsequent mycosis.

Herbivore-induced plant volatiles (HIPVs).—Lin et al. (2016, 2017) reported enhanced conidial performance (e.g., increased conidial germination and appressorial formation) and pathogenicity of *Lecanicillium lecanii* (current name: *Akanthomyces lecanii*; Hypocreales) as a result of HIPVs, in this case, damage caused by aphids. In contrast, working with tobacco, Brown et al. (1995) reported delayed germination of *Pandora neoaphidis* (Entomophthorales) conidia as a result of HIPVs; this effect is purportedly beneficial to the fungus because it provides additional time for the conidium to come in contact with the insect host. Finally, Hountondji et al. (2009) found that depending on the *Neozygites tanajoae* (Entomophthorales) strain, cassava HIPVs increased conidial or capilliconidial production. These papers serve as evidence for the importance and need of elucidating possible effects of HIPVs on Hypocrealean endophytic fungal entomopathogens.

Kairomones.—Another negative effect on insects from fungal entomopathogen endophytism may be alteration of kairomones, chemical signals produced by plants and used by insects to find the plant, i.e., favorable to the receiver but not to the emitter (Price et al. 2011). If an insect is using a kairomone to find its host plant and an endophyte is altering the chemical signals produced by the plant, this would make it more difficult for the insect to find the plant, which could have negative effects on insect behavior and fitness. In choice tests involving the nonfungal entomopathogen *Hypocrea lixii* (current name: *Trichoderma lixii*), Muvea et al. (2015) reported a preference by *Thrips tabaci* for *H. lixii*–free plants. The tests included use of a Y-tube olfactometer, and the results suggest the presence of repellent volatiles in the endophytic plants. In other experiments also involving a nonfungal entomopathogen, Daisy et al. (2002) detected the production of the volatile insect repellent naphthalene by the endophytic fungus *Muscodor vitigenus*.

Fungal secondary metabolites.—Because the vast majority of negative effects of fungal entomopathogen endophytism on insects do not involve mycosis, it has been proposed that negative effects could be a result of antibiosis and feeding deterrence mediated by in planta production of fungal secondary metabolites (Cherry et al. 2004; Akello et al. 2008b; Vega 2008; Vega et al. 2008a).

Although it does not involve fungal entomopathogens, Miller et al.'s (2002) work on rugulosin as a deterrent to spruce budworm (*Choristoneura fumiferana*) in white spruce (*Picea glauca*) is the best-documented case of deliberate and successful use of an endophyte to deter insect feeding.

Antibiosis could be tested by inoculating plants with fungal entomopathogens known to produce specific metabolites. The main obstacle is that there must be positive evidence that the fungus produces the metabolite in planta, i.e., it must be detectable. As stated by Fan et al. (2017), there is a "lack of information about the conditions under which fungal secondary metabolites are produced." In other words, the metabolite might not be produced in planta, even if the strain produces the metabolite in vitro. Ideally, the only difference between control and treated plants should be the presence of the metabolite, which would have to be detectable at levels within the range causing adverse effects on insects in laboratory bioassays. Only then would it be possible to infer whether a metabolite being produced by an endophytic fungal entomopathogen is affecting the test insect.

Another issue to consider is that "there is difficulty in establishing clear biological roles for many secondary metabolites" (Fan et al. 2017). For example, oosporein, a red dibenzoquinone produced by *B. bassiana* (Vining et al. 1962) and *B. brongniartii* (Strasser et al. 2000a), among many other fungi (see Feng et al. 2015), has insecticidal activity when topically applied (Amin et al. 2010). Feng et al. (2015) showed that it is involved in altering insect immunity, thus promoting infection. Fan et al. (2017) demonstrated that oosporein reduces bacterial competition after the insect dies, thus allowing the fungus to continue to utilize nutrients and eventually sporulate.

Leckie (2002) and Leckie et al. (2008) grew *B. bassiana* in liquid culture, removed the mycelia, and incorporated filtered broth into corn earworm diet as a proxy to determine the effects of unknown excreted metabolites. Overall, there was delayed development when insects fed on diets containing different concentrations of broth; at the highest concentration, there was a reduced percent pupation and a longer time to pupation. Beauvericin was detected in the broth cultures.

Three studies involving endophytic fungal entomopathogens analyzed the presence of fungal secondary metabolites, more specifically, destruxins (a cyclic hexadepsipeptide; Pedras et al. 2002). Golo et al. (2014) detected destruxins (DTX A, DTX B, DTX E) in cowpea plants endophytically colonized by *Metarhizium robertsii*, but not in endophytically colonized cucumber plants. Destruxins were not detected in cowpea or cucumber plants endophytically colonized by *Metarhizium acridum*.

Resquín-Romero et al. (2016) reported trace levels of DTX A in *Spodoptera littoralis* sprayed with a *Metarhizium brunneum* conidial suspension and then fed for 72 h on tomato leaves endophytically colonized by *M. brunneum*; DTX A was also detected in endophytically colonized melon and tomato leaves. Ríos-Moreno et al. (2016) detected DTX A in potato plants endophytically colonized by *M. brunneum*. Garrido-Jurado et al. (2017) detected DTX A in *Bemisia tabaci* nymphs (Hemiptera) fed on melon leaves endophytically colonized by *M. brunneum*.

In addition to the metabolites issues discussed above, it is important to consider whether introduction of fungal entomopathogens as endophytes might result in the introduction of their metabolites to the food chain. Regulation of metabolites produced by biocontrol agents is addressed by the European Union (1991), Strasser et al. (2000b), and the Organisation for Economic Co-operation and Development (2008),

among others. Related to this issue, Seger et al. (2005) developed a detection method for oosporein in potato tubers after application of a commercial formulation of *B. brongniartii* in potato fields, where it is used to control cockchafer larvae (*Melolontha melolontha*). Oosporein concentrations were below the levels of detection. Similar results were previously reported by Abendstein et al. (2000).

The sequencing and annotation of the genomes for *M. acridum* and *M. robertsii* (Gao et al. 2011), *B. bassiana* (Xiao et al. 2012), and *M. anisopliae* (Pattemore et al. 2014) provides useful information for determining their capacity to encode secondary metabolites, as discussed by Gibson et al. (2014).

Finally, based on the presence of such a wide diversity of endophytes in plants, and the fact that fungi are metabolite producers, it would be informative to determine how many different metabolites can be detected in planta, in tandem with a survey of what endophytes are present. Alternatively, it might be possible to amplify transcripts of the main gene encoding biosynthesis of the target metabolite. This would allow for a better understanding of whether there is a "metabolite soup" in planta or whether, despite the presence of so many fungi, metabolite presence is negligible. In addition, it is important to recognize that plants themselves could also produce metabolites that negatively affect fungal entomopathogens (Vega et al. 1997; Lacey and Mercadier 1998).

Plant defense induction.—Terpenoids are secondary plant metabolites with antiherbivore properties, among other properties (Gershenzon and Croteau 1991; Fürstenberg-Hägg et al. 2013). Tomato plants endophytically colonized by *B. bassiana* had significantly higher levels of two monoterpenes (δ-2-carene, sabinene) and three sesquiterpenes (δ-elemene, (*E*)-β-caryophyllene, α-humulene) than control plants, and the weight of beet armyworms (*Spodoptera exigua*) fed on colonized plants was significantly lower than in control plants (Shrivastava et al. 2015). The mechanism for increased terpenoid levels remains unknown. *Echinacea purpurea* plants colonized by *B. bassiana* exhibited no differences in contents of three sesquiterpenes ((*E*)-β-caryophyllene, germacrene D, and α-humulene) or two phenolic acids (cafteric and cichoric acid), in contrast to concentration of one alklylamede, which increased in plants colonized by *B. bassiana* growing at high phosphorus levels (Gualandi et al. 2014)

EXPERIMENTAL ISSUES

Sterilization of plant material.—Various methods are used to surface sterilize plants for subsequent isolation of endophytes. For reviews on the topic, see Schulz et al. (1993), McKinnon (2016), and McKinnon et al. (2017). When isolating endophytes from plants, it is imperative to verify that the surface sterilization method has been effective. McKinnon et al. (2017) reviewed sterilization techniques reported in 55 papers dealing with *B. bassiana* and found that 21 did not assess the efficacy of the sterilization protocol. One way to do this is to press the sterilized tissue onto agar (Schulz et al. 1998; Akello and Sikora 2012; Greenfield et al. 2015), followed by its removal and subsequent observation of the plates receiving the imprint. If fungal or bacterial growth is observed, then the sample should be discarded.

These methods have unexplored limitations. They do not adequately address the question of viability of fungal epiphytes that may form melanized multicellular hyphal structures on the plant surface, nor do they consider that some fungi, e.g., coprophilous fungi, can produce disinfectant-resistant ascospores designed to adhere to leaf surfaces. Such thick-walled, and often heavily pigmented, spores may resist common disinfection protocols. Another commonly used method involves the plating of aliquots of the water used to wash the tissues after the tissues have gone through the disinfectants, which are usually diluted bleach and ethanol (McKinnon et al. 2017). The latter method needs to be used cautiously, as aliquot plating might not yield growth, but imprints of the same material might, indicating that the aliquot method was not reliable (M. Greenfield, pers. comm.).

Soil sterilization.—Parsa et al. (2018) reviewed factors related to the use of sterile substrates in experiments involving inoculation of plants with endophytes. First of all, it is important to recognize difficulties encountered in sterilizing soil and that sterilization methods may have profound effects on the chemical and physical properties of the substrate (Parsa et al. 2018). It is also relevant to acknowledge that the use of sterile substrates, although necessary to limit the experimental variables, does not mimic field conditions. Therefore, to approximate field situations, nonsterile substrates should be included in experiments to gain a better understanding of the unconsidered variables of the inner workings in each system. The concept of external validity should become a beacon in future experiments, as espoused by Rosenheim et al. (2011), who state, "Narrowly controlled

environmental conditions of experimental studies give strong "internal validity" but may restrict the ability to extend conclusions to situations of different environmental conditions (i.e., limited "external validity")." Adopting this approach should help us design experiments with a higher chance of eventual field success.

PLANT COLONIZATION

Vertical transmission.—Vertical transmission of endophytes can be defined as the passage of an endophytic fungus from a plant to its progeny through seeds (Saikkonen et al. 2004). Clavicipitaceous endophytes of grasses are vertically transmitted (Carroll 1988; Schardl et al. 2004; Cheplick and Faeth 2009), and Philipson and Christey (1986) have described the mechanism, summarized by Scott and Schardl (1993): "Following floral meristem development, the mycelia invade the ovaries and developing ovules, eventually occupying the mature seed including the scutellum of the embryo. Infected seed gives rise to infected plants so that the endophytes are maternally transmitted into the next generation." Nonclavicipitaceous fungal seed endophytes (Bloomberg 1966; Vega et al. 2008b; Parsa et al. 2016) as well as bacterial seed endophytes (Truyens et al. 2015) have been widely reported. Morning glory endophytes also are seed transmitted (Beaulieu et al. 2015). Vertical transmission of endophytic fungal entomopathogens was reported for *B. bassiana* by Quesada-Moraga et al. (2014), Lefort et al. (2016), and Sánchez-Rodríguez et al. (2018). This is noteworthy because transmission of most nongrass endophytes has been assumed to be horizontal (Carroll 1988). The mechanism whereby *B. bassiana* enters seed needs further investigation.

It would be interesting to determine if fungal entomopathogens can be introduced to seeds by spraying inflorescences with a conidial suspension. Vega et al. (unpubl.) attempted this with *B. bassiana* and coffee inflorescences to determine possible effects on the coffee berry borer (*Hypothenemus hampei*), an insect that feeds on the coffee seed. Results from work done in Mexico were negative, i.e., *B. bassiana* was not detected in the ensuing coffee berries.

Studies focused in vertical transmission of fungal entomopathogens should also assess the presence of endophytes in pollen, because "the mycology of pollen is extremely poorly known" and pollen grains "provide good opportunities for the attachment of fungal spores" (Hodgson et al. 2014).

Transient colonization.—Several studies report transient endophytic colonization, i.e., the fungal entomopathogen detected in the plant for only a limited period of time (Posada et al. 2007; Biswas et al. 2012; Landa et al. 2013; Russo et al. 2015; Renuka et al. 2016; Garrido-Jurado et al. 2017; Rondot and Reineke 2018). Transient colonization implies that recovery declines with time. Theoretically speaking (no data are available), this could be a result of adverse conditions faced by the introduced fungal entomopathogen, including impeded intravascular movement, lack of adequate nutrients, pH, photoperiod, etc. Plant defense reactions might also negatively affect endophyte establishment (Schulz and Boyle 2005).

Fungal entomopathogens may be negatively affected by the presence of many other endophytes, by competition, mycoparasitism, or exposure to metabolites. Stone et al. (2004) compiled a list of 40 plant species for which the number of fungal endophytic species was reported. Evidence for the vast endophytic fungal diversity includes the recovery of 418 morphospecies in *Heisteria concinna* and *Ouratea lucens* (equivalent to ca. 347 distinct taxa; Arnold et al. 2000); 344 morphotaxa in *Theobroma cacao* (Arnold et al. 2003); and 257 unique ITS genotypes in *Coffea arabica* (Vega et al. 2010). Other studies revealed many endophytes in agricultural crops, including common beans (Parsa et al. 2016), lima beans (López-González et al. 2017), tomatoes (Larran et al. 2001), and wheat (Larran et al. 2002); three of these crops were subjects of studies aimed at introducing fungal entomopathogens as endophytes. Experiments testing the effects of naturally occurring endophytes on an introduced fungal entomopathogen demand the presence of only one endophyte, and even then, it would be difficult to determine what parameters to assess to determine possible negative effects. The complexity of endophytic biodiversity in plants is further complicated by the presence of viruses infecting endophytic fungi (Bao and Roossinck 2013), including *B. bassiana* (Herrero et al. 2012).

Assessments of the introduction of a fungal entomopathogen as an endophyte should identify other endophytes already present in the plant, as these will provide clues about the environment the fungal entomopathogen will face. It is also important to sample the plant as it matures, because endophyte diversity and richness can increase with age (Carroll et al. 1977; Arnold et al. 2003; López-González et al. 2017) and there can be a succession of endophytes as the leaf matures (López-González et al. 2017). The study by Rondot and Reineke (2018) is noteworthy because in addition to inoculating 7-wk-old grapevine plants (*Vitis vinifera*)

with *B. bassiana* conidial suspensions (foliar sprays), mature plants (planted in 1999) were also inoculated, resulting in recovery of *B. bassiana* from both treatments.

Localized colonization.—One common method to determine whether the introduced fungal entomopathogen has become endophytic is the fragment plating method (Torres et al. 2011), involving removal of leaves, stems, or roots followed by sterilization and plating of segments on culture media.

An alternative to the fragment plating method is dilution-to-extinction culturing, also known as extinction culturing, which exploits the microscale infection patterns of endophytes (Collado et al. 2007; Unterseher and Schnittler 2009). The technique is based on homogenizing the surface-sterilized plant material in a blender, followed by filtration and resuspension of particles between 100 and 200 μM. The suspension is centrifuged, the supernatant removed, and a dilution series prepared. The goal is to obtain a dilution for which a 10-μL aliquot contains 1–2 plant particles. This should result in recovery of no more than 1–2 endophytes. This technique reduces "intercolony interactions" (Collado et al. 2007) encountered when the plate fragment method or serial dilution is used, e.g., recovery of fast-growing endophytes at the expense of slow growers. Use of the dilution-to-extinction method also yields increased species richness (Collado et al. 2007; Unterseher and Schnittler 2009). The technique has never been used in studies solely involving endophytic fungal entomopathogens but was the only technique yielding endophytic *Cordyceps farinosa* in a study aimed at identifying endophyte biodiversity in European beech (*Fagus sylvatica*) (Unterseher and Schnittler 2010).

Fragment plating usually reveals that some fragments are positive for endophytes, whereas others are negative. This indicates localized colonization and could be a consequence of limited germination of and penetration by the conidia used for inoculation (e.g., after foliar sprays, see example of maize above). Working with *B. bassiana*, Landa et al. (2013) concluded that "fungal colonization was scarce and not uniform." Thus, localized endophyte colonization can be ascertained by the "extremely limited domains within plant tissues" (Carroll 1995), revealed through sampling. For example, it has been reported that size of sampled tissues influences the number of endophytes recovered, with larger leaf fragments yielding lower number of species than increasingly smaller fragments (Carroll 1995; Gamboa et al. 2002; Bayman 2006). Santamaría and Bayman (2005) found that "epiphytic and endophytic communities differed greatly on a single leaf, despite living only millimeters apart." Herre et al. (2007) include a figure depicting "a quilt-like patchwork" of endophytes species within 2-mm^2 leaf fragments. Similarly, Bissegger and Sieber (1994) identified up to six endophyte species in 1 × 1.5 cm sections of chestnut (*Castanea sativa*) phellem (the outer tissue of bark), and in one instance they isolated four endophyte species from a 0.2-cm^2 phellem area. Random landing of air spora on leaves could explain this patchwork pattern. Arnold and Herre (2003) sampled air spora in cacao plantations growing in the shade and reported that >36000 aerial propagules could come in contact with a leaf each day, in contrast to a clearing, where ca. 1100 propagules land on a leaf each day. Thus, determining an adequate size for plant fragments to be used in assessing colonization, and for sampling endophyte biodiversity, is worthy of consideration when planning a study. The detection of localized infections, as described above, can help visualize the close proximity of endophytic fungi within plant tissues and, consequently, the difficulty an introduced fungal entomopathogen would face in terms not only of competition but also of movement to points distant from the inoculation site.

An alternative way to sample plant tissues is to homogenize them, as done by Sasan and Bidochka (2012) and Behie et al. (2015).

Movement inside the plant.—The following papers report movement of the fungal entomopathogen from the inoculation site to other parts of the plant: Bing and Lewis (1991, 1992b), Wagner and Lewis (2000), Posada and Vega (2005, 2006), Gómez-Vidal et al. (2006), Tefera and Vidal (2009), Arab and El-Deeb (2012), Batta (2013), Landa et al. (2013), Ramírez Rodríguez and Sánchez-Peña (2016a, 2016b), and Jaber and Araj (2018).

Any seed inoculation or soil drench experiment with positive detection of the fungal entomopathogen in leaves or stems also serves as evidence for movement. Bing and Lewis (1992b) speculated on the movement of *B. bassiana* in maize as follows: (i) "The fungus colonized the plants and moved, primarily upward, within the pith, possibly along with plant photosynthates"; and (ii) "The fungus was isolated much more frequently from the node above the injection site than the node below,

indicating that it moved primarily upward from the injection site."

A seminal paper dealing with visualization and movement of endophytic *B. bassiana* in maize was published by Wagner and Lewis (2000). After topically inoculating corn leaves, they documented four sites for endophytic hyphae: leaf apoplast, xylem elements, stomatal openings, and air spaces between parenchyma. The authors speculate on how *B. bassiana* might move throughout the plant and provide valuable data on the fate of conidia landing on the leaf surface: "Approximately 3% of the conidia germinated, and less than 1% of these penetrated the leaf surface directly."

Working with date palm, Gómez-Vidal et al. (2006) reported *B. bassiana* endophytically colonized parenchyma and vascular tissue. They also observed that there was no conidial production. In opium poppy, Quesada-Moraga et al. (2006) reported *B. bassiana* colonization of xylem vessels, whereas Griffin (2007) observed hyphal growth in parenchyma and mesophyll tissues in cotton seedlings. Sasan and Bidochka (2012) provided visual evidence of *M. robertsii* colonizing root tissues, whereas Landa et al. (2013) concluded that colonization of opium poppy by *B. bassiana* was limited to intercellular spaces in the parenchyma. Maketon et al. (2013) reported *B. bassiana* colonization of parenchymal cells and vascular tissue in cowpea. Working with oilseed rape, Lohse et al. (2015) photographed intercellular hyphae of endophytic *B. bassiana* and confirmed species identification using a nested polymerase chain reaction (PCR) method developed by Landa et al. (2013). Finally, Lefort et al. (2016) reported *B. bassiana* hyphae in the intercellular spaces of the radicles of Monterey pine.

A noteworthy example of growth throughout the plant involved inoculation of coffee and cacao radicles with *B. bassiana*, with subsequent recovery from the stems, leaves, and roots, and eventual epiphytic growth (Posada and Vega 2005, 2006). Finally, even though it is not related to movement inside the plant, it is important to mention that Brownbridge et al. (2012) reported that *B. bassiana* moves from the plant and into the soil.

Effects on plant growth.—Thirty-four of the 85 papers (40%) examined plant responses to endophytism (TABLE 1), corresponding to 20 plant species (marked with an asterisk in SUPPLEMENTARY TABLE 1). Plant responses can be classified as (i) neutral (i.e., no differences between control and treated plants) in 12 studies; (ii) positive (i.e., at least one beneficial effect on plant health was observed) in 21 studies; and (iii) negative in 2 studies (one study reports both positive and negative effects).

Several studies unrelated to endophytism provide evidence for the involvement of fungal entomopathogens in promoting plant growth. Lee et al. (1999) tested 32 isolates of fungal entomopathogens against *Rhizoctonia solani* in cucumbers and found that 2 isolates promoted plant growth. Maniania et al. (2003) recorded a significant increase in onion yields in one of three trials when *M. anisopliae* was sprayed against onion thrips (*Thrips tabaci*) at weekly intervals. Growth of soybean seedlings in soil inoculated with *M. anisopliae* mycelium was significantly improved when compared with control plants (Khan et al. 2012). Liao et al. (2014) reported positive effects on various maize growth parameters when *M. anisopliae, M. brunneum*, and *M. robertsii* established an association with the roots.

Recent research results provided interesting insights on the involvement of fungal entomopathogens in the transfer of nitrogen to plants. For example, Behie et al. (2012) demonstrated the transfer of nitrogen by the mycelium from *M. robertsii*–infected greater wax moth (*Galleria mellonella*) to common beans (*Phaseolus vulgaris*) and switchgrass (*Panicum virgatum*). Even though endophytism was not demonstrated, the study provides evidence of a specific mechanism for plant growth promotion. In a subsequent experiment in which endophytism was not demonstrated, Behie and Bidochka (2014a) reported similar results using *M. acridum, M. brunneum, M. flavoviride, M. guizhouense, M. robertisii*, and *B. bassiana* and common beans, switchgrass, soybeans (*Glycine max*), and wheat (*Triticum aestivum*). *Akanthomyces lecanii* did not transfer nitrogen from the infected insect to the plant. Nutrient transfer in plant-fungal associations is discussed in detail by Behie et al. (2013) and Behie and Bidochka (2013, 2014b).

In a novel finding, Liao et al. (2017) found that *M. robertsii* produces the plant growth regulator indole-3-acetic acid (IAA; an auxin). This is the first report for a fungal entomopathogen producing a plant growth regulator. Other *Metarhizium* and *Beauveria* strains also produce IAA (Liao et al. 2017). These studies provide evidence that fungi traditionally referred to as "fungal entomopathogens" have other ecological roles, including a high potential to promote plant growth (Vega et al. 2009).

Several studies focused on possible mechanisms of *Metarhizium* species in promoting plant growth, in contrast to an absence of a similar focus for other fungal entomopathogens, suggesting that "Entomopathogenicity may not be the principal lifestyle

of *Metarhizium* spp." (Liao et al. 2014). Only one study examined effects of endophytic fungal entomopathogens on plant nutrients. Sánchez-Rodríguez et al. (2015) showed that *B. bassiana* can alleviate iron chlorosis symptoms in tomato and wheat.

DETECTION OF FUNGAL ENTOMOPATHOGENS AS ENDOPHYTES IN NATURE

Several fungal entomopathogens have been detected as naturally occurring endophytes in field-collected plants (TABLE 2). *Beauveria* was detected in nine plant families in China, Colombia, Ecuador, Indonesia, New Zealand, and USA. As more surveys for endophytes are conducted using field-collected plants, endophytic fungal entomopathogens may be recognized as being more prevalent than previously thought.

Molecular identification.—Numerous papers apply molecular techniques for detection of endophytic fungal entomopathogens (Leckie 2002; Ownley et al. 2004, 2008; Quesada-Moraga et al. 2006, 2014; Griffin 2007; Reddy et al. 2009; Biswas et al. 2012, 2013, 2015; Jia et al. 2013; Landa et al. 2013; Castillo Lopez et al. 2014; Jaber 2015; Lohse et al. 2015; Shrivastava et al. 2015; Gautam et al. 2016; Renuka et al. 2016; Garrido-Jurado et al. 2017). Specific aspects of molecular detection methods for endophytic fungal entomopathogens were reviewed by Garrido-Jurado et al. (2016) and McKinnon et al. (2017).

Visualizing fungal endophytism.—Using various microscopy techniques, a number of papers include photographs of endophytic fungal entomopathogens inside plants (Wagner and Lewis 2000; Gómez-Vidal et al. 2006; Quesada-Moraga et al. 2006; Griffin 2007; Sasan and Bidochka 2012; Landa et al. 2013; Maketon et al. 2013; Lohse et al. 2015; Lefort et al. 2016). Another visualization method involves the use of GFP-transformed fungal entomopathogens (Sasan and Bidochka 2012; Landa et al. 2013; Behie et al. 2015; Garrido-Jurado et al. 2017). In addition to scanning electron microscopy, clearing or staining plant tissues for fungal entomopathogen visualization would be valuable for understanding growth patterns, distribution, ultrastructure, and movement within plant tissues and would greatly increase the impact of studies in which detection is solely based on cultural methods. The papers by Atsatt (2003) and Atsatt and Whiteside (2014) should be consulted to be able to recognize the occurrence of mycosomes. For papers on stains used in endophyte studies, see Bacon and White (1994), Barrow and Aaltonen (2004), and Johnston et al. (2006).

COMMUNITY ECOLOGY

Establishment of endophyte communities.—Saunders et al. (2010) propose the use of community ecology to understand the assemblage of endophyte communities, whose establishment is a result of responses to (i) abiotic habitat filters such as ultraviolet (UV) radiation, nutrients, and moisture on the phyllosphere (Lindow 2006); (ii) biotic variables involving host plant–imposed habitat filters, such as host plant species, host plant genotype, biochemical defenses; and (iii) interactions with other microorganisms (Bayman 2006; Suryanarayanan 2013; Hardoim et al. 2015).

With respect to exposure to UV radiation, endophyte communities and colonization of fungal entomopathogens after foliar spray inoculation in the field will probably differ when plants are grown in full sun rather than shade. The phyllosphere is a biotic filter and the first point of contact between air spora and the plant. Phyllosphere associations with microbial communities have been extensively studied (Fokkema and Van den Heuvel 1986; Lindow et al. 2004; Bailey et al. 2006). Unfortunately, most studies dealing with introduction of fungal entomopathogens as endophytes ignore the phyllosphere.

Another component of the leaf surfaces is the stomata. Together with open wounds, stomata are known fungal entry routes (Agrios 2005). Posada et al. (2007) showed that *B. bassiana* foliar sprays resulted in the lowest colonization rates of coffee seedlings, compared with injections or soil drenches. This result may reflect the lack of stomata on the adaxial side of coffee leaves (Dedecca 1957) and the chemical components of the epidermis. Coffee leaves are covered by waxes (Stocker and Wanner 1975) and long-chain fatty acids (Holloway et al. 1972), making the leaf hydrophobic. Coffee cuticular waxes are toxic to *Colletotrichum coffeanum* (Steiner 1972; Lampard and Carter 1973; Vargas 1977), and their effect on fungal entomopathogens remains unknown. A basic understanding of the phyllosphere of plants subjected to the introduction of a fungal entomopathogen is necessary for this type of research. Only one study has examined the fate of a fungal entomopathogen in the phyllosphere. Du et al. (2014) examined the effects of *B. bassiana* conidial suspensions on the rice phyllosphere microbial community and reported no effects.

In conclusion, community ecology can help visualize the complexity of abiotic and biotic filters separating an airborne spore from the internal tissues of a plant.

Applying this global perspective to studies involving inoculations of fungal entomopathogens into plants should enable a more accurate understanding of the results obtained.

Effects on plant pathogens.—Antagonistic effects of endophytic *B. bassiana* on plant pathogens were reported by Ownley et al. (2008), Arab and El-Deeb (2012), Sasan and Bidochka (2013), Jaber and Salem (2014), and Jaber (2015). Ownley et al. (2008) proposed competition for space and induced systemic resistance as probable mechanisms for the antagonistic effects. Other possible mechanisms are discussed by Vega et al. (2009), Ownley et al. (2010), Ownley and Griffin (2012), and Jaber and Ownley (2018). Busby et al.'s (2016) review on endophytes as modifiers of plant disease is highly recommended.

Various fungal entomopathogens have a negative effect on plant pathogens in a nonendophytic context. Renwick et al. (1991) reported in vitro inhibition of *Gaeumannomyces graminis* by *B. bassiana* and detected production of chitinase and glucanases by the antagonist. Flori and Roberti (1993) tested 16 fungal strains, including three fungal entomopathogens (*B. bassiana, M. anisopliae, Paecilomyces farinosus* [current name: *Cordyceps farinosa*]) and found that all three were effective in reducing *Fusarium oxysporum* infection in onion bulbs. Working with two *Phoma* and three *Pythium* species, and with *R. solani* and *Septoria nodorum*, Veselý and Koubová (1994) reported lysis of the mycelium in dual culture with either *B. bassiana* or *B. brongniartii*; inhibition zones were also observed. Bark et al. (1996) observed that culture filtrates of *B. bassiana* have antagonistic effects on *Botrytis cinerea* and *F. oxysporum*, as evidenced by inhibited spore germination and reduced mycelial growth. Negative effects on morphogenesis were also reported, e.g., smaller spore size or abnormal hyphae. Similarly, Reisenzein and Tiefenbrunner (1997) found significant reductions in mycelial growth of *Armillaria mellea, F. oxysporum*, and *Rosellinia necatrix* grown in vitro with *B. bassiana*. Working with cucumbers, Lee et al. (1999) reported that 3 *Beauveria* isolates, among 32 *Beauveria* and *Metarhizium* isolates tested, none identified to species level, had activity against *R. solani*. In one of them, the compound responsible for the antagonistic effect was heat labile. Using in vitro tests, Shternshis et al. (2014) reported antifungal activity of *B. bassiana* on *Botrytis cinerea, F. oxysporum*, and *R. solani*, based on reduced growth. Gothandapani et al. (2015) described the inhibitory effects of *B. bassiana, M. anisopliae*, and *A. lecanii* (as *Verticillium lecanii*) on *Alternaria porri*. Treating wheat seeds with *Clonostachys rosea* in combination with *M. flavoviride* or *M. brunneum* greatly reduced infection by *Fusarium culmorum* (Keyser et al. 2016). Ownley et al. (2004) referred to *B. bassiana* as a "dual purpose biocontrol organism" based on its activity against insects and plant pathogens. A similar dual purpose was reported for *Akanthomyces* species (Askary et al. 1998; Benhamou and Brodeur 2000, 2001; Kim et al. 2007, 2008) and for combined treatments of *M. brunneum* and *C. rosea* in wheat seeds (Keyser et al. 2016). Science would benefit from focused studies aimed at elucidating specific mechanisms that explain how endophytic fungal entomopathogens antagonize plant pathogens in planta.

DISCUSSION

Just like humans and their indigenous microbiome, plants harbor an enormous internal microbial diversity (plant microbiome), whose role is just starting to be explored (Schlaeppi and Bulgarelli 2015; Van der Heijden and Hartmann 2016). What we see as a plant is a conglomeration of plant and microbial genes. The identification of endophytes in agricultural crops might reveal more than the presence of new fungal species (Peterson et al. 2005) and fungal metabolites (Smith et al. 2008; Suryanarayanan 2013); it might also lead to an improved understanding of what factors fungal entomopathogens face as they attempt to colonize plant tissues. Understanding that plants harbor a variable and seasonal fungal microbiome is a good justification for conducting surveys of endophytes in agricultural crops, at multiple times during a season, and within and across fields. If crops are growing in the shade (e.g., cacao, coffee), the trees providing the shade should be sampled, as well as air spora (Pedgley 1991; Petrini 1991) and rhizosphere fungi. This type of analysis, although labor-intensive, should illuminate the origins of endophyte populations. These data would also measure endophyte biodiversity as the plant grows, which clarifies ecological factors such as the plant age when endophyte diversity reaches a peak. All combined, these factors will provide a global perspective of actual field situations representing factors faced by the introduced fungal entomopathogen, more than is possible in the limited controlled conditions encountered in growth chambers or greenhouses.

Because ascomycetes are prolific producers of secondary metabolites, it is generally assumed that these metabolites play a role in endophytism, yet there is little experimental proof to support this assumption. Knowledge on metabolite production could be

extremely helpful for predicting the success of applied endophytes.

We also need to consider plant metabolites. For example, commercial maize varieties produce benzoxazinoids, whose by-products are active against insects and microbes, including fungi (Saunders and Kohn 2009). Several endophytic *Fusarium* species are tolerant to these compounds and occur at higher levels in plants producing them than mutants that do not, indicating that tolerance provides *Fusarium* with an advantage over other endophytic fungi (Saunders and Kohn 2009). This study demonstrates that plant chemistry can mediate interspecific competition, also shown by Arnold et al. (2003) and Bailey et al. (2006). Introducing fungal entomopathogens that lack tolerance to plant defense compounds prevalent in specific crops might decrease successful colonization and survival, compared with other endophytes tolerant to these compounds.

One area that has received insufficient attention is the cost to host fitness of harboring endophytes (Carroll 1991; Clay and Schardl 2002; Davitt et al. 2010; Suryanarayanan 2013). A better understanding of costs to the plant would be beneficial to scientists attempting to use endophytic fungal entomopathogens in biological control of insect pests and plant pathogens. One possible way to assess this is to consider gene induction following endophyte inoculation. Mejía et al. (2014) showed that inoculating endophyte-free cacao plants (defined by the authors as "generally <2% endophyte colonization") with the endophyte *Colletotrichum tropicale* induces the expression of hundreds of genes in the plant, some involved in plant defense. For fungal entomopathogens, only one study has tried something similar, undertaking a proteomic analysis of field-grown (i.e., not endophyte-free) date palm (*Phoenix dactylifera*) plants after field inoculation with *B. bassiana* or two *Akanthomyces* species (Gómez-Vidal et al. 2009; TABLE 1). This pioneering study revealed differences in protein accumulation, including some involved in plant defense, energy metabolism, and photosynthesis. Even though the cacao and date palms were asymptomatic, endophyte colonization resulted in a cost to the host, which points at the imperfect definition of the term "endophyte," which is largely based on lack of negative symptoms in the plant. Transcriptomic, proteomic, and metabolomic studies should include controls consisting of endophyte-free plants that can then be compared with plants inoculated with a fungal entomopathogen. Based on the difficulty in growing endophyte-free plants, it would be more realistic when setting an experiment to use plants for which the natural endophyte diversity has been identified. This endophyte diversity should be as consistent as possible among the plants used in the experiment. These plants could then be inoculated with fungal entomopathogens, and the results should allow us to discern the direct effect of the introduced fungal entomopathogen on plant responses.

Finally, in terms of mutualism, if the plant benefits by harboring a fungal entomopathogen, what would the benefit be for the fungal entomopathogen, other than a temporary haven? The findings of epiphytic *B. bassiana* growth in cacao and coffee after radicle inoculation (Posada and Vega 2005, 2006) are noteworthy because epiphytic growth would result in sporulation on the plant surface, which makes the infective propagule available for insect infection. Would similar results be obtained after radicle inoculation, suggesting systemic infection of the fungal entomopathogen, in other agricultural crops?

Two final considerations need to be mentioned. The first is obvious but is worth mentioning: the vast trove of literature on other endophytes, including on clavicipitaceous endophytes in grasses, is quite relevant to research on the introduction of fungal entomopathogens as endophytes. The second is the consideration of unusual impacts, such as sensory attributes. Rondot and Reineke (2018) noted the need to determine whether *B. bassiana* endophytism in grapevine plants influences "quality and sensory attributes" of must and wine.

The main challenge for field use of endophytic fungal entomopathogens as a pest management strategy is to manage reproducible fungal entomopathogen introductions into crops, and to predict the outcome of such introductions. The effectiveness of the technology needs to be proven in the field in order for growers to adopt it, and the results should have clear economic benefits to growers. Manageability and predictability are complicated by the inconstant fungal microbiome in plants, which might have different effects on fungal entomopathogens. Overall, research needs to focus more efforts on understanding mechanisms that facilitate, as well as those that impede, fungal entomopathogen endophytism.

Describing his paintings, Mark Rothko (1903–1970) once said: "I paint big to be intimate" (Thaw 1987). The same could be said about the topic at hand. The more we hunker down and focus on simple things, such as percent colonization, effect on an insect, and effect on a plant pathogen, the more likely we are to miss the big picture. Our palette is vast and includes entomology, mycology (including fungal metabolites), and botany (plant physiology, anatomy, morphology, pathology, chemistry). We can only become intimate with these complex interactions

when we collaborate with scientists in other disciplines who can help us understand the whole system and when we accept the importance of becoming familiar with endophytes in general, not just with endophytic fungal entomopathogens. Only then will we approach an approximation of how to make entomopathogenic endophytes work as a pest management strategy.

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