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MINIREVIEW

Fungal endophytes for sustainable crop production

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One sentence summary: An aniline-blue leaf sheath peel of an *Epichloe* species growing between plant cells.

Editor: Gerard Muyzer

ABSTRACT

This minireview highlights the importance of endophytic fungi for sustainable agriculture and horticulture production. Fungal endophytes play a key role in habitat adaptation of plants resulting in improved plant performance and plant protection against biotic and abiotic stresses. They encode a vast variety of novel secondary metabolites including volatile organic compounds. In addition to protecting plants against pathogens and pests, selected fungal endophytes have been used to remove animal toxicities associated with fungal endophytes in temperate grasses, to create corn and rice plants that are tolerant to a range of biotic and abiotic stresses, and for improved management of post-harvest control. We argue that practices used in plant breeding, seed treatments and agriculture, often caused by poor knowledge of the importance of fungal endophytes, are among the reasons for the loss of fungal endophyte diversity in domesticated plants and also accounts for the reduced effectiveness of some endophyte strains to confer plant benefits. We provide recommendations on how to mitigate against these negative impacts in modern agriculture.

Keywords: anti-insect compounds; anti-microbial compounds; biotic and abiotic stresses; commercialization; *Epichloë*; endophytic fungi; habitat adaptation; fungal secondary metabolites; plant adaption; volatile organic compounds

INTRODUCTION

Definition of 'endophyte'

We define endophytes based on the definition proposed by Hallmann et al. (1997), as 'those microorganisms that can be isolated from surface-disinfected plant tissue or extracted from within the plant, and that do not visibly harm the plant'. However, this definition is not perfect as, first, it does not account for endophytes that are unculturable and, second, it is not always easy to assess phytopathogenicity and distinguish latent pathogens from endophytes, particularly for non-culturable fungi that are part of the microbiome community (see Hyde and Soytong 2008; Card et al. 2015; Hardoim et al. 2015; Mercado-Blanco 2015; Card et al. 2016 for a discussion of the term 'endophyte'). Fungal

endophytes exhibit a range of symbiotic relationships with their hosts as well as various lifestyles; for example, some of these interactions can be mutualistic in which the long-term relationship is beneficial to both partners (reviewed in Card et al. 2016). In addition, some endophytes may only exhibit a mutualistic interaction for one plant species, but not for another (Hardoim et al. 2015). However, the scientific community generally agrees that endophytes are microorganisms that can be detected inside healthy plant tissues and are asymptomatic. Finally, for experimental purposes, in order to verify that a microorganism has an endophytic lifestyle, it must be demonstrated that it can be successfully reintroduced into disinfected seedlings as judged by microscopy, thereby fulfilling Koch's postulates (Hyde and Soytong 2008).

Aim of this review

After introducing some basic aspects of fungal endophytes, the primary aim of this paper is to provide insights into the means by which fungal endophytes can be effectively used for improving growth, health and adaptation of plants to stress conditions. Examples of fungal endophytes which are used in, or have promise for, agriculture and horticulture, will be presented. While several reviews have described aspects of fungal endophytes (Saikkonen et al. 1998; Strobel and Daisy 2003; Schulz and Boyle 2005; Arnold 2007; Rodriguez et al. 2008; Aly, Debbab and Proksch 2011; Porras-Alfaro and Bayman 2011; Friesen 2013; Johnson et al. 2013a), none have focused on the combined aspects of how:

- (i) fungal endophytes and their secondary metabolites improve plant health, plant performance and plant adaptation by using selected fungal endophytes;
- (ii) the commercialization of fungal endophytes and their products can be successfully achieved and;
- (iii) fungal endophytes and/or their plant-beneficial activities could have been lost or reduced from cultivated plants through plant breeding and use of fungicides and how this can be prevented in the future.

Occurrence

Within all plants, there is a diverse microbial community comprising bacterial, archaeal, fungal and protistic taxa (Hardoim et al. 2015). Blackwell (2011) discusses the classification, discovery and identification of fungi as well as estimations of the magnitude of fungal diversity present on earth which ranges from the conservative 1.5 million species by Hawksworth (2001) to the newer estimates of 5.1 million based on high-throughput sequencing methods. Researchers estimate there are at least 1 million endophytic fungal species (Strobel and Daisy 2003; Ganley, Brunsfeld and Newcombe 2004). A number of such magnitude signifies the importance of fungal endophytes as crucial constituents of fungal biodiversity. Endophytic fungi are predominantly ascomycetes that appear to be ubiquitous in nature as they have been recovered from plants adapted to a wide range of ecosystems that include hot deserts, Arctic tundra, mangroves, temperate and tropical forests, grasslands and savannahs, and croplands (Arnold 2007, 2008; Arnold and Lutzoni 2007). Present in all the major lineages of land plants, they are common to mosses and other non-vascular plants, ferns and other seedless plants, conifers and flowering plants (Arnold 2007). Our understanding of fungal endophyte diversity across large geographical areas is still developing but endophyte diversity in plants has been observed to decrease from the tropics to northern boreal forests (Arnold 2007; Arnold and Lutzoni 2007). Furthermore, endophyte communities from higher latitudes are characterized by relatively few fungal species, whereas tropical endophyte assemblages are dominated by a small number of classes but a very large number of different endophytic species (Arnold et al. 2000; Arnold and Lutzoni 2007). The spectrum of fungi within a plant varies in space, time and function. For example, leaves, stems and roots of alfalfa plants are colonized by distinct fungi that produce different ranges of secondary metabolites (Weber and Anke 2006; Porras-Alfaro and Bayman 2011). A compelling feature of fungal endophytes is their exceptional diversity both at a global scale and at the scale of individual leaves, plants and locations (Arnold 2008).

While acknowledging that the majority, if not all plants contain endophytes, many remain not studied for their endophytic diversity or function. However, the exponential increase in the use of endophytes for improving plant adaption, which has featured in recent reviews (Porras-Alfaro and Bayman 2011; Kivlin, Emery and Rudgers 2013; Johnson et al. 2013a; Hardoim et al. 2015; Card et al. 2016), indicates a growing recognition by the scientific community of the potential to exploit novel endophytes for the improvement of crop production.

Functional grouping of endophytic fungi

The differentiation of fungal endophytes into functional groups based on various criteria is a useful framework for clarifying the unique symbiotic and ecological functions of endophytic fungi and to aid researchers in answering fundamental biological questions about these organisms. Rodriguez et al. (2009) described different functional groups of endophytic fungi based on their phylogeny and life history traits. Class 1 endophytes are defined as the Clavicipitaceous endophytes (including *Balansia* spp. and *Epichloë* spp.). The *Epichloë* species form systemic associations with the aboveground tissues of grasses and are one of the most economically important examples of plant–endophyte interactions (Johnson et al. 2013a). The non-clavicipitaceous types are further separated into three subclasses, classes 2, 3 and 4. The diverse class 2 endophytes encompass both Ascomycota and a few Basidiomycota (the Dikarya). The most distinctive feature is their ability to colonize roots, stems and leaves and the formation of extensive plant infections (Rodriguez et al. 2009). Endophytes from class 3 are extremely diverse and form highly localized infections in aboveground tissues, such as in the leaves of tropical trees and non-vascular and vascular plants (Rodriguez et al. 2009). Class 4 endophytes are also referred to as the dark-septate endophytes (DSE) (Rodriguez et al. 2009) and these facultative biotrophic fungi colonize plant roots and have the distinguishing feature of having melanized dark septate hyphae (Jumpponen and Trappe 1998; Jumpponen 2001). We recommend that an additional class is needed to recognize the endophytic entomopathogenic fungi as symptomless endophytes of plants that have the unique ability to infect and colonize insects (Quesada Moraga, Herrero and Zabalgoceazcoa 2014; Vidal and Jaber 2015).

Transmission

The means of propagation or the transmission routes of many endophytic fungi remain to be determined; however, they can be transmitted either horizontally or vertically. The *Epichloë* species are one example where the endophyte can be propagated via both routes as long as the ability to produce ascospores on the host plant is retained. One of the well-studied examples of vertical transmission via seed has been described for the asexual *Epichloë* species. These fungi cannot produce reproductive structures on their hosts and are naturally propagated by growing into the embryo of a developing seed and, subsequently, as the seed germinates, hyphae colonize the young seedling (Philipson and Christey 1986). Moving vertically transmitted *Epichloë* endophytes from one plant to another can be manipulated in the laboratory (Latch and Christensen 1985) enabling the artificial inoculation of grasses for developing desirable grass–endophyte combinations for possible commercialization purposes (Johnson et al. 2013a). Another type of vertical transmission occurs via the seed coat and has been reported for class 2 endophytes (Rodriguez et al. 2009). A well-known example is that of *Curvularia protuberata* which confers high soil temperature tolerance

to its tropical grass host in a geothermal habitat (Redman et al. 2002).

In the case of horizontal transmission, propagation is usually dependent on the reproductive structures of the endophyte, such as spores, that move by wind or rain dispersal, or are moved by a vector, from plant to plant. This can occur via the soil, through air movement or by vectors, e.g. insects. For the class 2 and 3 endophytes, plant colonization likely occurs via infection structures such as appressoria or directly via the hyphal penetration of plant tissues (Ernst, Mendgen and Wirsal 2003; Gao and Mendgen 2006). The horizontal transmission of the sexual species of *Epichloë* (class 1) via ascospores has been well documented (reviewed in Schardl, Leuchtmann and Spiering 2004) and in contrast to the asexual *Epichloë*, their presence is not asymptomatic but forms external reproductive structures (stromata) on flowering tillers, preventing floral development and seed production (choke disease). These sexual species are obligately outcrossing (heterothallic) and a third mutualist partner, a fly species from the genus *Botanophila*, acts as the vector to transfer spermatia (conidia – mitotic spores) of one mating type to the stroma of the opposite mating type (Schardl, Leuchtmann and Spiering 2004). Ascospores are produced if mating partners are compatible and are ejected into the air and wind dispersed for mediating contagious infections (Chung and Schardl 1997; Brem and Leuchtmann 1999). Interestingly, the choice of transmission route that the sexual *Epichloë* species selects for propagation influences how beneficial or antagonistic the symbiotic outcome is for the host plant due to the direct effect the endophyte life cycle has on host reproduction.

Role in the ecosystem

Fungal endophytes play crucial roles in ecosystems by protecting plants against many biotic and abiotic stresses, increasing their resilience, and helping plants to adapt to new habitats (Strobel and Daisy 2003; Schulz 2006; Rodriguez et al. 2008; Aly, Debbab and Proksch 2011; Friesen 2013). Biotic stresses from which endophytes can provide protection include plant pathogens, insects and nematodes. Abiotic stresses include nutrient limitation, drought, salination and extreme pH values and temperatures. In return, plants provide spatial structure, protection from desiccation, nutrients and, in the case of vertical transmission, dissemination to the next generation of hosts (Schulz 2006; Aly, Debbab and Proksch 2011). Endophytes may also play a role in the ecosystem by affecting plant growth through antagonistic fungal–fungal interactions. An example is the interaction between the pathogen *Ustilago maydis* and the endophyte *Fusarium verticillioides* within their shared plant host (maize, *Zea mays*), whereby the endophyte is capable of reducing the rate of pathogen growth, possibly by secreting metabolites that break down plant compounds that limit *U. maydis* growth (Rodriguez Estrada et al. 2012).

A further role of some fungal endophytes in ecosystems may be to initiate the biological degradation of a dead or dying host plant which starts the process of nutrient recycling (Strobel and Daisy 2003; Zhang, Song and Tan 2006; Vega et al. 2010; Aly, Debbab and Proksch 2011; Boberg, Ihrmark and Lindahl 2011). A survey of grass endophytes by Vázquez de Aldana, Bills and Zabalgoitia (2013) shows that the dominate endophyte taxa of grasses are also found as common airborne fungi. They speculate that the transition from an endophyte to a saprobe requires sporulation after host senescence for some of these species to complete their life cycle. Being established in tissues, endophytes have immediate access to plant nutrients available

during plant senescence (Rodriguez et al. 2008; Aly, Debbab and Proksch 2011).

FUNGAL ENDOPHYTES FOR IMPROVING PLANT PERFORMANCE

Fungal endophytes are well known to contribute plant fitness benefits, enabling adaptation of the plant host to biotic and abiotic stresses (reviewed in Rodriguez and Redman 2008; Aly, Debbab and Proksch 2011; Franken 2012; Johnson et al. 2013a; Johnson, Alex and Oelmüller 2014; Card et al. 2016). These fungi have diverse secondary metabolites (Tan and Zou 2001; Gunatilaka 2006), some of which are bioactive compounds expressed as defensive weapons to protect the host plant from pests and diseases but also as metabolites for specific interactions and communications with the plant host. Habitat-adapted symbiosis, a term coined by Rodriguez et al. (2008), defines how certain fungal endophytes (class 2 types) adapt to stress in a habitat-specific manner under different environmental conditions. The molecular and biochemical mechanisms behind how this habitat-adapted symbiotic interaction results in plant tolerance to high stress are unknown. Endophyte-enhanced plant growth promotion is another conferred beneficial trait that at least for the root-colonizing endophyte *Piriformospora indica*, is likely achieved through enhanced nutrient uptake and translocation, and by the modulation of phytohormones involved in growth and development (Johnson, Alex and Oelmüller 2014).

Fungal endophytes for conferring pest and disease resistance

Epichloë endophytes (class 1) are found in some temperate grass species and their primary symbiotically important contribution is to produce bioactive metabolites in the host plant which act as deterrents to pests and herbivores. The common toxic endophyte, *Epichloë festucae* var. *lolii*, originally present in perennial ryegrass and introduced into New Zealand from Europe, produces three known major secondary metabolites:

- (i) Lolitrem B (Fig. 1A) is a neurotoxin now known to cause ryegrass staggers (Gallagher, White and Mortimer 1981; Gallagher et al. 1982; Fletcher 1993; Fletcher, Sutherland and Fletcher 1999).
- (ii) Ergovaline is a metabolite that causes vasoconstriction (Oliver 2005) resulting in heat stress (Fletcher 1993; Fletcher, Sutherland and Fletcher 1999) and in tall fescue it causes the condition referred to as fescue toxicosis (Hoveland 1993; Bacon 1995).
- (iii) Peramine (Fig. 1B) is an insect feeding deterrent (Rowan, Dymock and Brimble 1990; Rowan 1993).

While endophyte-free grass is not toxic to animals, it can become susceptible to insect pests. A novel *Epichloë* strain (AR1) was introduced 15 years ago which produces neither lolitrem B nor ergovaline but the production of peramine provides resistance to a major insect pest—Argentine stem weevil (ASW) (*Listronotus bonariensis*) (Johnson et al. 2013a). Later discoveries of other novel *Epichloë* endophyte strains identified another alkaloid present in endophytes, the epoxy-janthitrems (Finch, Fletcher and Babu 2012), which are a type of indole diterpene found only in perennial ryegrass infected with strain AR37 (Tapper and Lane 2004). AR37-infected grasses have broad pesticidal effects against five of the six major pasture pests present in New Zealand; however, the role of the epoxy-janthitrem

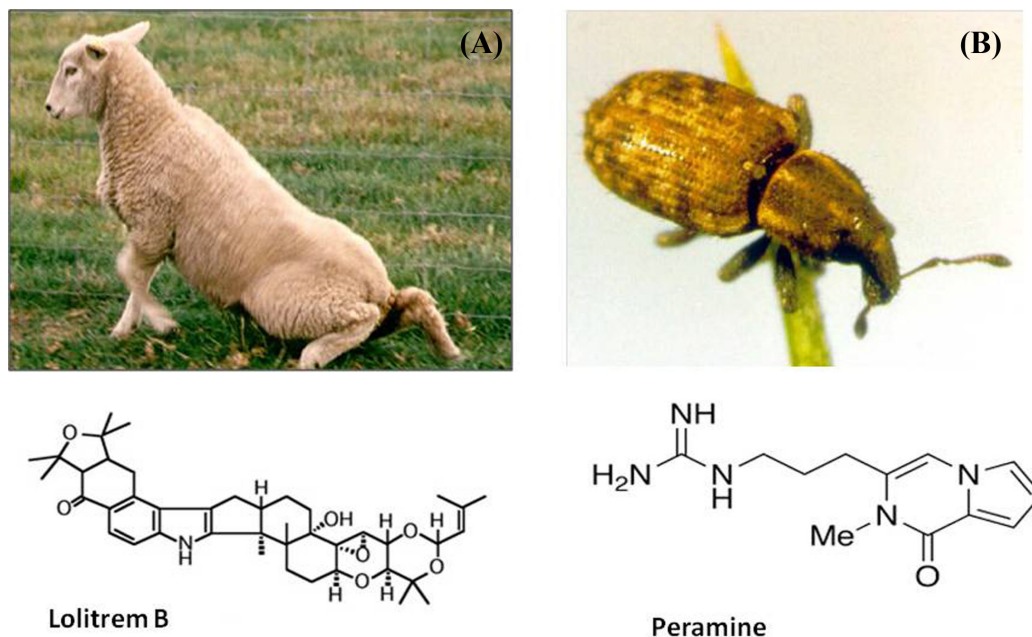


Figure 1. Molecular explanation of the ryegrass staggers story. Sheep grazing on perennial ryegrass can contract a neuromuscular condition designated as ryegrass staggers (A). The condition is caused by certain strains of *Epichloë* fungal endophytes. The offending endophyte could be removed by fungicide treatment. Grazing on endophyte-free grass did not cause the disease anymore but ryegrass persistence was seriously affected by damage caused by the insect ASW (B). Subsequent research has shown that the endophyte produced two major secondary metabolites: the neurotoxin **lolitrem B** which is causing ryegrass staggers and **peramine** as the major insect feeding deterrent. For details, see the sections 'Fungal endophytes for conferring abiotic stress, including impacts of climate change' and 'Anti-insect secondary metabolites'. Both figures have been used freely in the public domain for many years.

compounds in insect resistance has not yet been fully elucidated (Johnson et al. 2013a). Because of the advantage these novel endophytes provide through improving ryegrass persistence but without causing significant detrimental animal effects, the uptake by farmers has been rapid (Caradus, Lovatt and Belgrave 2013). A more comprehensive story on the importance of secondary metabolites present in *Epichloë* strains, and how they have been applied, can be found in Johnson et al. (2013a).

Epichloë endophytes have not only received worldwide recognition for their importance in pasture systems in delivering both economic and sustainable agricultural solutions, but they also represent an excellent model system for investigating various aspects of endophyte biology in addition to exploring both the ecological implications and the molecular basis of the symbiosis. *Epichloë festucae* in particular can be cultured on axenic media and is amenable to molecular functional studies. A growing number of studies characterizing *E. festucae* mutants have revealed that the maintenance of both reactive oxygen species (Takemoto, Tanaka and Scott 2006; Tanaka et al. 2006, 2008; Takemoto et al. 2011) and iron homeostasis (Johnson et al. 2013b) are essential factors for maintaining healthy mutualistic interactions with grasses.

The root-colonizing facultative endophyte *Piriformospora indica* forms beneficial symbioses with crop plants. It has the potential for use in agriculture, horticulture and floriculture (for reviews, see Oelmüller et al. 2009; Franken 2012; Qiang et al. 2012; Ansari et al. 2013; Oberwinkler et al. 2013; Unnikumar, Sree and Varma 2013; Johnson, Alex and Oelmüller 2014; Gill et al. 2016). *Piriformospora indica* was originally isolated by Verma et al. (1998) from the rhizosphere of several xerophytic plants located in the Indian Thar desert, India. This filamentous fungus belongs to the order Sebaciales in Basidiomycota (Weiss et al. 2004) and confers numerous host benefits, such as the promotion of plant growth, particularly in conditions of nutrient stress, and confers

tolerance to a wide range of abiotic stresses (drought, temperature extremes, water, salinity, heavy metals), as well as biotic (root and foliar pathogens) stresses (see above reviews for details). Original inoculation experiments showed the ability of *P. indica* to colonize plant roots (Verma et al. 1998) and to date, *P. indica* is renowned for its broad host range, forming symbiotic root interactions with both mono- and dicotyledonous plants, including the agriculturally important barley (*Hordeum vulgare*), and the model plants, tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (Johnson, Alex and Oelmüller 2014).

Plants colonized with *P. indica* show an enhanced tolerance to various root and foliar pathogens (see Table 2, Johnson, Alex and Oelmüller 2014 for a list of plant diseases controlled by *P. indica*). The potential of *P. indica* as a biocontrol solution to manage various root diseases in different crop species such as maize (Kumar et al. 2009), tomato (Fakhro et al. 2010), wheat (Serfling et al. 2007; Rabiey, Ullah and Shaw 2015) and barley (Waller et al. 2005) is evident. Systemic resistance to different foliar pathogens via *P. indica* interactions has also been demonstrated (Qiang et al. 2012). Fakhro et al. (2010) studied the effects of inoculation of tomato with *P. indica* and observed that in soil, *P. indica* colonizes the roots of tomato, increases the biomass of the leaves by up to 20% and reduces the disease severity caused by *Verticillium dahliae*, the causal agent of Verticillium wilt, by more than 30%. In hydroponics, *P. indica* increases fresh fruit biomass of tomato by up to 100%, results in higher numbers of fruits and increases the dry matter content by up to 20%. Moreover, at high light densities it represses Pepino Mosaic Virus which has been found widely in tomato greenhouses in many European countries, in Morocco, South and North America, and in China (Fakhro et al. 2010).

Many other fungal endophytes have been shown to protect host plants from pathogenic fungi. The endophyte *Epicoccum nigrum* isolated from sugarcane is especially known for its

biocontrol activity against pathogens, such as *Sclerotinia sclerotiorum* in sunflower, *Pythium* in cotton, phytoplasma bacteria in apple and *Monilinia* spp. in peaches and nectarines (Fávaro, Sebastianes and Araújo 2012). Murphy, Doohan and Hodkinson (2015b) isolated fungal endophytes from wild barley plants (*Hordeum murinum* subsp. *murinum* L.) and demonstrated that some were able to successfully suppress the activity of seed-borne fungal infections on seeds of cultivated barley. The tested seeds harbored some of the most devastating pathogens of barley, including species of *Cochliobolus*, *Fusarium*, *Pyrenophora* and *Rhynchosporium*. Two fungal endophytic isolates, including a *Penicillium brevicompactum* strain, gave 100% suppression of the infections. In addition to suppressing seedborne pathogens, this strain also suppressed growth of the soil-borne pathogen *Gaeumannomyces graminis* var. *tritici* (Murphy, Doohan and Hodkinson 2015b).

Fungal endophytes isolated from healthy *Theobroma cacao* tissues have been screened *in vitro* for antagonism against major pathogens of cacao including *Moniliophthora roreri* (causing frosty pod rot), *Phytophthora palmivora* (causing black pod rot) and *M. perniciosa* (causes witches broom) (Mejía et al. 2008). Field trials have assessed the effects of these endophytic fungi and have shown that treatment with *Colletotrichum gloeosporioides* significantly decreased pod loss due to black pod rot and that treatment with *Clonostachys rosea* reduced the incidence of sporulating lesions of *M. roreri* on cacao pods (Mejía et al. 2008).

Lastly, fungal entomopathogens are an important class of endophytes to consider for their anti-insect biocontrol properties (Quesada Moraga, Herrero and Zabalgoitia 2014). They have a unique ability to infect and colonize insects affecting insect survival and reproduction and many, if not all, have the ability to colonize plants (Vidal and Jaber 2015). Interestingly, most of the commercially produced entomopathogenic fungi can be endophytic for part of their life cycle and include the species of *Beauveria*, *Metarrhizium*, *Lecanicillium* and *Isaria* (Vidal and Jaber 2015). None of these commercial biocontrol products have made use of their endophytic mode of action however, but at least one such product is able to colonize plants endophytically (reviewed in Vidal and Jaber 2015). Wagner and Lewis (2000) first described in detail the penetration and colonization of plant tissues by the entomopathogenic fungus, *Beauveria bassiana*. Vidal and Jaber (2015) further review the colonization of plants by entomopathogenic fungi and discuss the variability and effectiveness in colonization rates observed when using different strain/cultivar combinations. Entomopathogenic fungi are effective against several root-feeding insects belonging to different orders, such as the root mealy bug, *Cataenococcus ensate*, the sugar beet root maggot, *Tetanops myopaeformis*, the diabrotic rootworms, and the white grubs *Melolontha melolontha* and *Popillia japonica* (Li et al. 2000). The coffee berry borer (*Hypothenemus hampei*) is the most devastating pest of coffee throughout the world. Vega et al. (2010) collected fungal endophytes of coffee plants from Hawaii, Colombia, Mexico and Puerto Rico. They found various genera of fungal entomopathogens, including *Acremonium*, *Beauveria*, *Cladosporium*, *Clonostachys* and *Paecilomyces*. *Beauveria bassiana* and *Clonostachys rosea* appeared to be pathogenic to the coffee berry borer (Vega et al. 2010). These fungal entomopathogenic endophytes also play a role in soil nitrogen cycling. Behie, Zelisko and Bidochka (2012) tested the ability of *Metarrhizium robertsii* to translocate insect-derived nitrogen to plants. They injected waxmoth larvae with ¹⁵N-labeled nitrogen and placed the labeled insects on haricot bean (*Phaseolus vulgaris*) and switchgrass (*Panicum virgatum*) and observed incorporation of ¹⁵N into amino acids of the plant, but only in the presence of *Me. robertsii*.

Fungal endophytes for conferring abiotic stress, including impacts of climate change

Fitness benefits conferred by mutualistic fungal endophytes contribute to, or are responsible for, plant adaptation to not only biotic stresses but also abiotic stress by increasing tolerance to drought and water stress, as well as tolerance to high temperature and high salinity (Aly, Debbab and Proksch 2011).

Habitat-adapted stress tolerance of plants conferred by habitat-specific symbionts is an intriguing phenomena hypothesized to be responsible for the establishment of plants in high stress environments (Rodriguez et al. 2008). A large number of class 2 endophytes have been found to be capable of habitat adaption providing tolerance to habitat-specific selective pressures such as soil pH, temperature and salinity (Rodriguez et al. 2009). Rodriguez et al. (2008) demonstrated that grass species adapted to coastal and geothermal habitats harbor symbiotic fungal endophytes that provide salinity and heat tolerance, respectively. *Leymus mollis* (dunegrass) plants from several coastal beach habitats in the USA were shown to be symbiotic with the endophyte *Fusarium culmorum* which, when inoculated into salt-sensitive non-coastal plants, conferred salt tolerance to those plants (Rodriguez et al. 2008). Similar experiments were carried out to demonstrate that endophytic fungi aid heat tolerance. The tropical panic grass, *Dichanthelium lanuginosum*, found growing in geothermal soils in Yellowstone National Park forms a mutualistic symbiotic interaction with the class 2 endophyte, *Curvularia protuberata* which confers heat tolerance (Redman et al. 2002). When root zones were heated up to 65°C, symbiotic plants infected with the endophyte tolerated and survived the heat but separately, neither the fungus nor the plant alone were capable of growing at soil temperatures above 38°C (Márquez et al. 2007). This relationship however is more complex as it was discovered that the endophyte harbors a virus in a tripartite mutualistic symbiosis and that the virus is an essential partner for bestowing heat tolerance to host plants, whereas virus-free endophytes were incapable of conferring this trait (Márquez et al. 2007). These habitat-specific symbionts also have a broad host range and have been shown to be able to confer habitat-specific tolerance to other plant species. For example, an endophyte from coastal plants can colonize the model monocot, rice, and confer salt tolerance, whereas endophytes from other habitats (agricultural, coastal and geothermal) can confer disease resistance but not salt or heat tolerance to the model dicot tomato (Rodriguez et al. 2008). Interestingly, all these endophytes conferred drought tolerance to plants regardless of the habitat of origin (Rodriguez et al. 2008).

Decreased water availability and increased soil salinization is a growing challenge to crop growth in many parts of the world (Egamberdieva et al. 2008; Egamberdieva and Lugtenberg 2014) and this problem is expected to increase due to climate change effects. The ability of fungal endophytes to confer stress tolerance to plants may provide a novel strategy for mitigating the impacts of global climate change on agricultural plant communities (Rodriguez et al. 2008). Redman et al. (2011) showed that some class 2 fungal endophytes can confer salt and drought tolerance to two commercial rice varieties which were not adapted to these stresses. Moreover, these endophytes reduced water consumption by 20%–30% while increasing growth rate, reproductive yield and biomass of greenhouse grown plants. They also conferred cold tolerance to growth chamber and greenhouse grown plants. These findings indicate that the incorporation of fungal symbionts may be a useful strategy in both mitigating impacts of climate change on major crops and

expanding agricultural production onto marginal lands (Redman et al. 2011).

Turning to endophytes that have the potential to promote plant growth, *P. indica* (introduced in the section 'Fungal endophytes for conferring pest and disease resistance') is known for its promotion of vegetative growth, early flowering, seed setting and seed germination which has been repeatedly observed with species from various plant families (Franken 2012). An extensive set of phytohormones and phytohormone signaling networks seem to be involved in mediating plant growth promotional activities which lead to increased early root growth promotion and finally to greater biomass. The extent of growth promotion is typically around 50%, but significant variation exists, due in part to variability in the environmental and experimental conditions in which the plants are grown. Considerable research has been carried out on the mechanism of salt stress tolerance conferred to plants colonized with *P. indica* and this trait has been demonstrated for barley as well as wheat, rice and tobacco and involves the induction of a high antioxidant environment for the detoxification of reactive oxygen species and an enhanced photosynthetic efficiency (Johnson, Alex and Oelmüller 2014).

Piriformospora indica's wide host benefits as well as its amenability for fundamental biological studies are attractive properties for research and its potential to transform the productivity of agricultural crops sustainably is exciting. With respect to barley, *P. indica* interactions have shown enhanced grain yield (Waller et al. 2005; Murphy, Doohan and Hodkinson 2014) and at low temperatures with higher nutrient input, *P. indica* also appeared to trigger flowering earlier and still increase grain yield. These results indicate that *P. indica* could be developed as an effective crop treatment in low temperature-stressed barley and may have the potential to increase crop yield under colder growing environments on the proviso that adequate nutrients are supplied (Murphy, Doohan and Hodkinson 2014). However, despite all of these described benefits, Franken (2012) states that it may be difficult to place *P. indica* on the market because (i) the fungus was first isolated in India and is protected by patent in many countries (international publication number—WO 99/29177) making any commercial prospect reliant on the patent owner to manufacturing it or licensing it for manufacture, and (ii) it sometimes has unexplained negative effects on plant growth. Nonetheless, a powder formulation of *P. indica* has been developed under the trade name 'ROOTONIC' and is currently deployed in field trials in India (Varma et al. 2013; Shrivastava and Varma 2014).

There are also other endophytes of barley that have the potential to be advantageous in agricultural settings. Murphy, Doohan and Hodkinson (2015a) isolated endophytic fungi from wild barley (*H. murinum* subsp. *murinum* L.) and in addition to various other biotic benefits previously described (in the section 'Fungal endophytes for conferring pest and disease resistance'), at least one of these isolates was able to increase grain yield in a nutrient-deficient barley cultivar (by up to 29%). The greatest impact one of these endophytic isolates had on grain yield and shoot dry weight was achieved under the lowest nutrient input. Such strains may therefore be able to help by reducing fertilizer inputs while maintaining acceptable yields.

Continuing with the theme of improving plant performance, the class 4 or DSE root-colonizing fungi, that are characterized by their darkly melanized septa, are an interesting group of endophytes that are present in a wide range of terrestrial ecosystems, but are especially common in polar and alpine habitats (Rodríguez et al. 2009). In these cold and water-stressed

environments, arbuscular mycorrhizal fungi, the typical mutualists of grass roots at lower altitudes and latitudes, are essentially absent (Newsham 2011) and it has been suggested that DSE species might act as surrogate mycorrhiza in these habitats (Bledsoe, Klein and Bliss 1990). Inoculation of monocotyledonous and dicotyledonous plant species with these fungi increases root and shoot biomass, and shoot nitrogen and phosphorous contents. Meta-analyses performed by Newsham (2011) on data from 18 research articles, in which plants had been inoculated with DSE species, indicated that they can enhance plant performance under controlled conditions, particularly when most of the nitrogen is available in an organic form such as proteins, peptides and amino acids in the rhizosphere. Plants apparently do not benefit from DSE species when roots can readily access inorganic nitrogen (Newsham 2011).

Fungal endophytes reducing animal health and welfare issues

The most intensively studied agricultural fungal endophyte associations are those formed between mutualistic asexual *Epichloë* endophytes of the family *Clavicipitaceae*, including the formerly classified anamorphic genus *Neotyphodium* (Leuchtmann et al. 2014) and the major pasture grass species in the *Festuca* and *Lolium* genera within the tribe *Poeae* (Johnson et al. 2013a). These asexual species do not manifest symptoms of disease and are characterized by their vertical transmission of hyphae via host seeds.

The grazing of ryegrass and/or fescue pastures is commonplace for dairy, beef and sheep production in New Zealand, parts of Australia, parts of South America and the USA. Sheep and cattle grazing on perennial ryegrass and tall fescue infected with the common-toxic (also known as wild-type) strains of *Epichloë* can develop either a neuromuscular condition known as ryegrass staggers (Fig. 1A) (Cunningham and Hartley 1959) or a restriction of blood flow also associated with heat stress and often designated as fescue toxicosis (Aiken et al. 2007), respectively. In Australia, the collective condition is referred to as perennial ryegrass toxicosis which can result in sheep deaths estimated to be in the hundreds of thousands in some years (Reed and Lean 2005). It was discovered that in all three countries the condition has been caused by infection with common-toxic strains of *Epichloë* fungal endophytes (Johnson et al. 2013a).

The obvious solution was to remove the offending *Neotyphodium* endophyte (now reclassified as asexual *Epichloë* species) from ryegrass and fescue (Latch and Christensen 1982). Indeed, grazing on endophyte-free grass did not cause the disease anymore but a new problem appeared. In New Zealand, ryegrass persistence was seriously affected by damage caused by invertebrate pests, especially by the Argentine stem weevil (*Listronotus bonariensis*) (Prestidge, Pottinger and Barker 1982) (Fig. 1B). In the USA, the persistence of tall fescue was also impacted (Hopkins and Alison 2006). In both regions, this issue was solved by introducing selected beneficial asexual *Epichloë* strains that do not cause animal health and welfare issues but still protect against insect pests and abiotic stresses (Bouton et al. 2000; Thompson, Stuedemann and Hill 2001; Young, Hume and McCulley 2013; Johnson et al. 2013a). In subsequent investigations, the chemical basis of these phenomena was discovered as explained in the section 'Fungal endophytes for conferring pest and disease resistance'. As a result, asexual *Epichloë* strains have been identified that produce animal safe bioactives that are still capable of providing a deterrence to insect pests ensuring plant persistence.

SECONDARY METABOLITES PRODUCED BY FUNGAL ENDOPHYTES

Fungal endophytes as a source of bioactive natural products

Due to the extraordinary biodiversity of fungal endophytes, they provide a largely untapped opportunity to discover novel natural products with unique chemical structures that have been optimized by (co-)evolution with higher plants. The recent development of screening technologies has revealed the great potential of fungal endophytes for producing novel biologically active compounds with promising medicinal or agricultural applications (Zhang, Song and Tan 2006; Aly, Debbab and Proksch 2011; Wu et al. 2015a). These molecules can play an important role in communication between organisms, in plant protection and plant adaptation to habitat and environmental changes. For reasons of safety and environmental problems, many synthetic agricultural agents have been, and will be, removed from the market. Secondary metabolites produced by fungal endophytes provide novel opportunities to control pests and pathogens (Strobel and Daisy 2003).

Overall, there is much to be gained from a better understanding of endophyte produced bioactives, some of which have been found to be novel anticancer agents, antibiotics, biofuels ('Mycodiesel'; see Strobel 2015) and antioxidants. Discovering plants that harbor these microbes capable of producing novel bioactive metabolites is the first step. In subsequent sections 'Anti-microbial Secondary Metabolites', 'Anti-microbial volatile compounds' and 'Anti-insect Secondary Metabolites', we will present examples of fungal endophytes and their useful secondary metabolites, but restrict discussion to those fungal secondary metabolites which have promise for, or are already being used in, agriculture and horticulture.

Discovering plants with microbial bioactive metabolites

Schulz (2001) described that certain microbial metabolites are characteristic of certain biotopes, on both an environmental and a taxonomic level. This study indicated that organisms living in unique biotopes or habitats that are subjected to constant metabolic and environmental interactions will yield even more secondary metabolites than organisms that are not (Schulz 2001). Therefore, the search for novel secondary metabolites should primarily center on organisms that inhabit unique biotopes.

Successful collection of plants harboring endophytes which produce novel and unique natural bioactives requires the identification of plants (i) from unique environmental settings, especially those with an unusual biology, and possessing novel strategies for survival; (ii) that have an ethnobotanical history (used by indigenous peoples) and that are related to the specific uses or applications of interest; (iii) that are endemic, that have an unusual longevity or that have occupied a certain ancient land mass; and (iv) that grow in areas of great biodiversity (Strobel and Daisy 2003). Expeditions to the Amazonian rain forest have led to the discovery of a wide range of secondary metabolites produced by endophytic fungi (Strobel and Strobel 2007).

Anti-microbial secondary metabolites

There are many examples of secondary metabolites from endophytic fungi which are being used, or have promise to be used, against pathogens and pests of horticultural and agricultural plants. This includes fungal endophytes which produce

promising but so far unidentified compounds through to well understood and described anti-microbial secondary metabolites. An overview of 230 metabolites produced by plant-associated microbial strains, including many fungal endophytes, was published by Gunatilaka (2006). Examples of some well-characterized anti-microbial secondary metabolites from endophytic fungi are presented below in alphabetical order. Several of these compounds have been described by Strobel and Daisy (2003). The chemical structures of selected compounds are shown in Fig. 2.

Some anti-microbial compounds produced by the sugarcane endophyte *Epicoecum nigrum* (Brown, Finlay and Ward 1987) have been characterized, such as epicorazines A-B (Baute et al. 1978), epirodines A-B (Ikawa et al. 1978), flavipin (Bamford, Norris and Ward 1961), epicoccines A-D (Zhang et al. 2007), epipiridones and epicocarines (Wangun and Hertweck 2007). Especially the compounds flavipin and epicorazines A-B have been associated with *E. nigrum* biocontrol activity (Brown, Finlay and Ward 1987; Madrigal, Tadeo and Melgarejo 1991; Madrigal and Melgarejo 1995).

Ambuic acid is an antifungal and anti-oomycete agent from the endophytic fungus *Pestalotiopsis microspora*, isolates of which have been found in many of the world's rainforests. It is active against several *Fusarium* species and against *Pythium ultimum* (Li et al. 2001). It also appears to be a quorum-sensing (as defined by Fuqua, Winans and Greenberg 1994) inhibitor (Gary Strobel, pers. comm.). Colletotrichic acid (Bills et al. 2002) is a compound from a *Colletotrichum* sp., isolated from *Artemisia annua*. It shows good anti-bacterial, anti-fungal and anti-algal activities (Hussain et al. 2014). Colletotrichic acid is a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus from *A. mongolica*. It displays antimicrobial activity against bacteria as well as against the fungus *Helminthosporium sativum* (Zou et al. 2000). Cordycepsidone A was isolated from the endophytic fungus *Cordyceps dipterigena* and has strong antifungal activity against the plant pathogenic fungus *Gibberella fujikuroi* (Varughese et al. 2012). Cryptocandin is a lipopeptide isolated from *Cryptosporiopsis quercina*, a fungus commonly associated with hardwood species in Europe. It is active against a number of plant-pathogenic fungi, including *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Strobel et al. 1999). It is related to known antimycotic compounds: the echinocandins and the pneumocandins (Walsh 1992). Cryptocin is produced by *Cr. quercina* (Li et al. 2000) and possesses the most potent activity against the fungus *Pyricularia oryzae*. It also shows strong activity against the plant-pathogenic fungi *Fusarium oxysporum*, *Geotrichum candidum*, *Rhizoctonia solarum* and *S. sclerotiorum*, as well as against the plant-pathogenic oomycetes *Py. ultimum*, *Phytophthora cinnamoni* and *Ph. citrophthora* (Li et al. 2000). Jesterone is produced by *Pe. jesteri* from Papua New Guinea and was isolated from an area with moist conditions in which plant pathogens, especially oomycetes, normally thrive. As anticipated, the compound has anti-oomycete activity (Li and Strobel 2001). Pestacin and isopestacin (Fig. 3) are anti-oxidants produced by *Pe. microspora*, an endophyte from *Terminalia morbensis* (Strobel et al. 2002; Harper et al. 2003). Both also have anti-microbial activity (Strobel and Daisy 2003). Phomopsichalasin is a secondary metabolite from the fungal endophyte *Phomopsis* sp. and has antibacterial activity against *Bacillus subtilis*, *Salmonella enterica* and *Staphylococcus aureus* (Horn et al. 1995). Scandenin was isolated from a *Derris scandens* plant from Pakistan and has strong anti-bacterial activity against *B. megaterium* and good anti-fungal and anti-algal properties (Hussain et al. 2015). It is remarkable that none of the anti-microbial secondary metabolites produced by these endophytic fungi

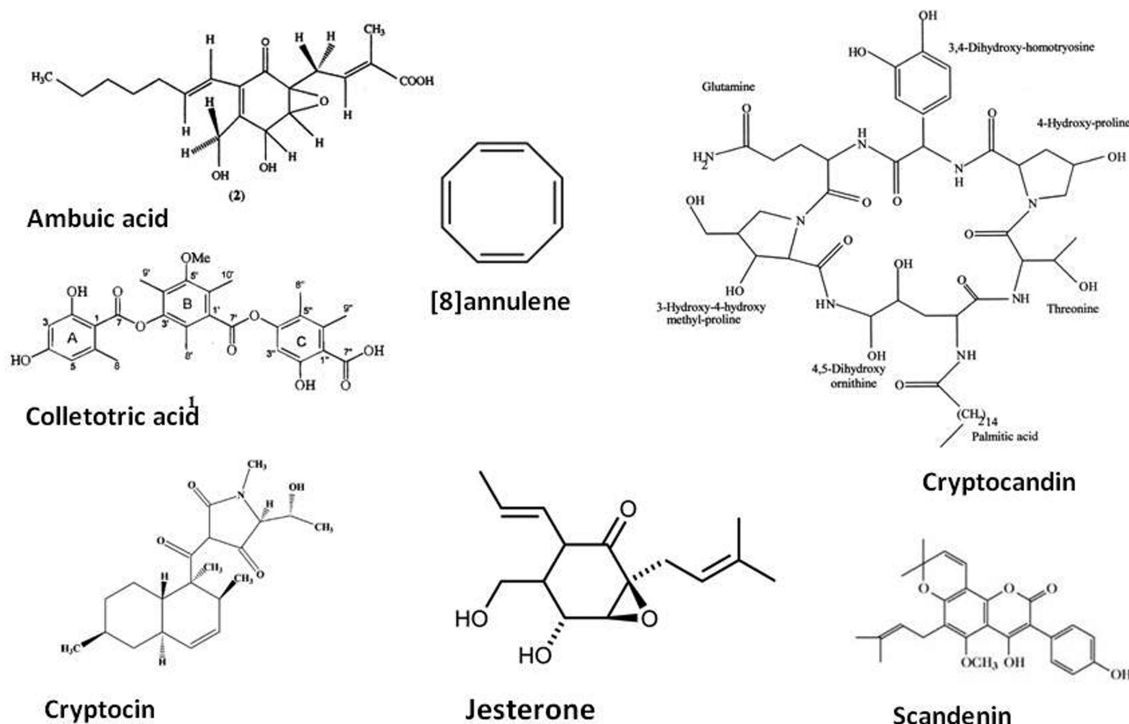


Figure 2. Examples of some well-characterized anti-microbial secondary metabolites from endophytic fungi.

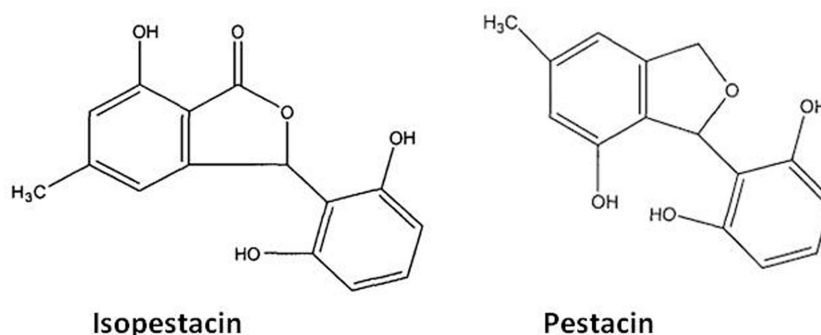


Figure 3. Examples of anti-oxidant secondary metabolites from endophytic fungi with anti-microbial activity.

has been found to be produced by bacteria which control plant diseases caused by microbes (Haas and Défago 2005; Lugtenberg and Kamilova 2009; Pliego, Kamilova and Lugtenberg 2011).

Anti-microbial volatile compounds

Although the topic of volatile antibiotics would perfectly fit into the previous section, they have been put it under a separate heading because special techniques are required to detect these compounds. Their anti-microbial activity is detected using air contact between the volatile-producing fungus and the target pathogen (see Fig. 4). The volatile mixture can subsequently be separated and identified using a combination of chemical profiles built by LC-HRMS (liquid chromatography-high resolution mass spectrometry) or NMR (nuclear magnetic resonance) and multivariate data analysis (Wu et al. 2015a).

The endophytic fungus *Muscador albus*, isolated from small branches of *Cinnamomum zeylanicum* (cinnamon tree) (Worapong et al. 2001), effectively inhibits and kills certain other fungi and

bacteria by producing a mixture of at least 28 volatile compounds (Strobel et al. 2001). Several individual compounds had some inhibitory effect against the test fungi and bacteria, but none was lethal. However, a strong synergistic effect was found and collectively they caused death of a broad range of plant- and human-pathogenic fungi and bacteria. Five classes of compounds were detected, namely alcohols, esters, ketones, acids and lipids. Initial studies showed that the most effective class of inhibitory compounds was the esters, of which isoamyl acetate was the most biologically active compound (Strobel et al. 2001). Subsequently, seven new *Mu. albus* strains were isolated which produce various novel mixtures of volatiles. However, none of the new isolates produced any of the esters known from the original *Mu. albus* isolate. All together, they produce 50 different volatiles with an impressive range of biological activities, such as growth inhibition of Gram-positive and Gram-negative bacteria and of plant-pathogenic fungi and oomycetes (Ezra, Hess and Strobel 2004). Interestingly, all isolates of *Mu. albus* isolated so far appear to make bioactive volatiles (Gary Strobel, pers. comm.).

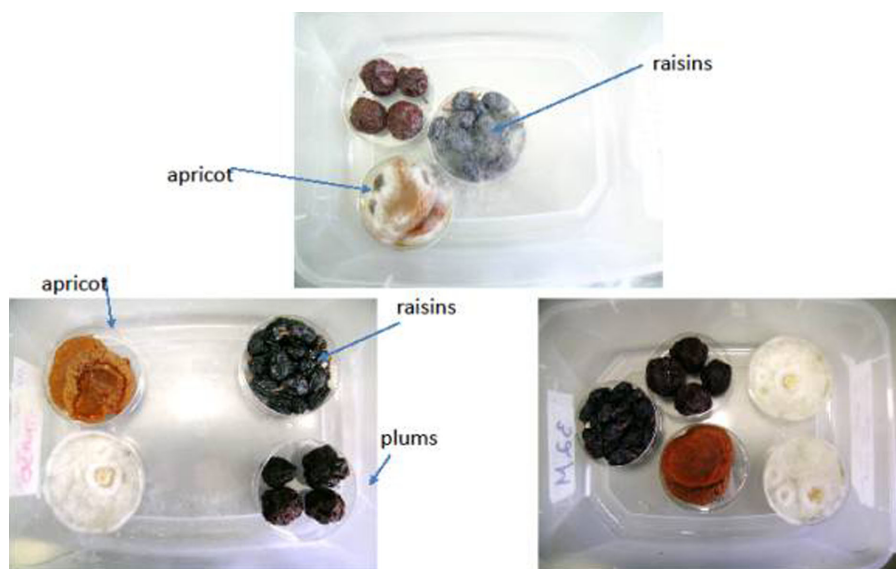


Figure 4. Prevention from rotting of dried fruits by volatiles produced by the endophytic fungus *Daldinia concentrica*. The volatile mixture consists of at least 28 compounds. This figure has not been published previously and was kindly provided by Dr David Ezra and Dr Orna Liarzi.

Muscador crispans is an endophytic fungus of *Ananas ananassoides* (wild pineapple) growing in the Bolivian Amazon Basin. The fungus produces a mixture of antifungal and antibacterial volatile organic compounds. *Pythium ultimum* appeared to be the most sensitive organism of the fungal and oomycete plant pathogens tested towards the volatile mixture of *Mu. crispans*. Other sensitive plant fungal and oomycete pathogens include *Alternaria helianthi*, *Botrytis cinerea*, *Fusarium culmorum*, *F. oxysporum*, *Phytophthora cinnamomi*, *Ph. palmivora*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Verticillium dahliae*. Also the plant-pathogenic bacterium *Xanthomonas axonopodis* pv. *citri* appeared to be sensitive (Mitchell et al. 2010).

Interestingly, in contrast to the situation with *Mu. albus*, the majority of the volatiles produced by *Mu. crispans* are on the US Food and Drug Administration's GRAS (Generally Regarded As Safe) list of harmless substances (see the FDA website for details: <http://www.fda.gov/>). For instance, no azulene or naphthalene derivatives were detected in the volatile mixture of *Mu. crispans*. When a mixture of the GRAS compounds produced by *Mu. crispans* was tested, the results were virtually identical to those of the natural mixture. As a result, this mixture was suggested to have potential utility in applications ranging from food preservation to agricultural, household and industrial uses (Mitchell et al. 2010).

A *Gliocladium* sp., an endophytic fungus of *Eucryphia cordifolia*, was discovered to be a volatile antibiotic producer. Of particular interest was that its most abundant volatile inhibitor was identified as [8]annulene (Fig. 2), which was formerly used as a rocket fuel and now discovered for the first time as a natural product produced by an endophytic fungus (Stinson et al. 2003).

The endophytic fungus *Daldinia concentrica*, isolated from an olive tree in Israel, produces at least 28 volatile organic compounds which are very promising for post-harvest control. These volatiles protect dried fruits of apricot, plum and raisin from rotting. Moreover, they protect peanuts against *Aspergillus niger*, oranges and tomato paste against *Penicillium digitatum* and grapes against *Botrytis cinerea*. Artificial mixtures of selected volatiles have great promise for application in food industry and agriculture (David Ezra and Orna Liarzi, pers. comm.).

Anti-insect secondary metabolites

The association of synthetic pesticides with ecological damage has led to research to discover powerful, selective and safe alternatives (Strobel and Daisy 2003). Until now, biopesticides are only a small part of the pesticide market but their impact is increasing. Several endophytes are known to produce secondary metabolites that have anti-insect properties. Some of their chemical structures are shown in Fig. 5.

Nodulisporic acids (Fig. 5) are indole diterpenes which exhibit potent insecticidal properties against the larvae of the blowfly. They act by activating insect glutamate-gated chloride channels. The first nodulisporic compounds were isolated from the endophyte *Nodulisporium* sp. from the plant *Bontia daphnoides*. Since this discovery the focus is on searching for more *Nodulisporium* spp. and for other producers of more-potent nodulisporic acid analogs (Demain et al. 2000).

Muscador vitigenus is an endophytic fungus of a liana from the rain forest of the Peruvian Amazon. It produces a single volatile, naphthalene (Fig. 5), which acts as an insect repellent which is the active ingredient of common mothballs. It repels the adult stage of the wheat stem sawfly *Cephus cinctus*. Starch in its growth medium is crucial for naphthalene production (Daisy et al. 2002).

Some new benzofuran compounds (5-hydroxy-2-(1'-hydroxy-5'-methyl-4'-hexenyl) benzofuran and 5-hydroxy-2-(1'-oxo-5'-methyl-4'-hexenyl)benzofuran) were isolated from an unidentified endophytic fungus from wintergreen (*Gaultheria procumbens*). Both show toxicity to spruce budworm, and the latter one is also toxic to its larvae (Findlay et al. 1997).

Peramine is a pyrrolopyrazine feeding deterrent against the insect pest Argentine stem weevil, and is produced by some *Epichloë*-grass associations (Rowan 1993; Johnson et al. 2013a). Indole-diterpenes are another class of alkaloids known to be produced by *Epichloë* endophytes. Lolitrem B, which is unique to *Epichloë*, is an infamous example as it is the major tremorgenic mycotoxin responsible for ryegrass staggers (Gallagher et al. 1984), but it also reduces the growth and development of ASW larvae (Prestidge and Gallagher 1985). As mentioned in the section 'Fungal endophytes for conferring pest and disease

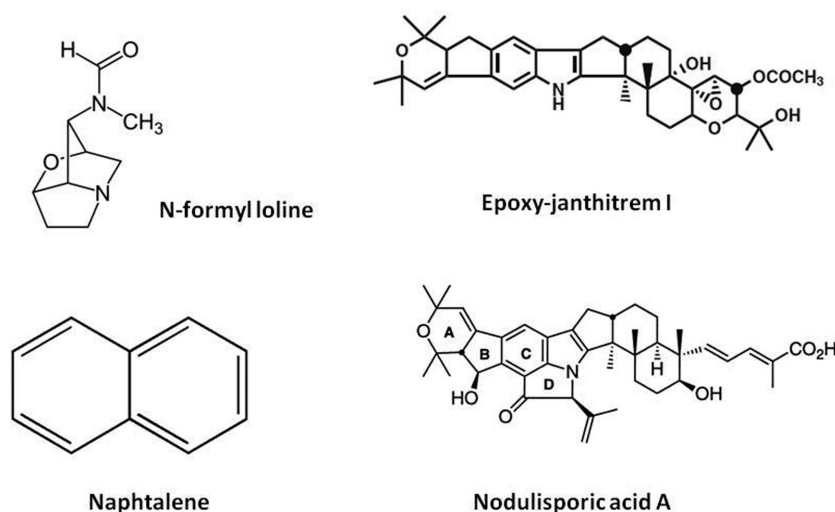


Figure 5. Examples of some well-characterized anti-insect secondary metabolites from endophytic fungi. See Fig. 1 for the structure of the insect feeding deterrent peramine. The structure of epoxy-janthitrem I is from Johnson et al. (2013a).

resistance', epoxy-janthitrems (Fig. 5), another type of indole-diterpene, are likely to have some pesticidal effect due to the broad spectrum insect resistance of the strain AR37 which is the only *Epichloë* strain known to produce these compounds (Tapper and Lane 2004; Finch, Fletcher and Babu 2012).

Similar discoveries were made in the USA where cattle grazing endophytic tall fescue pastures contracted fescue toxicosis caused by some strains of the fungal endophyte *Epichloë coenophialum*. These strains produce the alkaloid ergovaline which is responsible for the vasoconstriction of blood vessels leading to this debilitating animal health and welfare condition (Bacon 1993, 1995). While ergovaline in its own right does have a pesticidal effect (Popay et al. 1990; Rowan, Dymock and Brimble 1990), the associated effect of fescue toxicosis and heat stress means that ergovaline is not a useful candidate for controlling pests. However, other secondary metabolites which showed no mammalian toxicity were also discovered in this fungal endophyte host plant association. These included peramine and the lolines [of which N-formyl loline (Fig. 5) is the major one] which provide broad spectrum pest resistance (Siegel and Bush 1994; Schardl, Leuchtmann and Spiering 2004; Young, Hume and McCulley 2013; Johnson et al. 2013a).

Secondary metabolites with other plant-beneficial activities

Genomic sequencing of endophytic fungi has indicated that, despite the observation that these organisms contain gene clusters encoding certain secondary metabolites, some are not expressed under standard laboratory cultivation conditions. This raises the question as to which physiological and environmental conditions are required for endophytic fungi to express these secondary metabolites (Brakhage 2013; Netzker et al. 2015). In some cases, as observed with *Epichloë*, the host genome is required for significant expression of the endophyte's secondary metabolite pathways (Schardl and Panaccione 2005). For example, plant signaling is required to induce expression of the fungal gene clusters for lolitrem B biosynthesis by *E. festucae* var. *lolii* so that it is highly expressed in planta but expression is low or undetectable in culture-grown fungal mycelia (Young et al. 2006). The same applies to other secondary metabolites such as lolines and epoxy-janthitrems of *Epichloë* species that produce little or no

compound when grown *ex planta* (Porter 1994; Tapper and Lane 2004), despite attempts to circumvent this under in culture conditions as found with loline production (Blankenship et al. 2001).

In other cases, endophyte secondary metabolites may be triggered by the limitation of food sources, competition with other organisms, the presence of plant components (including exudates), the presence of other fungi and bacteria as well as their metabolites and their presence in plants attacked by pathogens and pests. Several examples of conditions under which the expression of secondary metabolites have been induced are published. For example, flavonoids present in root exudates of host plants induce the expression of *Rhizobium* Nod-factors (Van Brussel et al. 1986). Accordingly, the lack of a host stimulus in culture media may explain why the production of biomolecules by a nascent endophyte isolate is often severely attenuated through subculturing (Li et al. 1998).

Because microbes in nature live in communities, growth of various fungi in co-culture with other organisms has been tested. Co-culture can indeed result in activation of silent gene clusters or in strongly increased expression (Ola et al. 2013; Wu et al. 2015b). For reviews on the effects of co-culturing, we refer to Bertrand et al. (2014) and Wu et al. (2015a). An interesting observation is that in the case of co-cultivation between *As. niger* and actinomycetes, an intimate physical interaction is required to activate silent gene clusters in *As. niger* (Schroeckh et al. 2009).

COMMERCIALIZATION OF FUNGAL ENDOPHYTES AND THEIR PRODUCTS

Discoveries in the fields of fundamental microbial and plant ecology have resulted in commercial products that not only generate revenue and employment, but also provide a major contribution to food production and adaptation to environmental changes.

Commercialization of the obligate mutualistic *Epichloë* endophyte

Losses due to neuromuscular diseases and heat stress of sheep and cattle grazing on toxic temperate grasses containing asexual *Epichloë* species expressing lolitrem B or ergovaline (see the

section 'Fungal endophytes reducing animal health and welfare issues') are substantial (Johnson et al. 2013a). In the USA, the financial losses caused through fescue toxicosis from cattle grazing tall fescue infected with the ergovaline producing common-toxic endophyte were estimated to be US\$ 1 billion per annum to the beef cattle industry (Hoveland 1993). In New Zealand, the losses due to ryegrass staggers have been estimated to be worth US\$ 65 million (Imlach et al. 2008) and in Australia, the losses due to toxic *Epichloë* species causing perennial ryegrass toxicosis have been estimated to be US\$ 68 million in 2012 (Webb-Ware 2013).

Epichloë endophytes have been successfully commercialized in perennial ryegrass and tall fescue with different traits and pastoral benefits for New Zealand, Australia, South America and the USA (Young, Hume and McCulley 2013; Johnson et al. 2013a). The identification of novel *Epichloë* endophytes has resulted in grasses being developed and bred with animal-safe properties (Easton et al. 2001). They can enhance the plants' survival through protection from abiotic and biotic stresses and can therefore be utilized in agriculture. Presently, novel endophyte strains have been rapidly adopted by farmers (Caradus, Lovatt and Belgrave 2013) and are estimated to contribute approximately US\$ 130 million per annum to the New Zealand economy (Johnson et al. 2013a).

Commercialization of class 2 endophytes conferring habitat-adapted fitness benefits

Based on the observation that selected class 2 endophytes are able to confer disease, salt and heat tolerance to grasses (Rodriguez et al. 2008) and the subsequent finding that these tolerances can be transferred to agricultural plants (Rodriguez et al. 2008; Redman et al. 2011), these endophytes have been commercialized to improve agriculture in relation to climate changes, which may give rise to water, drought and salination stresses (<http://www.adaptivesymbioticttechnologies.com/executive-team.html>). The products include BioEnsure®-Corn and BioEnsure®-Rice. BioEnsure®-Corn is promoted to have a 25%–80% yield increase under heavy drought stress and even a 7% yield increase under low drought stress. These plants also use 25%–50% less water under normal conditions as well as under low drought stress. BioEnsure®-Rice is promoted to cause a yield increase under drought and salt stresses as well as a 25%–40% decreased water use. The products are sold as liquid formulations that are sprayed onto seeds by commercial seed treatment companies. The fungi remain dormant on the seed until germination when they establish a symbiotic association with seedlings (<http://www.adaptivesymbioticttechnologies.com/press-publications.html>).

Commercialization of secondary metabolites, including volatiles

Presently, a number of products based on secondary metabolites of endophytic fungi are on the market or close to market. The use of cryptocandin (Walsh 1992) and its related compounds has been effective against a number of fungi causing diseases of skin and nails (Strobel and Daisy 2003). Marrone Bioinnovations has licensed *Muscodor albus* and EPA approval for the release of this organism for use in agriculture is expected soon. It literally sterilizes the soil in which it has been placed. It is a potential replacement for methyl bromide (Gary Strobel, pers. comm.), which is now restricted from use in many countries. A strain is already on the market for decontamination of human

wastes (Strobel et al. 2001; Strobel and Daisy 2003). Potential applications of the volatiles produced by the new *Mu. albus* strains were suggested for treatment of seeds, fruits and cut flowers, as well as for soil (Ezra, Hess and Strobel 2004).

As discussed in the section 'Anti-microbial volatile compounds', the endophytic fungus *Daldinia concentrica* produces a mixture of volatile organic compounds which are very promising for post harvest control of dried fruits (see Fig. 4) and other plant products. Effective and safe mixtures can be assembled from the volatiles produced by *Mu. crispans*, and these mixtures could have commercial potential (Mitchell et al. 2010). In principle, the products can be used for food, flavoring and preservative purposes because each volatile component is on the FDA GRAS list. Presently, a major company is examining the volatile mixture for use as post harvest treatment of fruits and vegetables. In the meantime the product is being used to treat cheeses, and as a treatment of industrial surfaces (Gary Strobel, pers. comm.).

HOW CAN FUNGAL ENDOPHYTES BECOME LOST AND/OR THEIR PLANT-BENEFICIAL ACTIVITIES REDUCED IN DOMESTICATED PLANTS?

We postulate that some wild plants have fungal endophytes which are lacking in their domesticated relatives. Under conditions of variable environmental and physiological conditions, there will be selective pressure to maintain the endophyte within the plant and to keep its beneficial activity for the plant in optimal form. It seems reasonable to assume that plants can lose endophytes when they do not benefit from their presence, and also when pesticides and fungicides are frequently used. Also, in cases of low or no selective pressure, an endophyte may be less effective due to either the loss of secondary metabolites and/or because it will collect mutations against for which there is no selection. The result is a reduction in the efficacy of the endophyte and loss of beneficial activity.

The following experimental observations support these notions.

- (i) Weese et al. (2015) collected *Rhizobium* strains from two fields on which clover was grown. One field had been nitrogen-fertilized for 22 years, whereas the control field had not received nitrogen fertilizer. They compared the nitrogen-fixing activity of collected *Rhizobium* strains and found that the rhizobial soil community from the nitrogen-fertilized field was less mutualistic: the inoculated clover had 17%–30% less biomass and a reduced chlorophyll content.
- (ii) Redman et al. (2011) showed that inoculation of rice plants sensitive to salt and drought with selected fungal endophytes resulted in plants which had become tolerant to these stresses. It appeared that, when the plants were grown in the absence of these stresses, endophyte colonization decreased from 100% at planting to 65%. In contrast, plants grown under continual stress maintained 100% colonization levels.
- (iii) Chen, Gols and Benrey (2015) concluded that crop domestication can profoundly alter interactions among plants, herbivores and their natural enemies. Domestication consistently has reduced chemical resistance against herbivorous insects, improving herbivore and natural enemy performance on crop plants. They coined the term 'domestication

syndrome' which they defined as the 'suite of traits that commonly differ between wild and domesticated plants'.

- (iv) Finally, Li *et al.* (1998) proposed that the lack of host stimulus in culture media may explain why the production of biomolecules by a nascent endophyte isolate is often severely attenuated through subculturing.

Apparently, during domestication, both plant and endophyte can lose activities required for an optimal partnership. We hypothesize that the reasons for a loss or reduced effectiveness of endophyte(s) via the loss of certain secondary metabolites, for example, can include the following:

- (i) Growing plants with little or no selective pressure.
- (ii) Use of systemic fungicides during *in vitro* propagation in the plant breeding process: whereas fungicides are used to prevent fungal infection from the environment, the fungal endophytes within the plant material may also be deleteriously affected.
- (iii) Due to lack of selective pressure during the plant breeding process, activities such as endophyte-mediated nutrient acquisition and pathogen resistance may have been lost.
- (iv) Seeds can be 'cleaned' of pathogens present on and in the seeds by heat treatment. It seems likely that during this process some endophytes will also be killed.
- (v) Commercial seeds are likely to contain fungicides in their coating. The fungicides may kill endophytes during storage or during subsequent germination.
- (vi) The treatment of growing plants with systemic fungicides may kill all or part of the fungal endophyte population.

HOW CAN THE LOSS OF FUNGAL ENDOPHYTES BE FURTHER PREVENTED AND/OR RESTORED?

The solution to incorporating useful fungal endophytes in crop plants lies in being aware of how fungal endophytes might be lost and then mitigating against this loss through all stages of crop breeding and management. Those managing plant germplasm resources need to consider the following:

- (i) The lost part of the beneficial microbiome will be present in and on wild plants collected from the centers of origin of crop plants, the geographical area where a group of organisms was first domesticated and where they are native (Chen, Gols and Benrey 2015). The key is to conserve not only the plants but also the whole microbiome associated with these plants. Presently, the main focus of such studies is on bacteria, especially those from the rhizosphere (Wisniewski, Mazzola and Picard 2009; Bulgarelli *et al.* 2015; Perez-Jaramillo, Mendes and Raaijmakers 2016), but it is crucial that the focus is extended to fungi present in and on all parts of the plant.
- (ii) Seeds of plants from centers of origin of crop plants have often been collected by seed companies or germplasm centers. However, they generally only make seeds available to scientists after they have been propagated to increase the amount of seed available for evaluation. However, the risk associated with propagation in the absence of the required selective pressures is that endophytes may be lost. An additional concern is that when seed is sent across national borders biosecurity regulations often require a heat or fungicidal treatment to stop the importation of pathogens. In addition to killing fungal pathogens that might be present,

these treatments may also remove beneficial fungal endophytes.

- (iii) Manage the endophyte with the same level of importance that we manage the host crop plant. Despite the fact that the plant may naturally select its beneficial microbiome, it can only do this if the beneficial microbes are present for selection. Therefore, it may make sense to supply the beneficial endophyte by artificial inoculation, seed treatment or other means to the plant. In dealing with fungi that have an obligate endophytic lifestyle, it must be assumed that genes from both the host plants and the microbe are involved in establishing an optimal partnership. It is therefore crucial to select, identify and characterize these genes from both partners before they are lost by lack of selective pressure.

RECOMMENDATIONS FOR GERmplasm CENTERS, PLANT BREEDERS, SEED COMPANIES, MICROBIOLOGISTS, POLITICIANS, OPINION MAKERS AND THE PUBLIC

Primarily our recommendations have been targeted towards fungal endophytes, but in many instances the same will apply for bacterial endophytes.

To germplasm centers, we request that recognition is given to the likelihood that seed may contain numerous fungal endophytes that may have scientific and economic importance. Storing seed at low temperature and low humidity will not only benefit the longevity of the seed itself but also the viability of any fungal endophytes they may contain. In addition, ensure that high temperature and fungicidal treatment of seed are avoided.

To plant breeders, we request that they realize that their crop of importance is often benefited by fungal endophytes and suggest that they (i) check whether known beneficial microbes are lost during their breeding procedures, (ii) adapt their protocols in order to include the microbiomes, (iii) collect and sequence DNA from seeds of plants from the centers of origin before they are being propagated to identify the presence of potentially beneficial endophytes and (iv) check whether propagation and treatment of seeds from centers of origin results in the loss of beneficial microbes, and loss or mutation of genes.

To seed companies, we suggest that they (i) check whether heat treatment and the use of synthetic as well as biological fungicides in seed coatings kills or weakens beneficial microbes within or on the seeds, (ii) check whether substances present in seed coatings kill beneficial microbes during storage and during germination (iii) and if possible store seed containing beneficial and economically important fungal endophytes at low temperature and low humidity.

To plant microbiologists, we suggest that they (i) preserve and sequence all microbiomes of plants from the centers of origin, (ii) identify from these collections microbes beneficial in an agricultural and horticultural context and (iii) search for novel microbial beneficial secondary metabolites, especially from the centers of origin and from tropical rain forests.

To politicians, opinion makers and the public, we request that they have an increased awareness of the importance of (i) plant microbiomes for plant health and food production, and (ii) the preservation of wild plants and their microbiomes in their habitats. Annually, the loss of natural habitats through clearing, harvesting, fire, agricultural development, mining or other human-oriented activities results in the inevitable loss of not only plant species of importance but also of fungal endophytes.

Since these microbes may hold the key to future opportunities for a sustainable agriculture, we must focus on capturing the benefits of these beneficial microbes with some urgency.

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