Fungal endophytes are involved in multiple balanced antagonisms

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In order for endophytic fungi to grow asymptomatically in their plant hosts, a balance of antagonisms is presumed to exist between host defence and fungal virulence. However, *in planta*, endophytic fungi must deal with multiple organismal interactions, primarily with bacteria and other fungi. We hypothesize that the plethora of antibacterial and antifungal metabolites that endophytic fungi produce has the function of maintaining balances of antagonisms with microbial competitors, resulting in a compatible multipartite symbiosis. Results obtained from co-cultures of endophytic and rhizospheric fungi with *Pseudomonas aeruginosa* and of endophytic fungi with *Hymenoscyphus fraxineus*, pathogen of the European ash, corroborate this hypothesis.

Keywords: Fungal endophyte, *Hymenoscyphus fraxineus*, multipartite symbiosis, *Pseudomonas aeruginosa*, secondary metabolites.

Introduction

ENDOPHYTIC fungi, which colonize their host plants without causing visible disease symptoms, can grow inter- or intracellularly, systemically or locally within their hosts[1,2]. They have varied lifestyles; nevertheless, they must deal with the defence reactions of their various hosts. Thus, a key question relevant for all of these interactions is: How do endophytic fungi overcome host resistance, enabling asymptomatic growth within the host? There are some answers to this question: (i) secretion of metabolites toxic to their hosts[3,4], (ii) modulation of host phytohormones, e.g. endogenous concentrations of JA and SA may be influenced by endophytic colonization[5], (iii) detoxification of constitutive defence metabolites[6,7], and (iv) secretion of lytic enzymes[8–10]. These observations corroborate the hypothesis that the interactions between host and endophyte are asymptomatic as long as there is a balanced antagonism between host defence and fungal virulence[1,4,11].

This hypothesis explains the high proportion of herbicidal metabolites that endophytic fungi produce, but not the high proportion of metabolites that inhibit bacteria and fungi[4,12,13]. Sixty per cent of the culture extracts of fungal endophytes that we tested inhibited fungal test organisms and more than 30% inhibited bacterial test organisms[4]. This is the reason why so many researchers have become interested in the isolation of secondary metabolites from endophytic fungi[12–14]. What role do these metabolites play *in situ?* As Demain[15] reported in 1980, and subsequently elaborated[16], fungi would not expend energy to produce such metabolites if they did not have a function *in situ.* This means that the functions of such metabolites *in planta* may be entirely different from the ones envisaged by pharmaceutical and agrochemical industries in the past. We hypothesize that biologically active metabolites are synthesized not only *in vitro*, but also *in planta*, at concentrations sufficient to inhibit competitors. That this is indeed the case, has been found, for example, for *Phialocephala* sp., an endophyte of *Picea glauca* (white spruce) that synthesizes the insecticidal metabolite rugulosin *in planta* at concentrations toxic to *Choristoneura fumifurana* (spruce bud worm)[17,18].

We suggest that in order to grow asymptomatically within their plant hosts, fungal endophytes would need to not only maintain a balanced antagonism with their plant host, but also with bacterial and fungal inhabitants of the host. This would explain the *raison d'etre* for (at least some of) the antibacterial and antifungal metabolites that fungal endophytes synthesize.

We have studied the interactions of fungi with potential antagonists from the same ecological niche. Whereas the bacterium, *Pseudomonas aeruginosa*, is an opportunistic human pathogen, in nature *Pseudomonas* species are found both in the rhizosphere and as root endophytes[19,20]. We hypothesized that fungal root endophytes and rhizosphere fungi might secrete metabolites toxic to *P. aeruginosa* that could be developed into antibacterial agents. Rhizospheric and endophytic fungi of *Brassica napus* (canola) were tested in co-culture with *P. aerugionosa.* To study interactions between fungi from the same habitat, endophytes of *Fraxinus excelsior* (the European ash) were tested in co-culture with *Hymenoscyphus fraxineus*, the causal agent of ash dieback. Subsequently, culture extracts were analysed for novel metabolites and tested against the respective potential antagonists. Results presented here show that not only do the fungal and rhizospheric endophytes produce metabolites toxic to the potential antagonist, but also that antagonists often

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Table 1. Effects of co-culture of fungal isolates from the roots and rhizosphere of canola (*Brassica napus)* with *Pseudomonas aeruginosa* (*P.a.*)

			Co-culture extracts			Culture extracts				
						Inhibition of *P.a.* by fungal mono-culture extract[d]		Inhibition of *P.a.* by *P.a.*-fungal co-culture extract[d]		
Strain	Source	Taxon	Inhibition of *Pa.*[c]	Inhibition of fungal isolate	Altered pigmentation in co-culture[b]	PA01	DW5	PA01	DW5	Novel metaboltes in co-culture[b]
9682	Root	*Metarhizium* sp.	+	+	+		–	–	–	3
9683	Root	*Fusarium* sp.	+	?[a]	–	+	–	+	++	2
9684	Root	*Fusarium* sp.	+, Overgrown	–	+	+	+	++	+	3
9685	Root	*Microdochium bolleyi*	+	+	+	–	+	–	–	2
9686	Root	*Verticillium* sp.	+	+	+	+	+	+	+	3
9687	Root	*Gliocladium* sp.	+	+	+	–	n.t.	++	–	2
9688	Root	*Cercospora* sp.	+	+	+	n.t.	n.t.	n.t.	n.t.	n.t.
9690	Root	*Phialophora* sp.	+	+	–	n.t.	n.t.	n.t.	n.t.	n.t.
9692	Root	*Trichoderma* sp.	Overgrown	–	+	–	–	++	n.t.	5
9693	Rhizosphere	*Chalara* sp.	+	+	–	n.t.	n.t.	n.t.	n.t.	n.t.
9694	Rhizosphere	*Acremonium* sp.	–	?[a]	+	+	+	++	+	4
9695	Rhizosphere	*Verticillium cinnabarinum*	+	+	–	–	–	–	+	0
9696	Rhizosphere	*Phoma* sp.	+	+	+	n.t.	n.t.	n.t.	n.t.	n.t.
9697	Rhizosphere	*Cytospora* sp.	+	+	+	+	+	+	++	2

[a]Fungus grows very slowly; thus inhibition uncertain; [b]Pigments not present in mono-culture of *P. aeruginosa*; [c]PAO1 and DW5; [d]Tested in an agar diffusion assay; n.t., Not tested; –, No effect; +, Inhibition; ++, Strong inhibition.

secrete metabolites toxic to the endophytes and rhizospheric fungi. Of particular interest is the fact that some of these metabolites are only produced in co-culture.

Material and methods

Isolation of endophytic and rhizosphere fungi

Canola plants were sampled from two agricultural fields in December 2007: near Lucklum (52.203013, 10.684736) and near Lamme (52.276143, 10.455969), both in Lower Saxony, Germany. To isolate the endophytes, the roots were first thoroughly washed under running water, then immersed for 1 min in 70% ethanol, followed by 30–60 sec in 3% NaOCl and subsequently rinsed three times in sterile water. The roots were then cut with a sterile scalpel into 2 mm slices and plated on potato–carrot agar medium with antibiotics[21] and incubated for 3 weeks at 20°C. The emerging mycelia were taken into culture on potato–carrot agar medium and identified according to morphology.

For isolation of the rhizospheric fungi, 10 g of soil that had been attached to the roots was taken from each location and suspended in 30 ml of water for 30 min. From each concentration, 200 µl of a serial dilution of the suspension (10^0–10^5) was plated onto potato–carrot medium and incubated as above. Of the numerous isolates, 14 morphologically and taxonomically diverse endophytic and rhizospheric fungal isolates (Table 1) were retained to study the metabolic interactions between bacteria and fungi.

Endophytic fungi were also isolated from *F. excelsior* growing in the Elm forest, Lower Saxony, and identified according to morphology (Junker *et al.*, manuscript under preparation).

Pseudomonas aeruginosa

Three isolates were used for co-culture with the fungi: two isolates from gangrene infections – PAO1[22] and PA14 (UCBPP-PA14)[23], and DW5, an environmental isolate. Attempts made to isolate the bacterium from *B. napus* were unsuccessful presumably because isolations were conducted in winter and although *P. aeruginosa* can grow at lower temperatures, its optimum lies between 30°C and 34°C (ref. 24).

Hymenoscyphus fraxineus

The strains of *H. fraxineus* had been previously isolated from various locations in northern Germany and Poland[25].

Co-culture

P. aeruginosa and fungal isolate were inoculated simultaneously at a distance of 4.5 cm from each on biomalt[21] and/or CP agar medium (10 g yeast extract, 11 g D(+)-glucose, 1000 ml distilled water, 15 g agar, pH 6.2); cultivation was for three weeks at 20°C or at room temperature. *H. fraxineus* and endophytes from *F. excelsior* were tested in co-culture on solid media (Junker *et al.*, manuscript under preparation).

Culture extraction and agar diffusion assays

The cultures were frozen overnight at –70°C. The fungal mono- and co-cultures were subsequently lyophilized,

ground in a coffee grinder and extracted with ethyl acetate as described previously[4]. Due to the presence of a human pathogenic bacterium, the mono- and co-cultures with *P. aeruginosa* could not be lyophilized. After defrosting, these cultures were minced in a blender and extracted with ethyl acetate as above. After evaporation of the solvents, the dried extracts were defatted with petroleum ether and dissolved in methanol : acetone (1 : 1). For the agar diffusion assays, 1 ml of a suspension of *P. aeruginosa* was plated onto CP medium and subsequently dried; 50 µl (40 mg/ml) of culture extract was then pipetted onto a sterile test disc (0.45 cm) on the inoculated medium, and incubated at 30°C for two days before the radius of the inhibition zone was measured.

Thin-layer chromatography

Thin-layer chromatography (TLC) was performed on silica gel aluminum plates (Merck, Silicagel 60, F254) with 4% methanol in dichloromethane. Substances were visualized both with UV light at 254 and 366 nm, as well as following carbonization (spraying with 5% H_2SO_4 in ethanol and heating for 10 min at 110°C).

Determination of the minimal inhibitory concentration of culture extracts for inhibition of P. aeruginosa

Culture extracts of the mono-cultures (DW-5, PAO1, PA14, and the fungi 9683, 9684, 9686 and 9697) and of the co-cultures of the fungi (9683, 9684, 9686 and 9697) with the three *P. aeruginosa* strains were tested against the *P. aeruginosa* strains PAO1 and PA14. To test whether these culture extracts inhibited *P. aeruginosa*, overnight cultures of *P. aeruginosa* PAO1 and PA14 in MHB liquid culture medium (22 g Müller Hinton Broth 2 (Roth, Art.-Nr. X927.1), 1000 ml dH_2O) were washed twice in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PHO_4, 2 mM KH_2PO_4) and adjusted to 1×10^8 cells/ml in PBS buffer. Then 200 µl of the bacterial suspension was inoculated into each well of 96-well microtitre plates to which the culture extract with concentrations 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0 and 4.0 mg/ml was added. The microtiter plates were incubated for 4 h at 37°C and 300 rpm; the contents of each well were serially diluted down to a concentration of 10^5 cells/ml. From each dilution, 20 µl of each dilution were plated onto LB[26] agar medium and incubated for 20 h at 37°C. The number of cells was counted and expressed as the number per ml.

Results and discussion

Interactions of P. aeruginosa with endophytic and rhizospheric fungi

In co-culture, both bacteria and fungi secreted metabolites toxic to the respective partner (Figure 1). Most of the fungi (10 out of 14) inhibited *P. aeruginosa*. Secreted metabolites of *P. aeruginosa* inhibited most of the fungi, demonstrating the reciprocal antagonism between these inhabitants of the same environment (Table 1; Figure 1 *a* and *b*). Antifungal activity of *P. aeruginosa* could be due to the siderophores, pyoverdin and pyochelin, which *P. aeruginosa* is known to produce[27,28], or to one of the antifungal phenazines[29–31] that can also be secreted by *P. aeruginosa*. Alternatively, metabolites that are only produced in co-culture could be responsible for the inhibitions, as has been previously reported for numerous other interactions[32]. Not only was growth of *P. aeruginosa* inhibited in co-culture with the endophytes, but also the composition of its secreted pigments was sometimes altered. In co-culture, DW5 seems to have secreted less of the yellow–green pigment, pyoverdin, in relation to the blue–green pigment, pyocyanin (Figure 1 *a*). In the two interactions in which no inhibition of the bacterium was observed, the two fungal isolates, i.e. *Trichoderma* sp. and *Fusarium* sp. overgrew the bacterium.

All but one of the culture extracts of the co-cultures inhibited *P. aeruginosa* more than those of the mono-cultures (Table 1). Thin-layer chromatographs of culture extracts revealed that in all but one of the co-cultures, novel metabolites had been synthesized that were not found in mono-culture of either the bacteria or the fungi (Table 1). It is, of course, not clear whether these novel

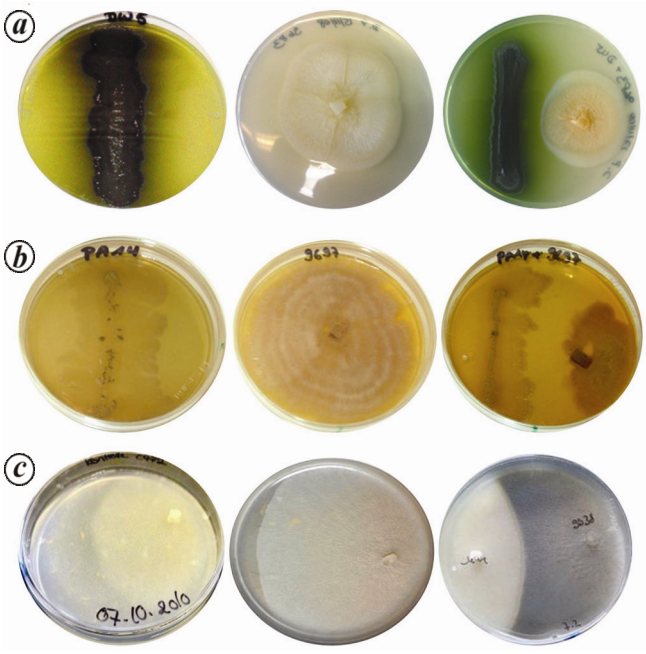

Figure 1. Mono- and co-cultures: In all interactions, growth of both organisms was inhibited in co-culture more than in mono-culture. ***a***, *Pseudomonas aeruginosa* DW 5 and *Fusarium* sp. 9697 (endophyte) in mono- and in co-culture. ***b***, *P. aeruginosa* PA14 and *Cytospora* sp. 9683 (rhizospheric fungus) in mono- and co-culture. ***c***, *Phomopsis* sp. (on left; endophyte) and *Hymenoscyphus fraxineus* (pathogen) in mono- and co-culture. All cultures were incubated for 3 weeks on potato–carrot agar medium at room temperature.

metabolites were synthesized by the bacteria or the fungi. Although co-cultures cannot replicate the situation *in situ*, particularly the fact that there are never just two partners *in situ*, it does nevertheless show that these fungi and bacteria that occupy the same ecological niche reacted metabolically to the presence of the other before they even had physical contact, as has also been shown for other interactions[32,33]. This suggests that in these interactions not only inhibitory metabolites, but also signal molecules must have been secreted, informing the two microorganisms of the presence of the other before they had physically contacted each other.

Culture extracts of both mono- and co-cultures that had inhibited PAO1 and PA14 in agar diffusion assays were selected to determine the minimal inhibitory concentrations (MIC) in liquid culture. In these tests, the only mono-culture that inhibited *P. aeruginosa* was that of 9697 (*Cytospora* sp.), inhibiting PAO1 moderately at concentrations >3.0 mg/ml (Figure 2 *a*). Of the co-cultures, that of DW 5 + 9683 (*Fusarium* sp.) inhibited PAO1 100% at a concentration of 1 mg/ml (Figure 2 *b*) and PA14 100% at a concentration of 1.5 mg/ml (Figure 2 *c*). Cultures of the respective mono-cultures exhibited no inhibitory effects (data not shown), showing that the metabolites that inhibited *P. aeruginosa* were only secreted in co-culture. Isolates of both genera, *Cytospora* and *Fusarium*, are known to produce biologically active secondary metabolites. For example, *Cytospora* sp. can produce the weakly antibacterial substance colletodiol and related macrodiolides[34] and two antibacterial benzyl γ-butyrolactone analogues[35]. *Fusarium* spp. are known to produce a plethora of biologically active metabolites[36,37].

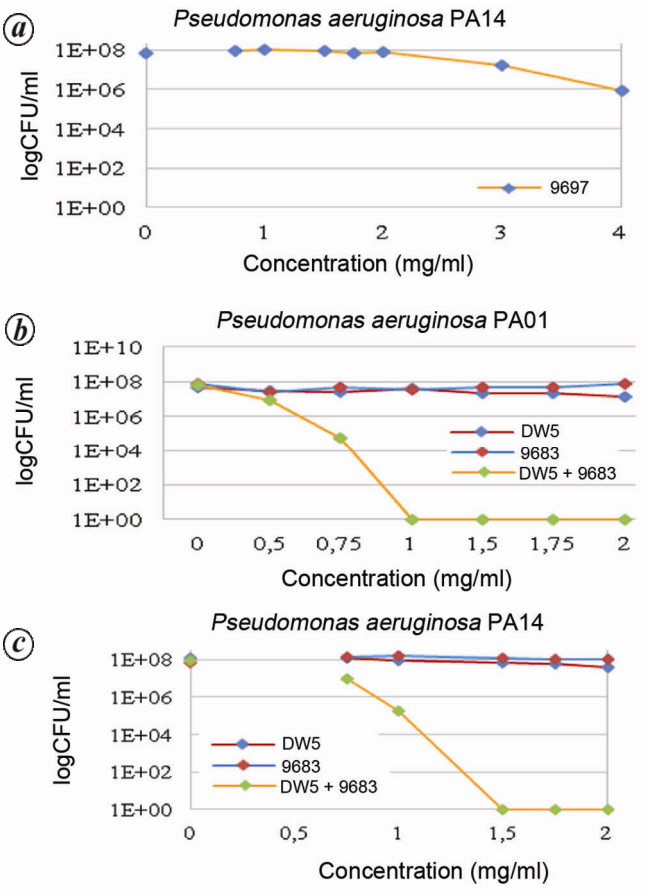

Figure 2. Minimal inhibitory concentration of growth of *P. aeruginosa* by culture extracts of mono- and co-cultures in liquid culture (4 h at 37°C and 300 rpm following addition of the culture extract). The only mono-culture extract that moderately inhibited *P. aeruginosa* was that of (***a***) *Fusarium* sp. 9697 against PA14. Inhibition by culture extracts of the co-cultures was greater: *Cytospora* sp. 9683 against (***b***) PAO1 and (***c***) PA14.

Interactions of H. fraxineus with endophytic fungi

One of our goals is to find a fungal endophyte that can be used for biocontrol of *H. fraxineus*, the causative agent of ash dieback, a disease that is threatening the existence of *F. excelsior* in Europe[38]. Thus, we isolated endophytes from healthy and diseased ash trees growing near Erkerode, in Lower Saxony, and initially tested them in co-culture for inhibition of *H. fraxineus* (Junker *et al.*, manuscript under preparation). Surprisingly, in co-culture, *H. fraxineus* inhibited most of the endophytes, and most of the endophytes inhibited *H. fraxineus*, as shown in Figure 1 *c* for the interaction between *H. fraxineus* and *Phomopsis* sp. Of the 59 endophytes, 57 inhibited *H. fraxineus*, 19 of them inhibiting the pathogen by >30% in comparison to the mono-culture control (Junker *et al.*, manuscript under preparation). *H. fraxineus*, in turn, inhibited 55 of the endophytes, 13 by more than 30% in comparison to the mono-culture (Junker *et al.*, manuscript under preparation; Figure 3). The inhibitions by *H. fraxineus* could be due to the phytotoxins viridiol[39] and a volatile lactone[40] that the pathogen is known to produce. TLC showed that novel metabolites were produced in all of the nine tested co-cultures that were not present in either of the corresponding mono-cultures. Additionally, in co-culture concentrations of the phytotoxins were reduced (Junker *et al.*, manuscript under preparation).

Function of endophytic antimicrobial metabolites

Microorganisms do not always secrete toxic metabolites in co-culture. For example, of the 58 actinomycetes that were co-cultivated with *Aspergillus nidulans*, only one was able to induce synthesis of orsellinic acid and biologically related compounds[33], suggesting that specificity was involved. In contrast, the endophytic fungi and *H. fraxineus* harboured by *F. excelsior* seem to specifically recognize each other. In co-culture, almost all of the fungi were reciprocally inhibitory. Furthermore, the fact that inhibitions occurred even before the co-cultured organisms established physical contact with each other suggests the involvement of signal molecules.

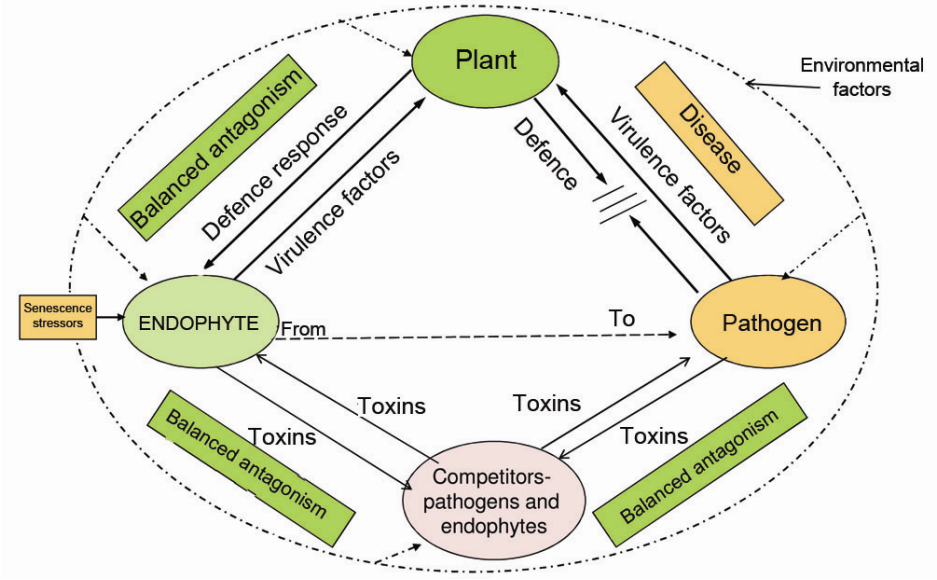

Figure 3. Hypothesis: In order to grow and survive *in planta*, endophytic fungi must maintain a balance of antagonism not only with their host plant, but also with microbial competitors, both endophytic and pathogenic bacteria and fungi. Secondary metabolites are important factors for maintaining these equilibria. The result is a compatible, multipartite symbiosis and a healthy plant.

In the two interactions that we report on, there was usually mutual antagonism in co-culture. This was due at least in part to the synthesis of the respective antibacterial and antifungal metabolites, including those synthesized only in co-culture. There are numerous reports of activation of secondary metabolism during co-cultivation of fungal endophytes and bacteria. Many of the researchers employing this technique are interested in inducing the synthesis of cryptic secondary metabolites[32,33,41,42]. Others strive to detect cryptic metabolites by co-culturing fungi with fungi[32,41,43]. For example, we had previously reported on co-cultures of *Fusicoccum betulae*, a pathogen of the birch (*Betula pendula*), with an endophyte of the tree, *Cryptosporiopsis* sp. The endophyte secreted substances that inhibited the pathogen, i.e. cryptosporiopsin, mycorrhizin A and mellein[44]. And yet others strive to find an endophyte that can be used for biocontrol of a microbial pathogen[45,46–49]. Although they had other goals, all of these investigations exemplify that microorganisms interact metabolically. There are, however, few other researchers that have (also) dealt with mutual metabolic interactions between the partners in co-culture or even *in planta*. This may be particularly pertinent in habitats with a high microbial burden, such as plant surfaces[42], but also in environments that presumably have a lower microbial burden, i.e. that of endophytes *in planta*.

Wang *et al.*[50] co-inoculated an endophytic bacterium, *Acinetobacter* sp., and an endophytic fungus, *Acremonium* sp., into their host plant, *Actractylodes lancea*. Interestingly, *in planta* host defence responses were reduced as was growth of the endophytic fungus. The authors suggest that since bacterial secreted metabolites inhibited fungal growth in *in vitro* co-culture, these might also be responsible for reduced growth of the fungal endophyte *in planta*. The authors also suggest that such constraints are necessary for maintaining a compatible and balanced symbiosis in the tripartite interaction involving plant host and its endophytes. The study of Mohandoss and Suryanarayanan[51] also provides presumptive evidence that multipartite interactions are involved. They showed that elimination of some endophytes from the leaves of *Mangifera indica* by fungicidal treatment results in colonization of the leaf by non-native endophytes, indicating an *in vivo* inter-specific competition among endophytes. And since *in planta* an endophyte must deal with more than one competitor, as well as host defence, we hypothesize that many, if not all, microbial inhabitants of hosts secrete antifungal and antibacterial metabolites *in planta* to inhibit competitors, assuring themselves adequate assimilates. An additional advantage for the plant would be that competitors would be less likely to become pathogenic, because their growth would be constrained.

Thus, in addition to dealing with host defence, the endophytes must secrete antibacterial and antifungal metabolites in order to maintain a balance of antagonism with competitors. Our hypothesis would explain the results obtained in these investigations: the antibacterial metabolites produced by the endophytic and rhizospheric fungi associated with *B. napus*, the antifungal metabolites synthesized by the endophytes and the pathogen from *F. excelsior*, but especially the metabolites that were only produced in co-culture. Additionally, it would clarify the unanswered questions regarding the roles of metabolites

in the interactions between endophytes and pathogens raised by Kusari *et al.*[14]. Accordingly, in order to grow asymptomatically in their hosts, fungal endophytes must maintain multiple balanced antagonisms – with the host plant and with the other microbial inhabitants of the host plant (Figure 3). This partly explains the capability of endophytic fungi to synthesize a plethora of antifungal and antimicrobial secondary metabolites. The recent findings that most of the secondary metabolite genes in fungi are generally silent in culture and are activated while being co-cultured with other microbes[33,52] lends further credence to our hypothesis that multiple antagonisms are involved in endophytism.

However, endophytes not only interact with bacterial and fungal competitors, but also with herbivores and insect predators of the host[53]. For example, entomopathogenic fungi such as *Pochonia chlamydosporia* and *Clonostachys* are not only found in the soil, but can also colonize as endophytes[54,55]. The results of Vega *et al.*[54] suggest that fungal metabolites are involved in the antibiosis. In the case of *Phialocephala* sp. in spruce, the inhibitory metabolite has been identified. Rugulosin is synthesized *in planta* by *Phialocephala* sp. at concentrations inhibitory to the spruce bud worm[17,18]. The classic case of an endophyte which secretes metabolites toxic to herbivores involves *Neotyphodium* spp.[56]. However, the tripartite interaction between the endophyte, herbivore and plant host is not as straightforward as previously assumed, e.g. herbivores have evolved to detoxify the alkaloids[57].

In conclusion, it is clear that in order to colonize their hosts asymptomatically, endophytes are involved in multipartite interactions. Also, much research remains to be done to elucidate the metabolic pathaways and signalling functions involved in these interactions.

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