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REVIEW

Endophytic fungi as bio-control agents: elucidating mechanisms in disease suppression

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ABSTRACT

Background: Fungal endophytes occur ubiquitously in plants and are being increasingly studied for their ability to support plant health and protect the host from diseases. Using endophytes in disease control provides potential advantages compared to other bio-control agents since they colonise the plant internally. A thorough understanding of their mechanisms is required in their mutualistic association with plants; both to optimise their efficacy and
for registration as plant protection products.
Aims: To provide a critical review on the mechanisms employed by endophytic fungi in biological disease control. Furthermore, we draw attention to gaps in our knowledge of the complex interactions between plant, pathogen and endophyte and discuss implications for future research.
Methods: Review of literature where endophytic colonisation during the specific interaction has been confirmed.
Results: Known disease-reducing mechanisms include direct inhibition of pathogen activity by competition, antibiosis and mycoparasitism and indirect inhibition by induced resistance, where the plant's own defence system is activated to combat the diseases. Relying on in vitro studies of alone can result in misleading conclusions.
Conclusions: We need to investigate nature and requirements for establishment of successful plant-endophyte interactions, for development of efficient bio-control agents.

KEYWORDS

Antibiosis; biological control; endophytic fungi; induced resistance; microbial competition; mycoparasitism; tripartite plant-microbe interactions

Introduction

Crop plant diseases cause considerable yield losses and therefore represent a major threat in agriculture. Owing to the development of fungicide resistance in pathogens and ecological and public health considerations, the number of available chemical fungicides is being reduced. In addition, the industry faces increasing costs for development and commercialisation of novel disease control products. For these reasons, there is an increasing need for development of alternative disease management products, which can offer environment friendly and economically feasible control of

important crop plant diseases. Along with selection of resistant plants and other management practices, biological control by beneficial microorganisms is increasingly considered an important element in integrated disease management strategies and there has been an intensified search for such micro-organisms in recent years.

Plants benefit from close associations with microorganisms, which for their own benefit, have developed mechanisms that increase their fitness and survival of their host. Plant-associated microorganisms colonise the surface of the plant as epiphytes, or interior tissues as endophytes. Recently, the concept of the plant micro-biome, comprising the collective genomes of all microorganisms associated with a plant, has led to a different view on plant evolution, in which plant and microbiome evolve together and the microbiome provides flexibility to plants as often sessile and slowly evolving organisms (Hardoim et al. 2015; Wani et al. 2015). Endophytic micro-organisms are particularly well adapted to the host plant, since they spend at least part of their life cycle internally colonising living plant tissue, without causing any immediate, overtly negative effects (Bacon and White 2000; Hardoim et al. 2015). In exchange for using the plant as a habitat and source of nutrition, some endophytic microorganisms benefit the host plant by stimulating growth, development, adaption and stress tolerance (Saikkonen et al. 1998; Wani et al. 2015). Specifically, endophytes have been shown to benefit their host by conferring protection against diseases, resulting in reduced levels of infection, suppression of growth or reduction of inoculum production of the pathogen (Aroca 2013; Bacon and White 2016).

Development and commercialisation of biological control agents (BCAs) is currently not a straightforward process and in practise, microorganism-based disease control is still limited. Many bio-control studies are carried out under controlled laboratory conditions, thereby circumventing complications associated with environmental influences, large-scale production and formulation (Spadaro and Gullino 2005). Therefore, when transferred to field conditions, BCAs frequently lack reliability in their activity due to the effects of varying environmental factors (Köhl et al. 2011), such as UV radiation and variations in temperature, humidity, water availability and soil pH. By using host-dapted endophytes as bio-control agents, some of the constraints faced by traditional BCAs can be minimised. Microorganisms with an endophytic lifestyle stay protected from environmental influence and fluctuations that could threaten their survival and reduce bio-control efficacy (Card et al. 2016). This circumstance makes endophytes likely to provide a more stable control effect and thus an ideal tool for biological control. For those endophytes that are seed transmitted, there is an additional advantage for commercialisation purposes, since there is no need to develop formulations and delivery techniques for their application. However, for the majority of the potential BCAs, cost-efficient delivery systems are yet to be developed. Understanding the mode of action of BCAs in disease control is essential in the context of product development in order to ensure reliable and durable efficacy (Minuto et al. 1997; Bardin et al. 2015), which can furthermore assist in screening for more efficient strains. Moreover, health and safety regulations require that the mode of action is known for registration of novel BCAs, to ensure their operational safety.

Challenges in studying the mechanisms of endophytic bio-control agents

In order to optimise the selection and utilisation of endophytes, an in-depth understanding of the general biology of the tripartite interaction between endophyte, host plant, and pathogen is required, as well as of the underlying physiological processes involved. The following four types of control principles are generally recognised for BCAs and thus also for endophytes: (1) competition for space and nutrients, (2) direct inhibition through antibiosis, (3) mycoparasitism as well as (4) induced resistance in the plant by activation of its own defence system (Zabalgogeazcoa 2008; Pessarakli 2010) (Figure 1). Often, several mechanisms are active at the same time. Growth promotion through nutrient acquisition

(nitrogen, phosphorous and essential minerals) or modulating plant hormone levels can also improve general plant health and thus protection against diseases (Kuldau G and Bacon C 2008; Franken 2012; Berthelot et al. 2016).

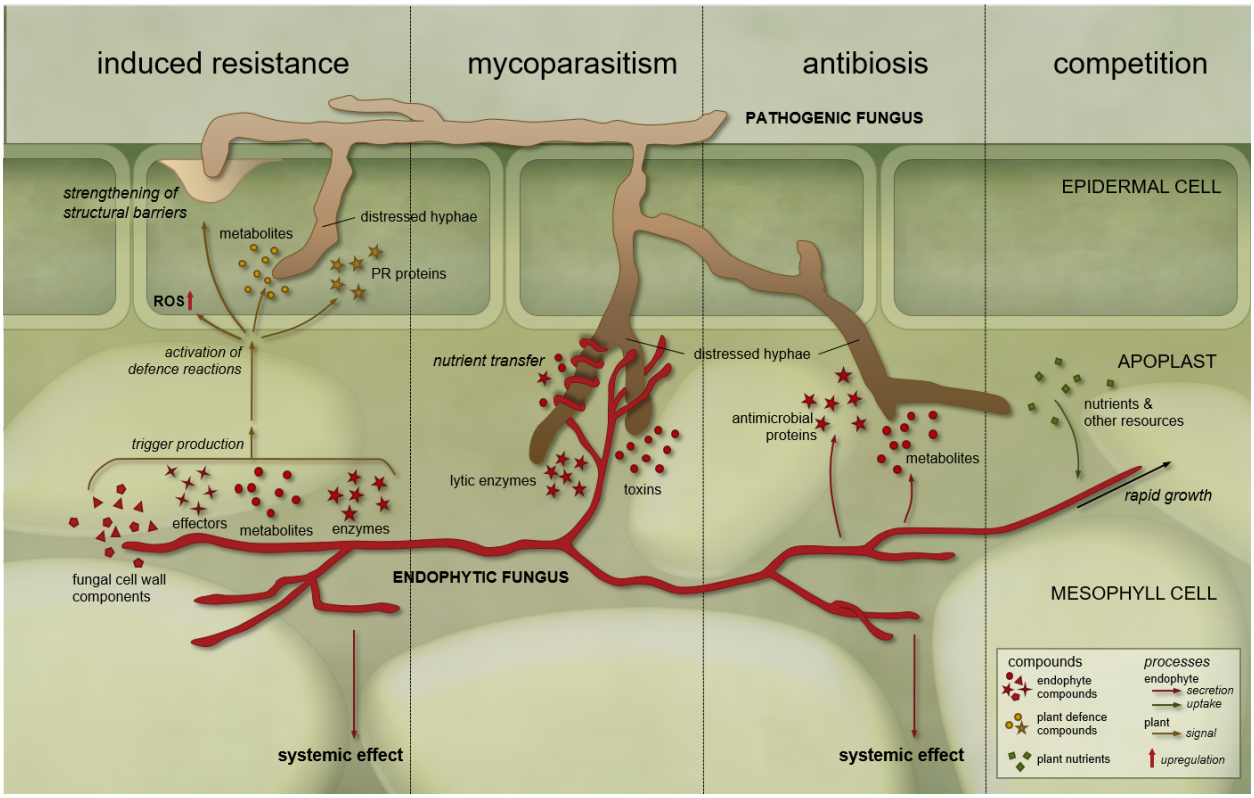

Figure 1. Mechanisms of fungal endophytes in biological control of phytopathogens. The anticipated mechanisms in the tripartite interaction between endophyte, host plant and pathogen include direct inhibition of the pathogen by the endophyte through competition, antibiosis or mycoparasitism, or indirect inhibition through induction of resistance. Induced resistance. Different compounds (effectors, metabolites, enzymes) secreted by the endophyte during the colonisation process and released from the cell wall (*e.g.*, chitin, β-glucans) can act as inducing agents and trigger production of a signal in the plant. Transmission and (systemic) translocation of a signal results in elevated activation of defence responses at the site of pathogen attack. Defence responses can include strengthening of structural barriers that halt pathogen penetration, generation of reactive oxygen species (ROS) and production of anti-microbial metabolites and proteins that inhibit pathogen growth. Mycoparasitism. Mycoparasitism of pathogens by the endophyte can be direct (coiling, penetration of prey) or indirect (no physical contact). In both situations the endophyte obtains nutrients from the pathogen by production of lytic enzymes and toxins. These compounds can also induce resistance after recognition by the plant. Antibiosis. The pathogen is inhibited by anti-microbial proteins and metabolites produced by the endophyte. Translocation of these compounds can provide a systemic protection of the plant. They can also induce resistance if detected by the plant. Competition. During the colonisation process, the endophyte grows inside the apoplast, taking up nutrients or other plant resources, and thereby competing for this niche with arriving pathogens.

The interaction between an endophytic BCA, host plant and pathogen and the mechanisms by which the pathogen is inhibited are complex and not trivial to study as outlined in Figure 1. Thus, several questions need to be raised. (i) What is the colonisation strategy and pattern of the potential BCA (Rodriguez et al. 2009; Hardoim et al. 2015; Card et al. 2016)? This is important in order to link inter- or intra-cellular endophyte structures to a specific disease-reducing mechanism. (ii) If a BCA has been isolated from surface sterilised tissue and categorised as an endophyte (Busby et al. 2016), does it, in fact, grow endophytically when applied in a specific bio-control set-up? Therefore, when assessing the mechanisms employed by an endophyte, it is important to evaluate critically whether the mechanisms are activated during endophytic colonisation (Suryanarayanan 2013). (iii) Studies of mechanisms of BCAs are often

conducted under in vitro conditions, but the question is whether the mechanisms active *in vitro* are, in fact, responsible for an effect observed in planta? Excluding one of the organisms from the tripartite interaction will significantly affect the interaction and may therefore not give much meaningful information. Therefore, relying on in vitro studies of endophyte-pathogen interactions alone can result in misleading conclusions. For example, in vitro experiments indicated that the epiphytic yeast *Pseudozyma flocculosa* inhibited *Blumeria graminis* f.sp. *hordei* in barley through antibiosis, but investigations of the transcriptome and cellular interactions by microscopy revealed that, in fact, mycoparasitism was the control mechanism (Laur et al. 2018). On the other hand, whereas the fungal BCA *Clonostachys rosea* showed no inhibitory effect on *Fusarium culmorum* growth in vitro, it efficiently controlled Fusarium seedling blight in barley (Knudsen et al. 1997). (iv) Finally, the mechanisms utilised by a BCA can differ depending on the pathosystem, exemplified by the different plant defence responses induced by *Serendipita indica* (formerly *Piriformospora indica*) when interacting with different host plants and pathogens (Felle et al. 2009; Molitor et al. 2011; Harrach et al. 2013). Sometimes, differences in bio-control performance of a BCA can even be observed depending on the host species cultivar (Smith and Goodman 1999; Xue et al. 2014). This paper aims at critically reviewing the evidence required to conclude which mechanisms contribute to biological disease control exerted by fungal endophytes during their endophytic lifestyle.

Mechanisms of endophyte-mediated disease suppression

Induced resistance

Kloepper et al. (1992) defined induced resistance to diseases as 'the process of active resistance dependent on the host plant's physical or chemical barriers, activated by biotic or abiotic agents'. The inducing agent triggers the formation of a translocatable signal in the host, which makes the host respond in a resistant manner to subsequent pathogen attack. This is an active process, in which the plant signal triggered by the inducing agent initiates differential expression of genes, protein synthesis and specific metabolic changes (Figure 1). These changes in plant metabolism alter the suitability of the plant as a host, which is reflected in a reduced disease level. Both living organisms and chemical compounds can serve as inducing agents and the response of the host can be local or systemic. The term 'priming' is increasingly used to describe the protection relying on activation of defence responses in the plant. Conrath et al. (2002) defined priming as: 'Induced resistance is often associated with an enhanced capacity to mobilize infection-induced cellular defence response s—a process called "priming"'. There is now a general understanding that priming denotes a situation where 'defence responses are not, or only slightly and transiently, activated by a given priming stimulus' (Martinez-Medina et al. 2016). However, the term is widely used without always taking this condition into account. Therefore, in this review, we refer to protection activated by beneficial fungi that relies on activation of plant defence responses, as induced resistance.

Endophyte-derived compounds inducing plant responses

Plants are able to recognise specific microbial components and identify them as foreign. Since these compounds can derive from potentially harmful microorganisms, the plant prepares for a rapid induction of defence responses. Comparison of the plant responses to pathogenic and endophytic organisms indicate that both are recognised in the same manner, but differ in induction of defence response (Wani et al. 2015; Xu et al. 2015). Therefore, it is very likely that induced resistance is one of the most important mechanisms employed by endophytes in disease control. Some of the compounds recognised by the plant are common among all fungi, such as certain cell wall components. Other compounds that are more specific to certain species include secreted proteins, certain lipids and specialised (also termed secondary) metabolites, molecules with hormonal roles and volatile

compounds. This paragraph provides a selective overview on endophyte-derived compounds that have been reported to or are likely able to induce plant defence responses, since they have been identified as inducers from fungi in general.
Compounds of microbial origin are frequently referred to as pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs), which are recognised by plant receptors and induce PAMP/MAMP-triggered immunity (Nürnberger T and Kemmerling B 2009). MAMPs recognised by plant receptors include the primary fungal cell wall components chitin and β-glucans (Lyon 2014), both of which have been found to trigger plant responses. Secreted proteins and peptides from endophytes have also frequently been described as inducing agents. Enzymes secreted during the infection and colonisation process, such as xylanases, cellulases and chitinases, can be recognised by the host directly or through their degradation products and induce defence responses (Rotblat et al. 2002; Belien et al. 2006; Druzhinina et al. 2011). Fungal effectors, often small cysteine-rich proteins, are secreted during both pathogenic and endophytic colonisation processes to promote compatibility with the host plant, by modulating defence responses and physiology (Bent and Mackey 2007; Rafiqi et al. 2013; Hacquard et al. 2016; Nogueira-Lopez et al. 2018). These small proteins can also induce plant responses when recognised by the host (Djonovic et al. 2007; Salas-Marina et al. 2015). Furthermore, there is evidence that some fungal compounds, produced to inhibit competing organisms, can also induce resistance (Luo et al. 2010). These examples reflect the diversity of endophyte-derived compounds that have potential to induce plant defence responses.

The complexity of hormonal signalling in induced resistance

Phytohormones play an important role in signal transmission of systemically induced resistance. Classically, two main types of systemic resistance are distinguished, 'systemic acquired resistance' (SAR) and 'induced systemic resistance' (ISR). In SAR, the hormone salicylic acid (SA) plays a prominent role, whereas the hormones jasmonic acid (JA) and ethylene (ET) are important in ISR (Pieterse et al. 2012, 2014). Cross-communication between SA, JA and ET signalling allows the plant to finely adjust the defence response (Pieterse et al. 2012, 2014) and JA/ET-dependent systemic resistance has, in fact, been found for plant-associated *Trichoderma asperellum*, *Penicillium* sp. and the endophyte *Serendipita indica* (Shoresh et al. 2005; Hossain et al. 2008; Stein et al. 2008). However, in other pathosystems, *S. indica* induced resistance independently of the JA/ET pathway (Waller et al. 2005) and plant-associated *T. asperellum* activated resistance in a SA-dependent manner (Yoshioka et al. 2012). This indicates that the roles of hormones and their potential interactions are complex and the application of a micro-organism to the plant is likely to change the whole hormone profile, instead of only affecting the levels of single hormones. Furthermore, the role of hormones may vary depending on the host and the strain of the inducing agent. This illustrates, despite the substantial progress in research on signal transduction in induced resistance, how much remains to be discovered about the specific roles of hormones in signal transduction in different systems. Therefore, we suggest using increased plant defence reactions as markers for activation of induced resistance and not increasing levels of specific hormones.

Plant defence reactions: markers for induced resistance

Induced resistance is the result of plant defence mechanisms that together make the plant less suitable as a host for a variety of pathogenic organisms. A multitude of defence responses are activated simultaneously and include strengthening of structural barriers, production of anti-microbial metabolites, synthesis and secretion of pathogenesis-related (PR) proteins, including defence enzymes and proteins with anti-microbial properties as well as production of reactive oxygen species and activation of the hyper-sensitive response (Kuć 2001; Van Wees et al. 2008). When studying whether an endophyte induces resistance, any set of defence responses can be examined. However, two criteria

should be fulfilled to classify any observed protection as induced resistance. Firstly, the defence responses should be able to inhibit the pathogen. Therefore, previous evidence that a particular response is effective against the pathogen is required and expression of the defence response should be correlated to arrest of pathogen infection/colonisation in order to link reduction of pathogen growth to increased expression of the response. Secondly, increased defence response expression should be observed after pathogen inoculation of previously endophyte-treated plants, in comparison to plants inoculated with the pathogen alone. Consequently, using the 'exclusion principle' is not a valid method for deducing whether induced resistance is involved in protection. Absence of direct in vitro inhibition, therefore, does not allow the assumption that induced resistance is responsible for disease reductions observed. Strengthening of structural barriers is a conserved mechanism of plants to prevent pathogen penetration and reinforced cell wall appositions have often been observed (e.g. Waller et al. 2005). This effect can also be a response to an inducing agent, as shown for different strains of plant-associated *Trichoderma* spp. Accordingly, *T. harzianum* (strain T22) enhanced expression of phenylalanine ammonia lyase (PAL) in maize (Shoresh et al. 2010), an enzyme involved in lignification, and *T. harzianum* strain T-203 strengthened epidermal and cortical cell walls of cucumber seedlings, possibly induced by endophytic intercellular colonisation (Yedidia et al. 1999). In addition to structural changes, plants are also 370 capable of producing metabolites and proteins with anti-microbial properties in response to pathogen exposure. Systemic changes induced by inducing agents are often associated with accumulation of phytoalexin-type compounds (Oliveira et al. 2016). Furthermore, PR -proteins, which are well known for their roles in defence and stress, possess antimicrobial properties. These PR proteins include enzymes and peptides such as thionins, lipid-transfer proteins and thaumatin-like proteins (van Loon and van Strien 1999; Sels et al. 2008). For example, β-1,3-glucanase (PR-2) was up-regulated in *Plasmodiophora brassicae* infected oilseed rape plants when colonised by the dark septate endophyte *Heteroconium chaetospira* (Lahlali et al. 2014). Likewise, endophytic *Fusarium solani* (strain Fs-K) induced systemic resistance in tomato against *Fusarium oxysporum* f.sp. *radicis-lycopersici* and was found to enhance expression of PR-5 (thaumatin-like) and PR-7 (endo-proteinase) genes (Kavroulakis et al. 2007). In another study, endophytic *Serendipita indica* induced resistance in barley against *Blumeria graminis* f.sp. *hordei* and the protein HvPR17b with hypothesised anti-fungal activity was highly up-regulated (Waller et al. 2008). However, in the barley-*S. indica-Fusarium graminearum* interaction, expression ofPR-genes (PR-1b and PR-5) was reduced (Deshmukh and Kogel 2007), showing that PR proteins are not involved in all systems.

Examples of fungal endophytes inducing resistance

In the following examples, endophytic colonisation was determined by microscopy in the specific endophyte-plant pathogen interaction. Furthermore, the biology of the pathogen was considered and reflected in the experimental design, implying that activity of a certain defence response is only a valid marker when the pathogen is known to be sensitive to the response.

The root endophyte *Serendipita indica* colonises the interior of a broad range of mono- and dicotyledonous plants and has been shown to confer enhanced growth and resistance in a variety of systems (Stein et al. 2008; Felle et al. 2009; Harrach et al. 2013; Varma et al. 2013; Nassimi and Taheri 2017). Whereas the endophyte exclusively colonises the roots, a systemic effect has frequently been observed, providing protection also in aerial plant parts. A substantial amount of research on the mechanisms of *S. indica* as inducer of plant resistance in the barley-powdery mildew system has been published (Waller et al. 2005, 2008; Felle et al. 2009; Molitor et al. 2011). According to their findings, *S. indica*-enhanced resistance in barley to powdery mildew is characterised by reduction of successful penetration events through an increase of papilla-formation and local cell death (Waller et al. 2005; Molitor et al. 2011). Only few changes in gene expression in roots were induced by *S. indica* root

colonisation alone, but up-regulation of the PR-gene HvPR17b in foliage was observed (Waller et al. 2008). Molitor et al. (2011) found that inoculation with *Blumeria graminis* f.sp. *hordei* resulted in induction of gene expression in the foliage and a set of genes involved in defence reactions, protein synthesis and apoptosis were up-regulated (notably PR1, PR2 and 435 PR5, Hsp70 and Barley chemically induced 7 [BCI-7]) (Molitor et al. 2011). These PR gene products are known for their direct or indirect anti-fungal effect. Furthermore, Felle et al. (2009) found rapid induction of systemic alkalinisation of the leaf apoplast, possibly facilitating the systemic response to the infection of *S. indica* colonised plants. In other pathosystems, *S. indica* enhanced resistance through different plant responses. Disease reduction of both root rot caused by *Fusarium culmorum* in barley and *Thanatephorus cucumeris* (anamorph *Rhizoctonia solani*) causing sheath blight in rice was achieved by increasing the anti-oxidant capacity of the host (Harrach et al. 2013; Nassimi Z and Taheri P 2017).
Using extensive microscopy and gene expression studies, Su et al. (2013) investigated the mechanisms by which the dark-septate endophyte *Harpophora oryzae* in rice roots suppressed rice blast caused by *Pyricularia oryzae*. *H. oryzae* colonisation protected roots from *P. oryzae* infection, prevented necrotisation of roots and limited foliar rice blast symptoms compared to the *H. oryza*e- free control. Furthermore, the SA-dependent transcription factor OsWRKY4 was up-regulated and it has previously been found to strongly enhance resistance against rice blast (Shimono et al. 2007). PR genes and JA-dependent genes were not differentially regulated (Su et al. 2013). A similar study on a dark-septate endophyte on oilseed rape was carried out by Lahlali et al. (2014), in which PR-2 (β-1,3-glucanase) and PAL was up-regulated when the plants were inoculated with the endophyte before inoculation with *Plasmodiophora brassicae*.

Antibiosis

Antibiosis denotes the inhibition of pathogens that can be attributed to compounds directly produced by a BCA (Dipietro 1995). Endophytic fungi represent a rich source of specialised metabolites and other compounds that may be inhibiting competing micro-organisms (Thines et al. 2004). Efforts have been made to identify and develop fungal metabolites or the producing organism towards commercial application in plant disease control and a range of different compounds have been investigated for their inhibitory activity against phytopathogens (Schulz et al. 2002; Thines et al. 2004; Suryanarayanan 2013; Daguerre et al. 2017). A great number of natural products possessing anti-microbial activities have been isolated from endophytes, including alkaloids, flavonoids, peptides, phenols, quinones, steroids, terpenoids, polyketides and volatile organic compounds with terpenoids and polyketides being the most purified anti-microbial-specialised metabolites, as recently reviewed (Yu et al. 2010; Mousa and Raizada 2013; Lugtenberg et al. 2016). The presence of different microbial species in the same plant can trigger metabolite production by both endophyte and/or the host to limit growth of potentially pathogenic organisms (Kusari et al. 2012). In some cases, host and endophyte share parts of a specific pathway and contribute partially to the metabolite production or one partner induces metabolism or metabolises products of the other (Aly et al. 2013; Kusari et al. 2013; Ludwig-Müller 2015) . A well-known example is the discovery that several fungal endophytes were associated with the production of the anti-cancer drug taxol in the Pacific yew tree *Taxus brevifolia*. However, Heinig et al. (2013) concluded that many of the endophyte strains were not actually independent producers.
There is an extensive body of research on the capability of endophytes for specialised metabolite production (Suryanarayanan 2013; Daguerre et al. 2017) and pathogen-inhibiting effects of endophyte cultures or crude extracts. Often, the effects of metabolites are assessed under in vitro conditions. Whereas this gives an indication of the potential of an organism to produce substances which can inhibit the pathogen, it does not give an indication pathogen inhibition will take place *in planta* (Köhl et al. 2011; Deketelaere et al. 2017; Lauret al. 2018). While direct application of *in vitro*-produced metabolites

may control disease in the plant by direct toxicity or by inducing resistance (Sinha and Trivedi 1969; Mathivanan et al. 2008), the endophyte may not actually produce these compounds in planta. This is due to major differences in nutrient levels, environmental conditions as well as interactions with other organisms in the *in planta* situation, with complex interactions between BCA, plant, inherent microbiome and pathogen. Even different kinds of artificial media or modification of other factors can give rise to production of different types of metabolites (Kusari et al. 2012). On the other hand, the biosynthetic machinery for some bio-active compounds produced by the BCA is only expressed in association with the plant or possibly in the tripartite interaction with plant and pathogen. For example, the gene cluster for the alkaloid lolitrem in endophytic *Neotyphodium lolii* is highly expressed in association with cold-season grasses, but only at low or undetectable levels under *in vitro* conditions (Young et al. 2006). Such endophytes would therefore be missed in in vitro-based screening systems for potential bio-control agents.

To demonstrate that an in planta produced antimicrobial compound is involved in bio-control induced by an endophyte, ideally it should be confirmed that the compound is in contact with the pathogen. This may be difficult to determine since the endophyte is localised inside the plant and the amount of metabolite produced may be very low. Furthermore, metabolites can be trans-located in the plant from the endophyte to the site of pathogen infection, whereas volatile organic compounds produced by a BCA can diffuse to the site of the pathogen infection, as reported in some studies (Lugtenberg et al. 2016; Gabriel et al. 2018). However, it remains to be tested whether the quantity of the active compound at the pathogen site is sufficient to control disease or whether the effect is through other mechanisms (Lugtenberg et al. 2016). Disruption of the biosynthetic pathway of the metabolite should result in loss of bio-control activity and thereby confirm dependence of the activity of the metabolite in question. To rule out the possibility of simultaneous induction of resistance by the fungal metabolite, differential expression of plant defence genes should not be induced the metabolite. Examples of fungal endophytes controlling diseases by antibiosis. In the following examples, endophytic colonisation was determined by microscopy in the specific endophyte-plant-pathogen interaction. Tian et al. (2017) investigated the role of an in planta secreted anti-fungal protein of Epichloë festucae in the control of *Sclerotinia homoeocarpa* in *Festuca rubra* ssp. *rubra*. *E. festucae* systemically colonises above ground parts of *F. rubra* ssp. *rubra*, a unique property that has not been shown for other fescues (Tian et al. 2017). A quantitative transcriptome study of *E. festucae*-infected *F. rubra* ssp. *rubra* identified the secreted protein Efe-AfpA which represented 6% of the fungal transcriptome (Ambrose and Belanger 2012) and which had similar characteristics as secreted antifungal proteins from Penicillium sp. and Aspergillus spp. (Tian et al. 2017). Furthermore, partially purified Efe-AfpA extracted from apoplastic fluid of endophyte-infected red fescue showed in vitro anti-fungal activity against *S. homoeocarpa*, as did the recombinant product of the Efe-afpA gene expressed in Pichia pastoris. By using a viability/vital stain, it was shown that Efe-AfpA targeted the cell membrane of *S. homoeocarpa*. Attempts to disrupt the gene in *E. festucae* have been unsuccessful so far.

Rafiqi et al. (2013) and Soliman et al. (2015) investigated the mechanisms of protection exerted by the endophyte *Paraconiothyrium* strain SSM001 associated with the taxol-producing yew tree (Taxus spp.) against harmful wood-decaying fungi. Yew trees typically hyper-branch from buds beneath the bark resulting in constant bark cracking and these cracks are an easy entry point for wood-decaying fungi. The endophytic strain SSM001 was found to grow towards tissue cracks and inflicted cuts. Eradication of the endophyte by fungicide treatment reduced taxol accumulation in the tree as well as transcript levels of two critical Taxus genes (taxadiene synthase and DXP reductoisomerase), important for taxol-production. This indicates that the endophyte was involved in at least part of the synthesis of taxol. In addition, *in vitro* studies by Soliman et al. (2015) showed that both treatment with taxol and with the endophyte SSM001 supressed growth of three wood-decaying fungal species (*Heterobasidion annosum*, *Phaeolus schweinitzii* and *Perenniporia subacida*), whereas strain SSM001 itself was not inhibited by

taxol. Furthermore, there was a significantly lower biomass of *P. schweinitzii* in yew plantlets colonised by strain SSM001 compared to plantlets in which endophyte colonisation was reduced by fungicide injection beforehand. An *in vitro* experiment showed that release of taxol by strain SSM001 was induced by the presence of *P. schweinitzii*, as well as by chitin and an elicitor from *P. schweinitzii*. It would be Interesting to identify the endophyte genes contributing to taxol biosynthesis and confirming that knockout of these would lead to loss of the control effect towards the fungi.

Mycoparasitism

Jeffries (1995) defined mycoparasitism as the situation where a fungus directly obtains nutrients from another fungus. This definition comprises a continuum of situations, from a biotrophic interaction where the host stays alive and the parasite obtains nutrients from within the host cells, to a necrotrophic Interaction where the parasite kills the host to live off the dead cells (Jeffries 1995; Horwitz and Viterbo 2010; Kim and Vujanovic 2016).

Verifying that mycoparasitism occurs *in planta* is a challenge, since confirmation of nutrient transfer is difficult and therefore many claims of mycoparasitism are based on circumstantial evidence (Jeffries 1995). The mere fact that two fungi grow in close association is not in itself evidence for mycoparasitism. If only a close association is seen between the organisms, they are described as fungicolous (Jeffries 1995). Mycoparasitism can be both indirect and direct, depending on whether the parasite produces compounds that aim to release nutrients from the prey at a distance, or is in direct physical contact (Jeffries 1995). In either case, the parasite produces compounds for nutrient release and acquisition, including cell wall degrading enzymes (CWDEs), antibiotics and toxins (Horwitz and Viterbo 2010; Kim and Vujanovic 2016). Kim and Vujanovic (2016) made a comprehensive survey of recent records of mycoparasites and listed a range of mycoparasites along with their modes of actions. Furthermore, the complexity of the interaction between parasite and prey is illustrated, for example in the studies of specialised metabolites produced in the interaction between *Stachybotris elegans* parasitising *Thanatephorus cucumeris* (Chamoun et al. 2015). This potential overlap between antibiosis and mycoparasitism often makes it difficult to distinguish clearly, which mechanisms are involved in the observed control, but illustrates that an organism may employ several mechanisms of inhibition of a pathogen at the same time.

Studies to verify mycoparasitic behaviour are generally conducted *in vitro* since it is easier to observe signs of parasitism in a Petri dish than on a plant. Using various microscopy techniques, it is often possible to see direct interactions between the partners. The mycoparasite may make direct contact, penetrate into the hyphae of the prey, coil around the hyphae and finally cause disruption/depletion of the prey hyphae. Examples of mycoparasitism by endophytic fungi include the work by Donayre and Dalisay (2016), who studied parasitism of *Geotrichum* sp. (isolate EF-ds104-16) on *Thanatephorus cucumeris*. The mycoparasite was isolated as an endophyte from the grass *Echinochloa glabrescens*, but the tests conducted *in vitro*. Likewise, Cao et al. (2009) tested three fungal isolates, isolated as endophytes from *Phragmites australis* against eight soil-borne pathogens by microscopy and found penetration followed by hyphal coiling around pathogen hyphae and degradation of cytoplasm. Furthermore, production of the extracellular CWDEs chitinases and β-1,3-glucanases was demonstrated. Common to above examples is that even though the mycoparasitic fungi were originally isolated as endophytes, they were tested *in vitro*, only in the presence of a plant pathogen, meaning that the plant was not there to interfere with the interaction. Verifying mycoparasitism for an endophyte, while growing endophytically, is not an easy task. For parasitism to occur, it would require that the parasite came sufficiently close to its prey inside the plant so that parasitism could be initiated. It is therefore likely that mycoparasitism as a control principle is not very important for endophytes, as also suggested by Card et al. (2016).

Competition

Competitive exclusion is a determining factor for the composition of the plant micro-biome and a probable mechanism by which inhabiting endophytes can prevent colonisation of the host by pathogens (Zabalgogeazcoa 2008; Martinuz et al. 2012). Fungal endophytes can colonise different plant tissues locally or systemically, either inter or intra-cellularly (Boyle et al. 2001). Through rapid colonisation and scavenging of available nutrients, they thereby occupy the niche that could otherwise be used by a pathogenic organism (Rodriguez et al. 2009). This is exemplified by the finding that elimination of specific endophytes in mango leaves by fungicide treatment allowed other fungi to colonise this niche (Mohandoss J and Suryanarayanan TS 2009), thus making space for potentially pathogenic microorganisms.

In biological control, the mechanism of competitive exclusion is most likely to occur in combination with other mechanisms rather than alone. Since the control effect is only local, it would require a systemic colonisation of the host plant part where the pathogen might attack. Correspondingly, colonisation of oilseed rape roots with the dark-septate endophyte *Heteroconium chaetospira*, measured by qPCR, was negatively correlated to clubroot symptoms (Lahlali et al. 2014). However, increased pathogen inoculum reduced the control effect, indicating the limitation of competition as a mechanism under high disease pressure. Arnold et al. (2003) found that foliar application of a mixture of frequently isolated endophytes from cacao tree leaves to endophyte-free seedlings locally reduced the disease symptoms of *Phytophthora* spp. on foliage, indicating competition as a mechanism. However, *in vitro* confrontation assays also showed metabolite production by the selected strains, suggesting that competition might not be the exclusive mechanism. Microscopy studies and quantification of endophyte biomass *in planta* in relation to disease severity, supplemented with microscopy of the extensive colonisation, appears to be a favourable approach to investigate competitive exclusion. However, as suggested by Card et al. (2016), competitive exclusion is probably not very important for endophytes as BCAs since colonisation at the expected entry point of the pathogen needs to be very compact.

Conclusion and perspectives

In recent years, there has been extensive research on fungal endophytes for control of crop plant diseases. Available technologies for studying mode of action have developed dramatically during the past decades, offering advanced molecular techniques for detailed investigations of the tripartite interaction between endophyte, plant and pathogen. 'Omics' techniques are a powerful tool to investigate such tripartite interactions and include genomics, transcriptomics, proteomics and metabolomics to elucidate which pathways are affected in the different organisms and their potential production of metabolites (e.g. Kaul et al. 2016; Laur et al. 2018). Recently, Laur et al. (2018) applied RNAseq as a tool to gain insight into the tripartite interaction of the yeast BCA *Pseudozyma flocculosa*, powdery mildew and barley. They studied the simultaneous responses of all three organisms at different time points of the interaction, and using light microscopy, scanning and transmission electron microscopy observations of the various phenotypes to support the RNAseq data. These methods allow a more detailed understanding of the interactions and the contributions of all participating partners. Other tools also allow insight into the molecular base of endophytic antagonists, such as gene expression studies, enzyme assays and use of knockout mutants or RNA interference. In order to investigate the nature of the interaction between pathogen and endophyte *in planta*, visualisation methods such as confocal and light microscopy can be applied when using fluorescently labelled fungal BCA and pathogen strains, as demonstrated by Karlsson et al. (2015). Recent development of Mass Spectrometry Based Molecular 3DCartography (Floros et al. 2017) offers great potential as a method for determining the distribution of specific metabolites in relation to microbes in situ and, if linked with molecular genetic studies, for clarifying the role of specific metabolites.

In conclusion, we need to investigate the nature of plant-endophyte interaction and the requirements for successful establishment. That involves determination of fungal roles and compounds required for the disease-reducing effect, but also investigations of plant responses induced by the interaction. Such knowledge will allow development of markers for the efficiency of a specific bio-control agent and testing the effects of plant genotype, inherent microbial community and the environment as well as providing a structured approach for discovery of novel endophytes with desired traits.

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