Entomopathogenic fungi as endophytes: plant–endophyte–herbivore interactions and prospects for use in biological control

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It is now evident that entomopathogenic fungi are able to colonize plant tissues as symptomless endophytes. Although most data so far published in this regard refer to *Beauveria bassiana* as an endophytic fungus, two other entomopathogenic fungi, viz. *Metarhizium anisopliae* and *Lecanicillium lecanii* have also been shown to colonize plant tissues endophytically. Several recent studies have also shown reasonable detrimental effects on herbivorous insects feeding on plants harbouring these fungi as endophytes. However, data published so far are highly variable and not consistent with regard to the underlying mechanisms which would allow explaining these effects. Growth conditions, specific cultivar features, or interactions with other microorganisms may impact the effect of these endophytic entomopathogenic fungi on the herbivorous insects. Furthermore, other fungi may block the systemic growth of the fungi in plant parts distant to the point of inoculation. Other parameters which need to be taken into account for using these fungi as biocontrol agents are the level of mycotoxins produced in plants, the level of pest reduction and the nature of formulations allowing a consistent colonization of the crop plants. This review discusses these and other problems related to the use of entomopathogenic fungi as endophytic biocontrol agents.

Keywords: *Beauveria bassiana*, *biocontrol*, colonization, *Metarhizium anisopliae*, *Lecanicillium lecanii*.

Introduction

PLANTS are commonly colonized by a wide range of endophytic microorganisms, such as bacteria and fungi[1,2]. Endophytes, a term first introduced by de Barry in 1866 (ref. 3), colonize internal plant tissues for at least a part of their life cycle without causing visible disease symptoms (i.e. retarded growth, discolouration, lesions, etc.) visible from the outside[4]. Although widespread and diverse in both natural and agricultural ecosystems[5] and present almost in all plant organs[2], the role of endophytes in shaping plant–environment interactions, especially plant–herbivorous insect interactions, has not been adequately appreciated. A few studies indicate that fungal endophytes may provide protection against herbivorous insects[6], plant diseases[7,8], or plant parasitic nematodes[9].

Endophytic fungal species comprise the well-studied Clavicipitaceous grass endophytes, which form an intimate association with their host plants because of vertical transmission of the endophytic fungus via the seeds and the production of specific alkaloids toxic to insects (class I endophytic fungi[10]). Much more abundant are the less specialized endophytes colonizing either above- or below-ground host plant tissues. Most species of entomopathogenic fungi belong to two divisions – Zygomycota and Ascomycota – and so far only in the latter division entomopathogenic species have been reported as endophytes in the order Hypocreales[11]. According to Rodriguez *et al.*[10], entomopathogenic endophytes should be classified as Class II endophytes, because they have been found colonizing both above- and below-ground tissues of their respective host plants[12,13].

The endophytic growth of *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (BB) in corn has already been reported by Bing and Lewis[14,15]. These authors not only proved the endophytic colonization of corn plant tissues by this fungus, but also found a higher mortality of larvae of the European corn borer (*Ostrinia nublialis* Hbn.) when feeding on the plants endophytically colonized by this fungus. Surprisingly, these data did not immediately prompt additional studies into these specific plant–entomopathogenic fungus interactions.

Interest in the use of this life-history trait of entomopathogenic fungi aiming at exploiting it for biological control strategy against pest insects of crop plants has gained more interest only in the recent years. Since the beginning of this century evidence accumulated that the findings of Bing and Lewis[14,15] were not outliers, but a common feature of these entomopathogenic fungi. The endophytic growth of BB is a common feature in corn cropping systems in USA. Although highly variable, the natural colonization of corn stalks in different US federal states ranged from zero to more than 60% of plants sampled[16], implying that entomopathogens as endophytes[1] may be

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quite common in other crop plants as well. For example, seeds of a common cultivar of oilseed rape grown in Germany, regularly contain a strain of BB (Jacobs-Schönwand, pers. commun.). Since the benchmark papers by Lewis and co-workers, many others were able to isolate entomopathogenic fungi and establish them either naturally or by artificial inoculation in many different plant species. Zimmermann[17] compiled papers reporting BB as an endophyte in a few plant species. Since then, there have been sporadic reports of BB, *Lecanicillium lecanii* (Zimmermann) Gams and Zare (LL), or *Metarhizium anisopliae* (since the revision of the genus *Metarhizium* spp.[18] it became apparent that this genus comprises more species than previously recognized. For convenience, I keep the old name *M. anisopliae*, although several studies from the past decades may in fact refer to other valid species within this genus) (Metchnikoff) Sorokin (MA) being able to grow endophytically in different host plants[19,20]. Plant species harbouring BB include cocoa[21], coffee[22], banana[23], date palm[24], sorghum[12], opium poppy[25], cotton, pumpkin and wheat[26], pine trees[27], jute[28], common bean[29] and artichoke[30].

There is growing evidence that most, if not all, entomopathogenic fungi are able to colonize tissues of at least some plant species. Depending on the plant species and the specific isolate of the endophytic entomopathogenic fungus (EEPF), these interactions could be beneficial to both the plant and the fungus, neutral or even antagonistic. This article aims to summarize the information on the extent of colonization of different plant parts by EEPF and also addresses the influence of such colonization on plant host metabolism. Recent findings on the preference and performance of herbivorous insects on these plants will be reviewed. Options and problems using these EEFs within biocontrol strategies will be discussed.

Colonization of plants by endophytic entomopathogenic fungi

The penetration and growth of an entomopathogenic fungus (BB) isolate into plant tissues were described for the first time in detail by Wagner and Lewis[31]. Briefly, conidia form germ tubes, which gradually elongate into hyphae, enter the plants via either natural openings or directly with the aid of enzymes and mechanical pressure through the epidermal cell walls. With regard to insects, the infection processes constituting adhesion and germination of conidia, and appressorium differentiation are closely linked to fungistatic and nutritional compounds found in the insect cuticle[32]. Whether specific compounds on the plant surface also contribute to the incidence of endophytic colonization of entomopathogens in fungus–plant interactions has so far not been investigated in detail.

Inside the maize plants, the hyphae grow through the air spaces between parenchyma cells, and sometimes also within xylem vessels. Though the potential of a systemic growth of the hyphae from leaves to stalks leading to colonization of entire plants was suggested[31], this hypothesis was not corroborated in sorghum plants where application of endophytic BB inoculum via the leaves did not result in colonization of the roots when the plants were grown in non-sterile soil, sterile soil or vermiculite[12]. Given these findings, it can be speculated that a systemic colonization of plant tissues is facilitated by acropetally growing hyphae when plants are colonized via the seeds or the roots. On the other hand, the highest colonization rates were found in plants grown in sterile soil or in vermiculite, pointing to the importance of the rhizosphere environment crucial for the initial establishment of the endophyte[12]. The growth of the hyphae within opium poppy tissues has also been described by Landa *et al.*[33]. They reported a pronounced decrease in the colonization of inner tissues in poppy plants after 10–15 days, following an inoculation of the fungal isolate via the leaf surfaces. When applied on the leaf surfaces, colonization rates of conidia were low and formation of appressorium-like structures was not found[31,34]. Interestingly, MA isolates seem to be less capable of colonizing different plant species. Akutse *et al.*[35] were not able to establish infection by MA isolate ICIPE30 in *Vicia faba* or *Phaseolus vulgaris* cultivars by soaking seeds in a conidial suspension of 10^8 for 2 h. On the other hand, Batta[36] reported a high recovery rate of a MA isolate applied onto leaves of an oilseed rape cultivar, both from leaves (not previously inoculated) or petioles (>70%) and stems (>30%) after 4 weeks and high colonization rate of two MA isolates was also observed in roots of faba beans[37]. Gurulingappa *et al.*[26] were not able to establish *L. lecanii* or BB in roots, stems or leaves of wheat and cotton when the inoculum was applied directly in the soil. The highest post-inoculation recovery was reported when coffee plants were inoculated with a BB isolate by direct injection[22]. Taken together, these data provide evidence that fungal isolate–host plant interactions play an important role for the potential establishment of the entomopathogens in the plants; successful colonization may depend on a specific character of the cultivars used and may be enhanced when soil conditions provide an 'enemy-free space'.

In our study, for evaluating the potential of BB isolates to grow endophytically in crop plants, we used 14 different BB isolates or strains isolated from different herbivore species and in different regions of the world (Table 1), to systematically test their potential to colonize oilseed rape (*Brassica napus* var. Favorite, DSV – Reinsaat KG, Germany) or faba bean (cultivar Hangdown Grünkernig, Gevo – Germany) plants. For each isolate/strain, a stock suspension was prepared in sterile 0.1% Tween 80 solution containing about 1×10^8 conidia ml^{-1} and 4 ml of this suspension was applied to the upper and lower surface of two opposite leaves assigned on each *B. napus* plant, and to the third leaf pair on each *V. faba* plant.

Table 1. *Beauveria bassiana* isolates/strains screened for endophytic establishment in *Brassica napus* and *Vicia faba*

Isolate/strain[a]	Geographic origin	Insect host[b]	Plant host[c]
Bb03032	Colombia	–	Coffee berries *Coffea arabica* L. (N)[d]
EABb04/01–Tip*	Spain	Stem–borer *Timaspis papaveris* (Kieffer) larva	Opium puppy *Papaver somniferum* L. (I)[24]
ATP01	Ethiopia	Stem borer *Busseola fusca* (Fuller)	–
ATP02	Ethiopia	Stem borer *Busseola fusca* (Fuller)	Sorghum *Sorghum* spp. (I)[12]
ATP03	Ethiopia	Sorghum chafer *Pachnoda interrupta* (Olivier)	–
ATP04	Ethiopia	Sorghum chafer *Pachnoda interrupta* (Olivier)	–
ATP05	Ethiopia	Sorghum chafer *Pachnoda interrupta* (Olivier)	–
Bb64	Austria	Codling moth *Cydia pomonella* L. larva	–
Bb101	The Netherlands	Black vine weevil *Otiorhynchus sulcatus* (Fbr.) adult	–
Bb135	Germany	European spruce bark beetle *Ips typographus* L. adult	–
Bb1022*	Canada	Pine shoot moth *Rhyacionia buoliana* (Schiff.)	–
Bb1025*	Canada	Insect (unidentified)	–
Bb1555*	Canada	–	Dead leaf (unidentified)
Naturalis® (strain ATCC74040–based bioinsecticide)	USA	Cotton boll weevil *Anthonomus grandis* (Boh.)	–

[a]Only isolates that have been characterized by molecular or biological means from other studies are referred to as strains (marked with an asterisk)
[b]Insect host from which the isolate/strain was originally isolated. [c]Plant host on which the isolate/strain has been reported as an endophyte.
[d]An (N) or (I) following the host plant indicates whether *B. bassiana* was reported as a naturally occurring endophyte (N) or introduced into the plant via artificial inoculation (I).

Plants in the control treatment received the same amount of sterile 0.1% Tween 80 solution applied in the same manner. Each treatment was replicated 10 times. Seven days after inoculation, plant colonization by different isolates/strains of BB was determined through re-isolation of the fungus from surface-sterilized, inoculated leaves using a method described previously[39]. Twelve leaf discs (approximately 2 mm^2) per plant replicate were cut from surface-sterilized, inoculated leaves using a sterile cork-borer. Thus, a total of 120 leaf discs were obtained per treatment combination. Leaf discs were evenly plated onto BB selective medium[39] in 55 mm petri dishes. In order to determine whether the surface-sterilization method was successful in eliminating epiphytic microorganisms or BB spores remaining viable on the leaf surfaces, 20 µl aliquot from 10^{-3} dilution of the final rinse water was plated onto petri dishes containing the selective medium. The petri dishes were sealed and incubated for two weeks at 25°C, after which all leaf discs were examined visually for fungal growth. BB was identified based on morphology and microscopic observations[40]. For each isolate/strain, per cent colonization was calculated using the formula: % colonization = number of leaf discs showing BB outgrowth divided by the total number of incubated leaf discs × 100 (ref. 41).

All screened BB isolates/strains, except Bb101 and Bb1555, were able to colonize inoculated leaves of oilseed rape and faba bean plants (Table 2). Colonization significantly varied among the screened isolates/strains within each host plant ($F_{14,270} = 70.060$; $P < 0.0001$). For example, colonization of *B. napus* by BB was significantly higher when the plants were inoculated with isolates/strains ATP02, ATP04, and ATCC74040 (BB-based Naturalis®) in contrast to ATP05, Bb03032, Bb1022 and Bb1025 ($P < 0.05$; Tukey's HSD test with Bonferroni correction for multiple testing; Table 2). On the other hand, colonization of *V. faba* plants by ATP02 was significantly higher than that by ATP03, ATP05, Bb03032, and ATCC74040 (BB-based Naturalis®; $P < 0.05$). Isolates ATP03 and strain ATCC74040 (BB-based Naturalis®) colonized *B. napus* plants more consistently than *V. faba* plants, while strains Bb1022 and Bb1025 colonized *V. faba* plants better than *B. napus* plants ($P < 0.05$).

Influence of endophytic entomopathogens on herbivorous insect performance

Information available so far on the effect of an endophytic entomopathogenic fungus on the performance of eggs, larvae or adults of herbivorous insects is inconsistent. Lepidopteran larvae (*Ostrinia nubilalis* or *Sesamia calamistis*) exhibit reduced tunnelling in corn and sorghum plants inoculated by BB isolates[14,42–44]; however, mycosis was not observed (or not reported) in these studies, indicating an indirect effect of the fungal colonization of plant tissues on larval performance (Table 3). In banana inoculated with a BB isolate, banana root borer larvae exhibited a higher mortality, and both eggs and adults showed mycosis[45]. Inoculation of opium poppy with endophytic BB reduced larval abundance of the gall wasp *Iraella luteipes* up to 73%, although without any mycosis[25]. These authors[25] also reported that the poppy

Table 2. Colonization (%) of *B. napus* and *V. faba* plants by 14 *B. bassiana* isolates/strains seven days after inoculation of plants with a sterile 0.1% Tween 80 conidial suspension containing 1×10^8 conidia ml^{-1} of each isolate/strain. Control plants were treated with sterile 0.1% Tween 80 solution. Colonization (%) represents the number of colonized segments divided by the total number of cultured segments × 100

Treatment *Beauveria bassiana* isolate	Colonization (%) ± SE	
	Brassica napus	*Vicia faba*
ATP01	76.36 ± 9.01 A[a], abc[a]	83.64 ± 3.79 A, ab
ATP02	92.73 ± 2.27 A, a	91.82 ± 2.12 A, a
ATP03	61.82 ± 9.17 A, bc	36.36 ± 7.17 B, c
ATP04	89.09 ± 3.26 A, ab	88.18 ± 3.61 A, ab
ATP05	53.64 ± 10.18 A, c	69.09 ± 4.92 A, b
Bb03032	55.46 ± 5.50 A, c	68.18 ± 7.33 A, b
EABb04/01-Tip	71.82 ± 5.15 A, abc	79.09 ± 5.43 A, ab
Bb64	70.91± 9.66 A, abc	81.82 ± 3.03 A, ab
Bb101	00.00 ± 0.00 d	00.00 ± 0.00 d
Bb135	64.04 ± 7.67 A, bc	75.46 ± 3.85 A, ab
Bb1022	54.55 ± 3.83 A, c	78.18 ± 4.54 B, ab
Bb1025	52.73 ± 4.66 A, c	72.73 ± 7.55 B, ab
Bb1555	00.00 ± 0.00 d	00.00 ± 0.00 d
Naturalis® (strain ATCC 74040-based bioinsecticide)	83.64 ± 2.27 A, ab	68.18 ± 5.29 B, b
Control	00.00 ± 0.00 d	00.00 ± 0.00 d

[a]Different uppercase letters refer to means (± SE) significantly different within rows; different lowercase letters refer to means (± SE) significantly different within columns ($P < 0.05$, Tukey's HSD test with Bonferroni correction for multiple testing after two-way ANOVA).

plants were colonized by the endophyte following seed treatment throughout the growth of the plants from the rosette stage via the capsule formation to the seeds of the second generation[13]. Larvae of the leaf mining fly *Liriomyza huidobrensis* suffered significantly when developing on BB endophyte-inoculated faba or common bean plants, resulting in less pupation; moreover, emergence of adults from pupae developing on inoculated plants was reduced. However, mycosis was never observed in more than 6000 cadavers recovered from inoculated faba plants[35]. Gurulingappa *et al.*[26] were able to establish both *L. lecanii* and BB in cotton, wheat, bean, tomato, corn and pumpkin plants when these crops were inoculated via the leaves; but recovery rates in almost all cases significantly declined within three weeks after the initial inoculation.

A significantly reduced reproduction rate was reported in two aphid species (*Aphis fabae* and *Acyrthosiphon psium*) confined on the leaves of faba plants, inoculated by soaking seeds in a spore suspension of two BB and MA isolates (additionally tested endophytic fungi not discussed here). Colonization of the roots, though variable, was reported to be over 95% after one month, but systemic growth into aerial parts of the plants was not assessed[37].

In most of the studies published so far on endophytism of entomopathogenic fungi, mycosis has either not been tested or not observed (Table 3). Mycosis of herbivore developmental stages feeding on inoculated banana plants has been reported with more than 60% mycosed adults[45]. Mycosed larvae of *Helicoverpa zea* were also reported from BB-inoculated tomato plants, but no difference in acute larval mortality or longevity was found[46]. Thus, the mode of action of EEPFs in most of the studies remains obscure.

We tested the virulence of endophytic BB strains on third instar larvae of the American bollworm (*Helicoverpa armigera*), using inoculated faba bean plants, a host plant species for this herbivore. Only BB strains/isolates that were able to endophytically colonize faba plants (Table 2) were used in this study. Plants were treated as described above and seven days past-inoculation, a clip-on cage containing a single larva was attached to one of the leaflets of the uninoculated fourth leaf pair of each plant to ensure that any effect against the introduced larvae was ensuing from fungal growth within plant tissues. The larvae were monitored daily and the time taken for them to die was recorded. In cases where almost all leaf material within the cage was consumed, the clip-on cage containing the larva was moved to the adjacent leaflet of the fourth leaf pair and kept on the plant until death or pupation. Dead larvae were transferred to petri dishes lined with moistened filter paper to monitor outgrowth of the respective BB isolate/strain. Mycosis of larval cadavers (i.e. cadavers showing external mycelial growth) was monitored daily for 14 days.

While all *H. armigera* larvae fed upon plants of the control treatment remained alive until pupation, larval mortality was observed on faba plants inoculated with the fungus in all other treatments (Table 4). However, only plants inoculated with the isolates ATP01, ATP02, and Bb03032 resulted in a significantly higher larval mortality compared to control plants (one-way ANOVA, $P < 0.05$; Tukey's HSD test with Bonferroni correction). While

Table 3. Studies reporting establishment of endophytic entomopathogenic fungi and effects on herbivorous insect feeding or development

Entomopathogenic fungus	Strain	Plant species colonized	Plant parts treated – assessed for colonization	Herbivore species targetted	Effect	Mycosis	Reference
Beauveria bassiana (Balsamo) Vuillemin	ARSEF 3113	*Zea mays* L.	Leaves – stems	*Ostrinia nubilalis* (Hbn.)	Larval tunnnelling reduced	Not reported	14
	Five different isolates	*Zea mays* L.	Leaf sheets – no final assessment	*Sesamia calamistis* (Hampson)	Larval tunnnelling reduced	Not reported	42
	Six different isolates	*Zea mays* L.	Stems – no final assessment	*Sesamia calamistis* (Hampson)	Fewer larvae, less dead hearts per 20 stems	Not observed	43
	G 41	*Musa acuminata* × *M. balbisiana* Colla	Roots; rhizomes–pseudostems	*Cosmopolites sordidus* (Ger.)	Reduced larval survivorship	<5% of eggs; <60% of adults	45
	GHA; BB 11-98	*Solanum lycopersicum* L.	Seeds, roots – stems, leaves, lateral shoots	*Helicoverpa zea* (Boddie)	No differences in acute larval mortality or longevity	<6% of larvae	46
	ITCC 4688	*Sorghum bicolor* (L.) Moench	Leaves – stems	*Chilo partellus* (Swinhoe)	Larval tunnneling reduced	Not reported	44
	EABb 04/01-Tip	*Papaver somniferum* L.	Leaves – leaves	*Iraella luteipes* (Thoms.)	Reduced larval numbers	Not reported	25
	GenBank AN GU953211, AN GU953212	*Gossypium hirsutum* L.; *Triticum aestivum* L.; *Phaseolus vulgaris* L.; *Solanum lycopersicum* L.; *Curcubita maxima* L. *Zea mays* L.	Leaves – leaves	*Aphis gossypii* Glover; *Chortoicetes terminifera* (Wlk.)	Reduced aphid reproduction and locust growth rate	Not observed	26
	Eight different isolates	*Vicia faba* L.; *Phaseolus vulgaris* L.	Leaves – leaves	*Helicoverpa armigera* (Hbn.)	Reduced larval survivorship	Observed, but not in all isolates tested	This paper
	Three different isolates	*Vicia faba* L.; *Phaseolus vulgaris* L.	Seeds – leaves	*Liriomyza huidobrensis* (Blanchard)	Fewer pupation, reduced emergence and adult survival	Not observed	35
	G1LU3; S4SU1	*Vicia faba* L.	Seeds – roots	*Acyrthosiphon pisum* (Harris), *Aphis fabae* L.	Reduced population growth	Not observed	37
Metarhizium anisopliae	N1LT6; S4ST7	*Vicia faba* L.	Leaves – roots	*Acyrthosiphon pisum* (Harris),	No effect on population increment	Not observed	37
(Metchnikoff) Sorokin	YB 150	*Brassica oleracea* L.	Leaves, leaf-petioles, stems	*Aphis fabae* L. *Plutella xylostella* L.	Higher larval mortality	Not reported	36

Table 4. Virulence of 12 *B. bassiana* endophytic isolates/strains against third instar *Helicoverpa armigera* larvae fed on leaves of inoculated *V. faba* plants

Treatment *B. bassiana* isolate	Parameter sampled ± SE		
	Mortality (%)	Mycosis (%)	Survival time (days)
ATP01	70.00 ± 0.11 ab[a]	00.00 ± 0.00 b	10.36 ± 0.82 b
ATP02	85.00 ± 0.08 a	100.00 ± 0.00 a	6.41 ± 0.58 a
ATP03	10.00 ± 0.07 cd	00.00 ± 0.00 b	23.5 ± 1.50 cd
ATP04	30.00 ± 0.11 bcd	16.76 ± 0.17 b	21.33 ± 0.67 cd
ATP05	40.00 ± 0.11 abcd	37.50 ± 0.18 b	20.63 ± 0.59 cd
Bb03032	55.00 ± 0.11 abc	54.55 ± 0.16 a	18.64 ± 0.64 c
EABb04/01-Tip	45.00 ± 0.11 abcd	66.67 ± 0.17 a	19.11 ± 0.63 c
Bb64	40.00 ± 0.11 abcd	50.00 ± 0.19 ab	20.25 ± 0.59 c
Bb135	25.00 ± 0.10 bcd	40.00 ± 0.25 ab	20.80 ± 0.66 cd
Bb1022	30.00 ± 0.11 bcd	00.00 ± 0.00 b	21.00 ± 0.97 cd
Bb1025	35.00 ± 0.11 bcd	28.57 ± 0.18 b	20.29 ± 0.67 c
Naturalis® (strain ATCC74040-based bioinsecticide)	25.00 ± 0.10 bcd	22.22 ± 0.15 b	21.11 ± 0.68 cd
Control	00.00 ± 0.00 d	00.00 ± 0.00 b	24.60 ± 0.83 d

[a]Means (± SE) followed by the same letter within a column are not significantly different at $P < 0.05$ (Tukey's HSD test with Bonferroni correction for multiple testing).

none of the larval cadavers collected from plants inoculated with isolates/strains ATP01, ATP03 and BB1022 displayed BB mycosis, between 16.76 and 100% of the cadavers recovered from plants inoculated with the remaining isolates/strains showed mycosis (Table 4). Survival time varied significantly among larvae fed upon plants inoculated with different BB isolates/strains (one-way ANOVA, $F_{12,99} = 60.847$; $P < 0.0001$). Isolate ATP02 followed by isolate ATP01 caused significantly earlier larval mortality compared to the remaining isolates/strains (Table 4).

Endophytic entomopathogenic fungi–host plant–herbivorous insect interactions

Although it is known that entomopathogenic fungi could colonize plants endophytically, many questions regarding this specific endophyte–plant interaction remain to be answered. The high variability of the results reported above (Tables 2 and 4) calls for a more holistic approach to understand these plant–fungus interactions.

At least several isolates of BB are able to grow endophytically in both monocotyledonous and dicotyledonous angiosperms as well as in gymnosperms. Whether MA or LL isolates are also able to grow in a diverse array of different plant classes and divisions still remains to be tested. The results from the tests with 14 different BB isolates reported above indicate that a high variability, both in colonization efficiency and in adverse effects on herbivorous insects, can be expected. Specific plant species (or even cultivar) – EEPF relationships may in one instance result in a high colonization incidence, whereas in other cases only low or even no colonization of plant tissues may be found. The role of the inoculation methodology and environmental conditions which the plants are exposed to, already addressed above, may also play a crucial role in these interactions. These specific conditions have not been taken into account in most of the studies published so far, and we hypothesize that climatic conditions and the nutritional status of the plants may also contribute to the incidence of successful establishment of EEPFs. Given the low colonization rate of a BB endophyte in plant tissues[33], host plants may also upregulate their defence metabolism, perceiving the endophytic organisms as adverse intruders.

It has already been pointed out previously that hardly any information exists on interactions between endophytic fungi and other ecological groups of fungi co-occurring in the same plant tissues[47]. Plants are commonly colonized by a diverse array of endophytic organisms[48]. It can therefore be expected that antagonistic interactions between these fungi are the rule rather than the exception. When EEPFs are starting to grow systemically from the point of inoculation to other plant parts, they inevitably have to confront other fungi already established. Since studies so far most probably have not used axenic plants, they are highly likely to already harbour endophytic fungi at the time of executing the experiments, thus adding a component of interspecific interaction to the already complex set-up in these experiments. In line with this hypothesis, a recent paper by Yan *et al.*[49] reported almost no systemic growth of endophytic fungi in *Silene dioica* (L.) Clairv., a non-mycorrhized forb, because most of the fungi were starting to exhibit antagonistic interactions when plated together in a kind of competitive setting on a growth medium.

Recovery of EEPFs from inoculated plants is taken as evidence for their active colonization of the plant tissues above or below the point of inoculation. However, in most of the studies demonstrating an antagonistic activity

of EEPFs to herbivorous insects, mycosis has not been observed. In these cases the effect of the EEPF may have been mediated by a change in the metabolism of the host plant or by the activation of specific metabolites, aiming at out-competing other endophytic organisms. Direct colonization of an insect pest by entomopathogenic fungi via ingestion of hyphae or spores seems to be unlikely, or at least, has to be demonstrated *in vivo*. We are not aware of any study demonstrating that ingestion by an insect of entomopathogenic fungal hyphae growing as endophytes results in mycosis.

Beauvericin, the most important virulence factor of BB effective against herbivorous insects, has not yet been reported to be produced in plant tissues. Similarly, with reference to MA, the most important virulence factor protease Pr1A (ref. 50), has not yet been reported to be upregulated or even identified in plant tissues colonized by this fungus. However, destruxins were found in cowpea endophytically colonized by a *Metarhizium robertsii* J.F. Bisch., Rehner and Humber isolate 12 days after inoculation[51]. Thus, more focused studies need to unequivocally demonstrate that the anti-insect effect is due to the fungus or by metabolites originating from the fungus, and not by fungus-mediated changes in host plant metabolism. Most of the studies cited above do not quantify the tissues colonized by the EEPF isolates, with one exception[33]. Though PCR methodology could be useful here, care should be exercised to ascertain the specificity of the primers used. False positive results may be not uncommon, but also false negative results are probable, because the methodology used for the assessment of the fungal isolates in the plant tissues will have a strong impact on the results, both qualitatively and quantitatively[52].

Options for using endophytic entomopathogenic fungi for pest control

Several problems and caveats need to be addressed before the potential of using entomopathogenic fungi as endophytes as a biological control strategy targetting herbivorous pests on crop plants can be fully capitalized. To date, more than 700 species of fungi have been determined to be pathogenic to insects and mites and about 170 insect biocontrol agents, based on different fungal entomopathogenic species, have been commercialized worldwide; however, over 75% of these products are based on the hypocrealean fungi BB, MA, *Isaria fumosorosea* (Wize) A.H.S.Br. and G.Sm. and *Beauveria brongniartii* (Sacc.) Petch[53]. Two-thirds of these commercialized products are based on aerial conidia preparations of BB and MA, although the drawbacks of aerial applications of these formulations are known for long[54]. Reasons for the variable efficacies reported in most field studies are the environmental instability of fungal spores when exposed to solar irradiation[55]. Moreover, weather conditions prevailing during application periods also affect the efficacy; rainfall will immediately wash-off the applied spores from the plant surface resulting in low colonization[56]. None of the commercialized products so far made use of the endophytic mode of action of entomopathogenic fungi, although at least one product is able to colonize plants endophytically (Table 3).

Several of the shortcomings, most probably contributing to the lack of commercialization, have already been addressed above. We currently do not understand how and to what extent plant tissues are colonized by EEPFs, and whether the colonization per se or changes in plant metabolism mediated by these fungi, contribute to the reduced herbivore damage. For use as a biocontrol agents, the efficacy of the product must be guaranteed, and following an application, pest abundance reductions need to be consistent and at a comparable level to chemical insecticides. An additional major hurdle is the potential of some of the entomopathogenic fungi to produce a wide array of compounds such as mycotoxins with biological activity against other organisms, including humans[57]. The specific human toxicity of beauvericin or destruxin has been described and discussed previously[58]. Currently, we do not have sufficient data to understand whether genes responsible for the production of these toxins are expressed differentially in insects and plants.

An optimal and probably the most effective option for making use of the endophytic growth of entomopathogenic fungi would be the inoculation of host plants with these fungi at the start of the germination of seeds, either by producing seeds already containing these fungi, or by coating the seeds with spores and protecting against adverse environmental conditions for their survival in the soil. Quesada-Moraga *et al.*[34] and Biswas *et al.*[28] used conidia as a seed treatment and were able to isolate the inoculated BB strains from most plants used in their studies, but there is most probably much room left for improved formulations.

Currently, the prospects for using endophytic entomopathogenic fungi as biocontrol agents are difficult to assess, given the many open research areas. However, the question raised by Hyde and Soytong[59] regarding 'how much do we really know about fungal endophytes, especially the non-grass endophytes?' (now also including the entomopathogenic fungi) deserves much more work before an answer can be given.

1. Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D. and Kursar, T. A., Are tropical fungal endophytes hyperdiverse? *Ecol. Lett.*, 2000, **3**, 267–274.
2. Stone, J. K., Bacon, C. W. and White Jr, J. F., An overview of endophytic microbes: endophytism defined. In *Microbial Endophytes* (eds White Jr, J. F. and Bacon, C. W.), Marcel Dekker, New York, 2000, pp. 3–29.
3. Carroll, G. C., The biology of endophytism in plants with particular reference to woody perennials. In *Microbiology of the Phyllosphere* (eds Fokkema, N. J. and van den Huevel, J.), Cambridge University Press, Cambridge, 1986, pp. 205–222.

4. Schulz, B. and Boyle, C., The endophytic continuum. *Mycol. Res.*, 2005, **109**, 661–686.
5. Meyling, N. V. and Eilenberg, J., Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. *Biol. Control*., 2007, **43**, 145–155.
6. Azevedo, J. L., Maccheroni Jr, W., Pereira, J. O. and de Araújo, W. L., Endophytic microorganisms: a review on insect control and recent advances on tropical plants. *Electron. J. Biotechnol*., 2000, **3**, 40–65.
7. Arnold, A. E., Mejıa, L. C., Kyllo, D., Rojas, E. I., Maynard, Z., Robbins, N. and Herre, E. A., Fungal endophytes limit pathogen damage in a tropical tree. *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 15649–15654.
8. O'Hanlon, K. A., Knorr, K., Jørgensen, L. N., Nicolaisen, M. and Boelt, B., Exploring the potential of symbiotic fungal endophytes in cereal disease suppression. *Biol. Control*, 2012, **63**, 69–78.
9. Waweru, B., Turoop, L., Kahangi, E., Coyne, D. and Dubois, T., Non-pathogenic *Fusarium oxysporum* endophytes provide field control of nematodes, improving yield of banana (*Musa* sp.). *Biol. Control*, 2014, **74**, 82–88.
10. Rodriguez, R. J., White Jr, J. F., Arnold, A. E. and Redman, R. S., Fungal endophytes: diversity and functional roles: Tansley review. *New Phytol*., 2009, **182**, 314–330.
11. Roy, H. E., Steinkraus, D. C., Eilenberg, J., Hajek, A. E. and Pell, J. K., Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. *Annu. Rev. Entomol*, 2006, **51**, 331–357.
12. Tefera, T. and Vidal, S., Effect of inoculation method and plant growth medium on endophytic colonization of sorghum by the entomopathogenic fungus *Beauveria bassiana*. *Biocontrol*, 2009, **54**, 663–669.
13. Quesada-Moraga, E., López-Díaz, C. and Landa, B. B., The hidden habit of the entomopathogenic fungus *Beauveria bassiana*: first demonstration of vertical plant transmission. *PLoS ONE*, 2014, **9**, e89278.
14. Bing, L. and Lewis, L. C., Suppression of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuill. *Environ. Entomol*., 1991, **20**, 1207–1211.
15. Bing, L. A. and Lewis, L. C., Endophytic *Beauveria bassiana* (Balsamo) Vuillemin in corn: the influence of the plant growth stage and *Ostrinia nubilalis* (Hübner). *Biocontrol. Sci. Technol*., 1992, **1**, 29–47.
16. Arnold, A. E. and Lewis, L. C., Ecology and evolution of fungal endophytes and their roles against insects. In *Insect – Fungal Associations – Ecology and Evolution* (eds Vega, F. E. and Blackwell, M.), Oxford University Press, Oxford, 2005, pp. 74–96.
17. Zimmermann, G., Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol. Sci. Technol*., 2007, **17**, 553–596.
18. Bischoff, J. F., Rehner, S. A. and Humber, R. A., A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia*, 2009, **101**, 512–530.
19. Vidal, S., Entomopathogenic fungi as endophytes: a new plant protection strategy? *IOBC/WPRS Bull*., 2011, **66**, 91–97.
20. Ownley, B. H. and Griffin, M. R., Dual biological control of insect pests and plant pathogens with fungi in the order hypocreales. In *Biocontrol: Management, Processes and Challenges* (ed. Brar, S. K.), Nova Science Publ., Hauppauge, 2012, pp. 133–152.
21. Posada, F. and Vega, F. E., Establishment of the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte in cocoa seedlings (*Theobroma cacao*). *Mycologia*, 2005, **97**, 1195–1200.
22. Posada, F., Aime, M. C., Peterson, S. W., Rehner, S. A. and Vega, F. E., Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). *Mycol. Res.*, 2007, **111**, 748–757.
23. Akello, J., Dubois, T., Coyne, D. and Kyamanywa, S., Effect of endophytic *Beauveria bassiana* on populations of the banana weevil, *Cosmopolites sordidus*, and their damage in tissue-cultured banana plants. *Entomol. Exp. Appl*., 2008, **129**, 157–165.
24. Gómez-Vidal, S., Lopez-Llorca, L. V., Jansson, H. B. and Salinas, J., Endophytic colonization of date palm (*Phoenix dactylifera* L.) leaves by entomopathogenic fungi. *Micron*, 2006, **37**, 624–632.
25. Quesada-Moraga, E., Munoz-Ledesma, F. J. and Santiago-Alvarez, C., Systemic protection of *Papaver somniferum* L., against *Iraella luteipes* (Hymenoptera: Cynipidae) by an endophytic strain of *Beauveria bassiana* (Ascomycota: Hypocreales). *Environ. Entomol*., 2009, **38**, 723–730.
26. Gurulingappa, P., Sword, G. A., Murdoch, G. and McGee, P. A., Colonization of crop plants by fungal entomopathogens and their effects on two insect pests when in planta. *Biol. Control*, 2010, **55**, 34–41.
27. Reay, S. D., Brownbridge, M., Gicquelb, B., Cummings, N. J. and Nelson, T. L., Isolation and characterization of endophytic *Beauveria* spp. (Ascomycota: Hypocreales) from *Pinus radiata* in New Zealand forests. *Biol. Control*, 2010, **54**, 52–60.
28. Biswas, C., Dey, P., Satpathy, S., Satya, P. and Mahapatra, B. S., Endophytic colonization of white jute (*Corchorus capsularis*) plants by different *Beauveria bassiana* strains for managing stem weevil (*Apion corchori*). *Phytoparasitica*, 2013, **41**, 17–21.
29. Parsa, S., Ortiz, V. and Vega, F. E., Establishing fungal entomopathogens as endophytes: towards endophytic biological control. *J. Vis. Exp.*, 2013, **74**, 50360.
30. Guesmi-Jouini, J., Garrido-Jurado, I., López-Díaz, C., Ben Halima-Kamel, B. and Quesada-Moraga, E., Establishment of fungal entomopathogens *Beauveria bassiana* and *Bionectria ochroleuca* (Ascomycota: Hypocreales) as endophytes on artichoke *Cynara scolymus*. *J. Invertebr. Pathol*., 2014, **119**, 1–4.
31. Wagner, B. L. and Lewis, L. C., Colonization of corn, *Zea mays*, by the entomopathogenic fungus *Beauveria bassiana*. *Appl. Environ. Microbiol*., 2000, **66**, 3468–3473.
32. Butt, T. M., Ibrahim, L., Clark, S. J. and Beckett, A., The germination behavior of *Metarhizium anisopliae* on the surface of aphid and flea beetle cuticles. *Mycol. Res*., 1995, **99**, 945–950.
33. Landa, B. B., Lopez-Diaz, C., Jimenez-Fernandez, D., Montes-Borrego, M., Munoz-Ledesma, F. J., Ortiz-Urquiza, A. and Quesada-Moraga, E., In-planta detection and monitorization of endophytic colonization by a *Beauveria bassiana* strain using a new-developed nested and quantitative PCR-based assay and confocal laser scanning microscopy. *J. Invertebr. Pathol*., 2013, **114**, 128–138.
34. Quesada-Moraga, E., Landa, B. B., Munoz-Ledesma, J., Jiménez-Diáz, R. M. and Santiago-Álvarez, C., Endophytic colonisation of opium poppy, *Papaver somniferum*, by an entomopathogenic *Beauveria bassiana* strain. *Mycopathologia*, 2006, **161**, 323–329.
35. Akutse, K. S., Maniania, N. K., Fiaboe, K. K. M., van den Berg, J. and Ekesi, S., Endophytic colonization of *Vicia faba* and *Phaseolus vulgaris* (Fabaceae) by fungal pathogens and their effects on the life-history parameters of *Liriomyza huidobrensis* (Diptera: Agromyzidae). *Fungal Ecol*., 2013, **6**, 293–301.
36. Batta, Y. A., Efficacy of endophytic and applied *Metarhizium anisopliae* (Metch.) Sorokin (Ascomycota: Hypocreales) against larvae of *Plutella xylostella* L. (Yponomeutidae: Lepidoptera) infesting *Brassica napus* plants. *Crop Prot.*, 2013, **44**, 128–134.
37. Akello, J. and Sikora, R., Systemic acropedal influence of endophyte seed treatment on *Acyrthosiphon pisum* and *Aphis fabae* offspring development and reproductive fitness. *Biol. Control*, 2012, **61**, 215–221.

38. Arnold, A. E., Maynard, Z. and Gilbert, G. S., Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. *Mycol. Res*., 2001, **105**, 1502–1507.
39. Chase, A. R., Osborne, L. S. and Ferguson, V. M., Selective isolation of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* from an artificial potting medium. *Fla. Entomol*., 1986, **69**, 285–292.
40. Humber, R. A., *Fungi: Identification. In Manual of Techniques in Insect Pathology* (ed. Lacey, L. A.), Academic Press, London, 1997, pp. 153–185.
41. Fisher, P. J. and Petrini, O., Location of fungal endophytes in tissues of *Suaeda fruticosa*: A preliminary study. *Trans. Br. Mycol. Soc.*, 1987, **89**, 246–249.
42. Cherry, A. J., Lomer, C. J., Djegui, D. and Schulthess, F., Pathogen incidence and their potential as microbial control agents in IPM of maize stem borers in West Africa. *Biol. Control*, 1999, **44**, 301–327.
43. Cherry, A. J., Banito, A., Djegui, D. and Lomer, C., Suppression of the stem-borer *Sesamia calamistis* (Lepidoptera: Noctuidae) in maize following seed dressing, topical application and stem injection with African isolates of *Beauveria bassiana*. *Int. J. Pest Manage*., 2004, **50**, 67–73.
44. Reddy, N. P., Ali Khan, A. P., Devi, U. K., Sharma, H. C. and Reineke, A., Treatment of millet crop plant (*Sorghum bicolor*) with the entomopathogenic fungus (*Beauveria bassiana*) to combat infestation by the stem borer, *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae). *J. Asia-Pacific Entomol.*, 2009, **12**, 221–226.
45. Akello, J., Dubois, T., Coyne, D. and Kyamanywa, S., Endophytic *Beauveria bassiana* in banana (*Musa* spp.) reduces banana weevil (*Cosmopolites sordidus*) fitness and damage. *Crop Prot.*, 2008, **27**, 1437–1441.
46. Powell, W. A., Klingeman, W. E., Ownley, B. H. and Gwinn, K. D., Evidence of endophytic *Beauveria bassiana* in seed-treated tomato plants acting as a systemic entomopathogen to larval *Helicoverpa zea* (Lepidoptera: Noctuidae). *J. Entomol. Sci*., 2009, **44**, 391–396.
47. Suryanarayanan, T. S., Endophyte research: going beyond isolation and metabolite documentation. *Fungal. Ecol*., 2013, **6**, 561–568.
48. Arnold, A. E. and Lutzoni, F., Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? *Ecology*, 2007, **88**, 541–549.
49. Yan, J. F., Broughton, S. J., Yang, S. L. and Gange, A. C., Do endophytic fungi grow through their hosts systemically? *Fungal Ecol*., 2014, **13**, 53–59.
50. Schrank, A. and Vainstein, M. H., *Metarhizium anisopliae* enzymes and toxins. *Toxicon*, 2010, **56**, 1267–1274.
51. Golo, P. S. *et al.*, Production of Destruxins from *Metarhizium* spp. fungi in artificial medium and in endophytically colonized cowpea plants. *PLoS ONE*, 2014, **9**, e104946.
52. Unterseher, M. and Schnittler, M., Dilution-to-extinction cultivation of leaf-inhabiting endophytic fungi in beech (*Fagus sylvatica* L.) – different cultivation techniques influence fungal biodiversity assessment. *Mycol. Res.*, 2009, **113**, 645–654.
53. Faria, M. and Wraight, S., Mycoinsecticides and Mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biol. Control*, 2007, **43**, 237–256.
54. Jackson, M. A., Dunlap, C. A. and Jaronski, S. T., Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol. *Biocontrol*, 2010, **55**, 129–145.
55. Cohen, E. and Joseph, T., Photostabilization of *Beauveria bassiana* conidia using anionic dyes. *Appl. Clay Sci.*, 2009, **42**, 569–574.
56. Inglis, G. D., Goettel, M. S., Butt, T. M. and Strasser, H., Use of hyphomycetous fungi for managing insect pests. In *Fungi as Biocontrol Agents – Progress, Problems and Potential* (Butt, T. M., Jackson, C. and Magan, N.), CABI Publishing, Oxfordshire, 2001, pp. 23–69.
57. Vey, A., Hoagland, R. E. and Butt, T. M., Toxic metabolites of fungal biocontrol agents. In *Fungi as Biocontrol Agents – Progress, Problems, and potential* (eds Butt, T. M., Jackson, C. W. and Magan, N.), CABI Publishing, Wallingford, 2001, pp. 311–346.
58. Strasser, H., Vey, A. and Butt, T. M., Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of Metarhizium, Tolypocladium and Beauveria species? *Biocontrol. Sci. Technol*., 2000, **10**, 717–735.
59. Hyde, K. D. and Soytong, K., *The fungal dilemma; Fungal Divers*., 2008, **33**, 163–173.

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