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Fungi with multifunctional lifestyles: endophytic insect pathogenic fungi

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Abstract This review examines the symbiotic, evolutionary, proteomic and genetic basis for a group of fungi that occupy a specialized niche as insect pathogens as well as endophytes. We focus primarily on species in the genera *Metarhizium* and *Beauveria*, traditionally recognized as insect pathogenic fungi but are also found as plant symbionts. Phylogenetic evidence suggests that these fungi are more closely related to grass endophytes and diverged from that lineage ca. 100 MYA. We explore how the dual life cycles of these fungi as insect pathogens and endophytes are coupled. We discuss the evolution of insect pathogenesis while maintaining an endophytic lifestyle and provide examples of genes that may be involved in the transition toward insect pathogenicity. That is, some genes for insect pathogenesis may have been co-opted from genes involved in endophytic colonization. Other genes may be multifunctional and serve in both lifestyle capacities. We suggest that their evolution as insect pathogens allowed them to effectively barter a specialized nitrogen source (i.e. insects) with host plants for photosynthate. These ubiquitous fungi may play an important role as plant growth promoters and have a potential reservoir of secondary metabolites.

Keywords Insect pathogenic fungi · Plant endophytes · Symbiosis · Evolution · Nitrogen · Plant health · Plant growth promotion · Protease · Secondary metabolite · Biocontrol

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Introduction

In terms of ecological interactions and evolutionary history, fungi that infect and kill insects are plainly fascinating. Most infect the host insect by transgressing the cuticle, that is, they do not have to be ingested to cause infection in the insect. This allows infection of insects with sucking mouthparts such as aphids (Chandler 1997) and adult mosquitoes (Blanford et al. 2005). The breadth of variety of insect pathogenic fungi is tremendous, a group that comprises over 100 fungal species. There are examples of insect pathogenic fungi found in most major fungal taxonomic groups from chytridiomycetes to basidiomycetes. However, there is no underlying phylogenetic relationship that suggests a basal group from which all insect pathogenic fungi arose. Even within the Hypocreales, the taxonomic group with the largest number of insect pathogenic fungi, there is little evidence to suggest that there is a singular common origin (Humber 2008). Some of these insect pathogenic fungi are obligate pathogens while many are facultative. Adding further intrigue into their ecology is a subset of insect pathogenic fungi that additionally function as endophytic symbionts of plants.

Two genera of insect pathogenic fungi that fall within the category of endophytes are *Metarhizium* and *Beauveria*. There is considerable divergence within the genus *Metarhizium* and some species (e.g. *M. acridum*) have restricted insect host ranges while other species (e.g. *M. robertsii*) have broad host ranges, and not all species show equivalent endophytic capabilities. The potential of *Metarhizium* and *Beauveria* to control insect pests in agroecosystems has been known since the early 20th century (Madelin 1963), and numerous formulations of *Metarhizium* and *Beauveria* have been approved for use in crop protection (Faria and Wraight 2007; Castrillo et al. 2011).

Phylogenetic analysis has shown that *Metarhizium* and *Beauveria* are related to the fungal grass endosymbionts *Claviceps* and *Epichloë* (Spatafora et al. 2007). Furthermore, comparative genomic analyses have shown that *Metarhizium* spp. are more closely related to endophytes and plant pathogens than to animal pathogens (Gao et al. 2011), and that *Metarhizium* lineages diverged from the lineage of the mutualistic plant endophyte *Epichloë festucae* approximately 88–114 MYA (Gao et al. 2011). This strongly suggests that *Metarhizium* evolved from fungi that were plant associates and that insect pathogenicity is a more recently acquired adaptation (Fig. 1). *Metarhizium* may have evolved from a plant symbiont lineage and subsequently acquired the ability to infect and kill insects. This is supported by genomic analysis that shows a large number of genes for plant degrading enzymes within *Metarhizium* genomes (Gao et al. 2011).

Genes involved in insect pathogenicity may have been co-opted from genes involved in plant colonization or from horizontal gene transfer (Screen and St. Leger 2000). Figure 2 shows hypothetical mechanisms by which genes involved in insect pathogenesis may have been co-opted, evolved, or acquired by horizontal gene transfer from a plant-associated fungus. The evolution of insect pathogenic *Metarhizium* spp. must have involved adaptations that enabled degradation of insect cuticle and host body components, as there exists numerous proteases, lipases, and chitinases within the *Metarhizium* and *Beauveria* genome. Furthermore, comparative analysis of plant and insect adhesin genes in *Metarhizium* spp. suggest that, while other abiotic and biotic factors cannot be excluded in contributing to divergence within the genus, plant relationships, rather than insect host, have been a driving factor in the divergence of the genus *Metarhizium* (Wyrebek and Bidochka 2013).

Why evolution toward insect pathogenicity? There is some speculation of interkingdom jumping by these fungi from plants back to arthropods and then back to plants (Humber 2008). We suggest that many of these fungi have never left the role as plant symbionts and subsequently gained the ability to infect insects. We hypothesize that insect pathogenicity is an adaptation that allowed certain species of endophytic fungi to access a specialized source of nitrogen (i.e. insects), or other insect derived nutrients, and effectively barter these insect-derived nutrients for access to plant carbohydrates.

Insect pathogenesis

Metarhizium, *Beauveria*, and related insect pathogenic fungi, transgress the insect cuticle and infect insect hosts through a combination of mechanical penetration and degradation of cuticular components by employing enzymes such as proteases, esterases, *N*-acetylglucosaminidases, chitinases and lipases (St. Leger et al. 1996; Schrank and Vainstein 2010; Pedrini et al. 2013). The infection strategies of insect pathogens, such as *Metarhizium* and *Beauveria*, are well documented and understood (Small and Bidochka 2005; Ortiz-Urquiza and Keyhani 2013). During the initial stages of infection, hydrophobic conidia adhere to the insect cuticle and germinate to form a germ tube and holdfast infection structures termed appressoria (Small and Bidochka 2005; Holder and Keyhani 2005; Holder et al. 2007), similar to those found in plant pathogenic fungi (Deising et al. 2000).

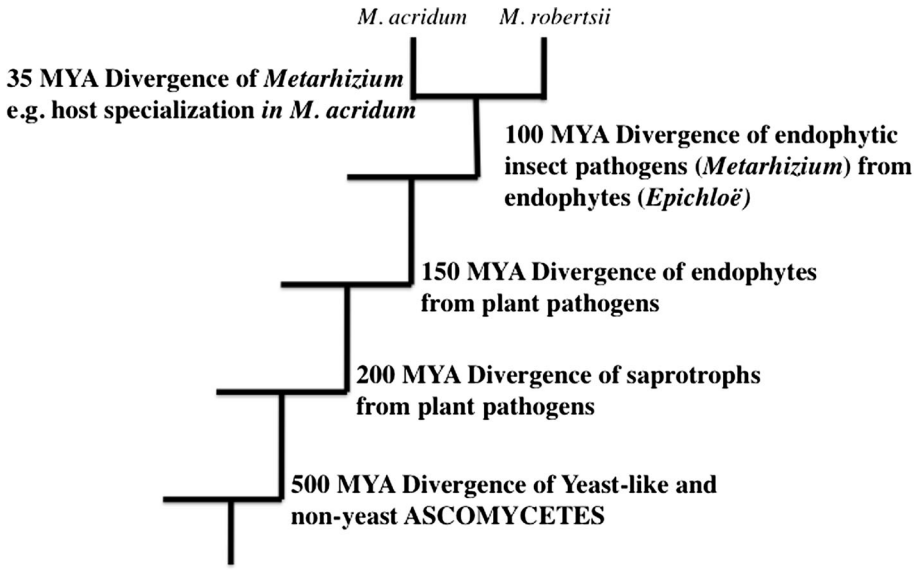

Fig. 1 Major transitions in the evolution of *Metarhizium* species

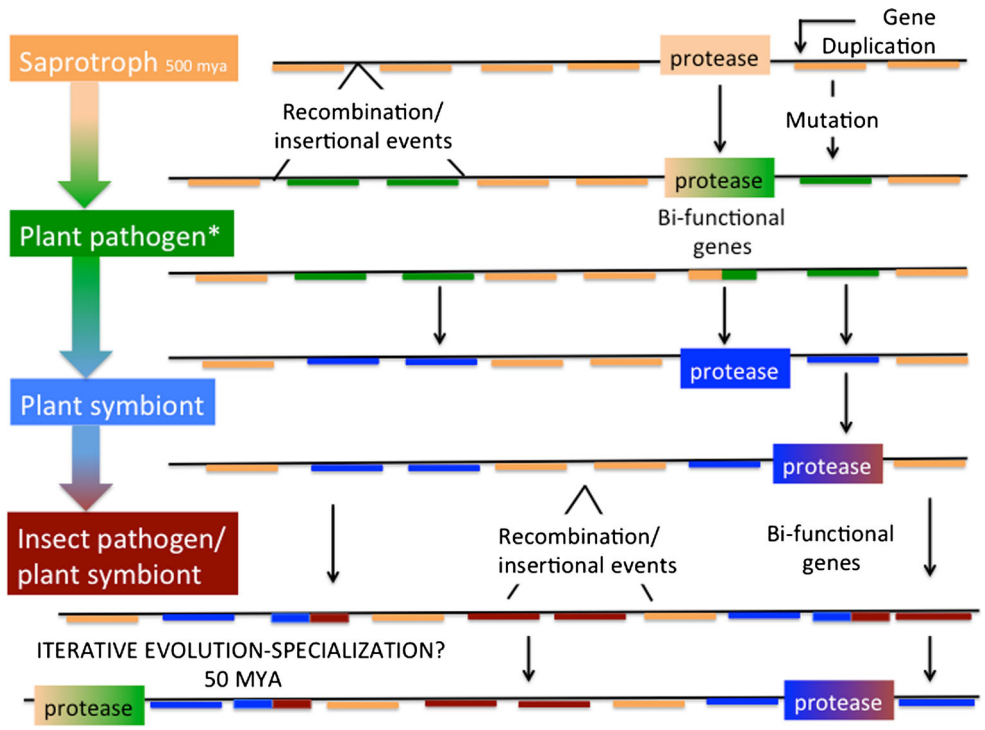

Fig. 2 Potential model for the evolution (*from top to bottom*) of genetic events that lead to multifunctional lifestyles of EIPF. The accumulation of plant pathogenic genes potentially allowed for the root colonizing ability of saprotrophs; selection pressure could have then pushed plant pathogens toward symbiosis with the plant host, ultimately resulting in a mutualistic relationship; symbiotic pressure from the plant to receive nutrients (e.g. nitrogen) from the fungus could have lead to the evolution of insect pathogenesis—an extension of its ability to breakdown organically bound nutrients or the co-option of genes involved in plant pathogenesis/symbiosis. Proteases are illustrated as an example of one of the gene families that likely allowed for the evolution of EIPF. In *Metarhizium* spp. they are one of the most numerous and diverse enzymes that allow this fungus to adapt to various habitats and enables its multifunctional lifestyle. The occurrence of plant pathogenic homologous proteases in *Metarhizium* may be utilized in initial endophyte colonization and/or immune evasion but does not lead to pathogenesis. Coloured bars indicate genes involved in saprotrophic (*orange*) lifestyle, plant pathogenesis (*green*), plant symbiosis/endophytism (*blue*), and insect pathogenesis (*red*). The *asterisk* indicates a lifestyle not observed with the organisms discussed as the focus of this review (i.e. *Metarhizium*, *Beauveria*), but is a theoretical evolutionary transitional lifestyle

Two of the critical genes involved in facilitating conidial adherence to the insect cuticle are identified as *Metarhizium* adhesion protein 1 (*Mad1*) and *ssgA*, a hydrophobin (Wang and St. Leger 2007a; St. Leger et al. 1992a), and the hydrophobin genes, *hyd1* and *hyd2*, in *Beauveria* (Zhang et al. 2011). Fungal penetration of insect cuticle is primarily facilitated through enzymatic degradation by proteases, and by the expression of different isoforms of endoproteinases. During growth on insect cuticle, the major proteases produced by *Metarhizium* includes cuticle degrading subtilisin-like protease (Pr1A), a thermolysin-like metalloproteinase, a trypsin like serine protease (Pr2) and other exo-acting peptidases (St. Leger et al. 1998). Expressed sequence tag (EST) analysis showed that *M. anisopliae* expressed 11 subtilisin-like proteases during growth on insect cuticle (Bagga et al. 2004). Furthermore, proteases also play a significant role in nutrient acquisition, evasion of host defense by degrading antifungal proteins, and regulation of micro-environmental pH (St. Leger et al. 1999).

Beneath the appressoria, hyphae penetrate through the insect cuticle and once in the insect hemolymph, the hyphae differentiate into yeast-like bodies termed blastospores (Small and Bidochka 2005; Lewis et al. 2009; Wanchoo et al. 2009). Enzymatic degradation as well as mechanical pressure has been implicated in cuticular penetration. For example, the expression of *Mpl1* (perilipin) is implicated in the transport of lipid bodies to the appressoria thereby increasing turgor pressure (Wang and St. Leger 2007b). Once in the hemocoel, *Metarhizium* evades the insect immune system by expression of a collagen-like protein (*MCL1*) (Wang and St. Leger 2006) and adapts to the osmotic pressure in the hemolymph through expression of *Mos1*, an osmosensor-like protein (Wang et al. 2008). The expression of genes involved in insect pathogenesis is coordinately expressed as microarray analysis on mutant strains lacking *Metarhizium* protein kinase A1 (*MaPKA1*) showed the down-regulation of 244 genes involved in cuticular infection processes (Fang et al. 2009). The

complexities of signaling pathways and genes involved in stress and virulence responses in *Beauveria*, are examined by Ortiz-Urquiza and Keyhani (2015). *Metarhizium* kills insect hosts within 3–7 days by producing toxins and absorbing nutrients. Once hemocoelic nutrients are depleted, hyphae emerge from the insect cadaver and conidiate, resulting in the mummification of the insect host (Small and Bidochka 2005; Schrank and Vainstein 2010).

Metarhizium is an excellent example of a fungus with a multifunctional lifestyle. It is an insect pathogen, a saprobe, and an endophyte. *Metarhizium* displays genotypic plasticity when exposed to dissimilar environments, thereby enabling the fungus to effectively persist saprobically or as a colonizer of plant or insect hosts (Pava-Ripoll et al. 2011; Wang and St. Leger 2005; Wang et al. 2005). For instance, *Metarhizium* uses two different proteins, MAD1 and MAD2 to facilitate adherence on insect and plant surfaces, respectively (Wang and St. Leger 2007a). EST and cDNA microarray analysis revealed that *Metarhizium* expressed different, yet overlapping, subsets of genes when grown on different insect cuticles, insect hemolymph, or in root exudate media (Wang and St. Leger 2005; Wang et al. 2005; Freimoser et al. 2003).

Notable in *Metarhizium* is the large number of proteases that it produces, and of these, 18 out of 43 protease genes are differentially expressed on insect cuticle and in root exudate (Wang et al. 2005). One significant exception is the subtilisin-like protease, *pr1A*, which was highly expressed in insects and root exudate (Wang et al. 2005). This suggests that *pr1A* is an example of a gene for a multifunctional life style. Similar differential gene expression has been reported in *B. bassiana* (Luo et al. 2015). Ortiz-Urquiza et al. (2015) examine the multitude of genes implicated in insect pathogenesis of both *Metarhizium* and *Beauveria* from a mycoinsecticide perspective and highlight that the success and survivability of these biocontrol agents in the environment is tightly connected to their capacity as plant symbionts. The adaptation of these fungi to insect and/or plant hosts could be the result of gene duplication or horizontal gene transfer events (Fig. 2; Bagga et al. 2004; Screen and St. Leger 2000; Xiao et al. 2012). A detailed understanding of the molecular mechanisms involved in fungal colonization of plant roots could provide an overall description of genes required by a fungus as a plant symbiont as well as an insect pathogen. That is, it could provide evolutionary insight into genes used for plant colonization that have been co-opted for insect pathogenicity.

Plant root colonization by insect pathogenic fungi

A number of insect pathogenic fungi are also plant endophytes (Vega 2008; Sasan and Bidochka 2012; Ownley et al. 2010). We term these fungi endophytic, insect pathogenic fungi (EIPF). Colonization of plants roots by EIPF may have evolved as a way to survive in soils, in the absence of an insect host (Hu and St. Leger 2002). However, a more likely explanation is that the *Metarhizium* evolutionary lineage initially adapted as an endophyte (>100 MYA), and insect pathogenesis is more recently acquired trait (<100 MYA). While there is little information on the specific evolutionary history of EIPF, recent work has suggested evolutionary pressure to maintain broad-range insect pathogenesis, indicating strong selection by the plant host to acquire nutrients from the broadest range of soil insects (Hu et al. 2014). However, under certain environmental conditions several *Metarhizium* spp. have evolved as specialists to certain insect species (i.e. *M. acridum*).

The ability of EIPF to infect insects is predicated on adherence to the insect cuticle (Wang and St. Leger 2007a), a mechanism that holds true for plant colonization as well, as successful association is dependent on adherence to the plant surface (Nicholson and Epstein 1991). For example, in *M. robertsii* the gene *Mad2* encodes a plant adhesin that is crucial for attachment to plant roots and is up regulated when *Metarhizium* is grown in root exudate media (Wang and St. Leger 2007a). An orthologue of *Mad2* was found within the genome of *B. bassiana*, suggesting *Mad2* plays a role in *Beauveria* plant adhesion as well (Xiao et al. 2012).

Metarhizium is capable of growing internally within plant tissue (Sasan and Bidochka 2012), and evidence has shown *M. robertsii* endophytically colonizes the roots of switchgrass (Sasan and Bidochka 2012) as well as wheat, haricot bean, and soybean (Behie et al. 2015). In field conditions in Ontario, three species of *Metarhizium* (*M. robertsii*, *M. brunneum*, and *M. guizhouense*) were found to associate with grasses, shrubs, and trees, respectively (Wyrebek et al. 2011). *B. bassiana* is capable of endophytic colonization of roots, stems, and leaf tissues of tomato, cotton, snap bean, and haricot bean (Ownley et al. 2008; Behie et al. 2015). Typically, it has been thought that *B. bassiana* gains entry through naturally occurring openings (e.g. stomata), however evidence of distortions in the cell wall of corn (*Zea mays*) around penetration sites suggests enzymatic activity, similar to that observed during cuticular invasion of corn earworm (*Heliothis zea*), may play a role in plant invasion (Wagner and Lewis 2000; Pekrul and Grula 1979).

Proteases are a key component of cuticular penetration during insect infection but may also play a role in plant colonization. The Pr1 subtilisin-like protease of *Metarhizium* and a protease produced by *B. bassiana* are homologous to the fungal protease, At1, from *Acremonium typhinum*, a grass endophyte (Reddy et al. 1996). At1 is believed to facilitate symbiotic development by aiding in the degradation of the plant cell wall and/or apoplastic

proteins, to allow fungal colonization (Reddy et al. 1996). Homologs of At1 are also observed in the mycoparasite *Trichoderma harzianum* and the nematode-trapping fungus *Arthrobotrys oligospora* (Geremia et al. 1993; Tunlid et al. 1994), both of which are also endophytes (Bordallo et al. 2002). Sequence variations in regions coding for substrate specificity would yield proteases with differing substrate specificities and may reflect evolutionary changes allowing fungi to adapt to various lifestyles as pathogens or endophytes (St. Leger et al. 1992b).

Protease functionality is highly dependent on environmental pH (Mayerhofer et al. 2015) and may help partition fungi into different ecological niches based on utilizable proteins. *Metarhizium* is able to grow over a wide range of pH (2.5–10.5) (Hallsworth and Magan 1996), and can modulate the pH of its immediate environment through the production of ammonia (St. Leger et al. 1999). The alteration of environmental pH allows for optimum activity of the extracellular subtilisin-like proteases (St. Leger et al. 1999), and may be a factor in the success of this ubiquitous EIPF.

Once inside the plant, endosymbiotic EIPF must avoid plant host defense. Plants are able to detect the presence of a pathogen and increase defense pathways, resulting in the expulsion, suppression, or death of the invading fungus (Dangl and Jones 2001). Fungal endophytes and other fungal root colonizers however, are able to communicate with the plant, indicating they are not pathogens. The arbuscular mycorrhizal fungus, *Glomus intraradices* releases a diffusible factor that primes the plant for root colonization. This diffusible communication molecule, named myc (mycorrhizal) factor, was identified as a lipochitooligosaccharide (LCO), and resembles Nod factors found in rhizobia (Maillet et al. 2011). Myc factors have been shown to prepare the root for fungal colonization by inducing transcriptional changes, such as those that activate the SYM (symbiotic) signaling pathway, and by inducing morphological changes that increase contact between plant roots and hyphae, such as increased root hair growth (Oldroyd et al. 2009; Kosuta et al. 2003). *M. robertsii* root colonization causes extensive root hair development in switchgrass, a notable indication of root priming (Sasan and Bidochka 2012), suggesting *Metarhizium* releases a myc-like factor prior to root colonization.

Nutrient exchange between EIPF and their plant hosts

In most ecosystems, soil nutrients are limited, thus competition among plants for nutrients is high (Clark and Zeto 2000). The majority of plant species are able to overcome this insufficient supply of nutrients by forming symbiotic associations with soil bacteria, mycorrhizal fungi, and fungal endophytes (Clark and Zeto 2000). Typically, in plant-fungal symbioses, fungal partners transfer limiting soil nutrients, such as phosphorus and nitrogen (Behie and Bidochka 2014; Guether et al. 2009; Govindarajulu et al. 2005) to their host plant in exchange for plant-derived carbohydrates (Bonfante and Genre 2010).

The dual life cycle of *Metarhizium* and *Beauveria*, suggested that insect pathogenesis and plant root colonization are coupled to provide plant hosts with insect derived nitrogen. EIPF are able to infect insects and subsequently translocate insect derived nitrogen to a host plant (Behie et al. 2012; Behie and Bidochka 2014). In this manner, plants colonized by EIPF have access to a specialized nitrogen reservoir present in soil ecosystems, organically bound in insects, and are able to reacquire nitrogen lost through insect herbivory.

What is the fungus gaining in return for providing insect-derived nitrogen to the plant? Freely available carbon is very difficult to access in the soil and is generally bound into complex carbohydrates such as cellulose and lignin. We hypothesized that EIPF gain access to simple plant carbohydrates in return for nitrogen. To confirm this we used plants colonized with EIPF and tracked ^{13}C, through the introduction of $^{13}CO_2$ in plant growth chambers, into plant carbohydrates and ultimately into fungal carbohydrates in the root/endophyte complex (trehalose and chitin; unpublished data). *Metarhizium* mutants deficient in a raffinose transporter gene (*mrt*) showed reduced rhizosphere competency, suggesting that fungal carbon acquisition is critical to *Metarhizium*/plant symbioses, and that MRT is a potential a route for the uptake of plant derived carbohydrates (Fang and St. Leger 2010).

EIPF as plant growth promoters

Not only have EIPF been shown to be involved in plant acquisition of nitrogen, plants grown in the presence of these fungal partners have shown increased growth and productivity (Behie and Bidochka 2014). Plants colonized by *Metarhizium* showed significantly greater number of lateral roots and root hair formations when compared to untreated plants (Sasan and Bidochka 2012), and increased leaf collar formation and foliage biomass have been reported in corn seeds treated with different *Metarhizium* strains (Liao et al. 2014). The effect of *Metarhizium* on the growth of tomato plants has also been evaluated, and *Metarhizium*-colonized plants showed significantly greater plant height, root length, and shoot/root dry weights (Elena et al. 2011). Kabaluk and Ericsson (2007) reported highest corn yield when corn plants were treated with both

Metarhizium and a conventional insecticide as compared to corn treated with just insecticide. These studies suggested that *Metarhizium* confers plant growth promotion properties beyond protection from insects, and help increase primary production in agricultural systems.

EIPF also confer protection to plants against microbial pathogens. *Metarhizium* is antagonistic towards the bean root rot fungus *Fusarium solani* (Sasan and Bidochka 2013) and *Beauveria* was found to stimulate plant defense responses (Ownley et al. 2008, 2010). *Metarhizium* also promoted soybean growth during salt stress, where *Metarhizium* treated plants showed significantly greater biomass and comparatively lower levels of the plant stress hormone abscisic acid (Khan et al. 2012).

Potential source of economically important secondary metabolites

Genomic data of *Metarhizium* and *Beauveria* showed that these EIPF are rich in secondary metabolite gene clusters when compared to fungi with other trophic associations (Gibson et al. 2014). *Metarhizium* spp. produce secondary metabolites that are toxic to insects as well as other microbes (Carollo et al. 2010). There are 85 and 52 core genes putatively involved in secondary metabolite biosynthesis in *M. robertsii* and *M. acridum*, respectively (Gibson et al. 2014). Both pharmaceutically active and insecticidal secondary metabolites are reported from *Metarhizium* and include compounds such as destruxins, fusarin-like compounds (NG39x), cytochalasin, and swainsonine (Gibson et al. 2014). In *Beauveria*, the best-studied secondary metabolite is beauvericin, which was shown to have antibacterial, anti-tumor, antifungal and insecticidal activities (Wang and Xu 2012). Furthermore, in vitro studies revealed beauvericin has cytotoxic activity against human cell lines, reverses multidrug resistance phenotype in yeast, and flucanazole resistance phenotype in *Candida albicans* (Gibson et al. 2014).

Several EIPF have been identified in the biotransformation of various chemical substrates and of these, *Beauveria* spp. have been frequently used for such purposes (Grogan and Holland 2000). Specific gene clusters, predicted in *Metarhizium* spp. and *Beauveria* spp., show potential to be exploited for biotransformation, or as biocatalysts, that may be utilized in bioremediation and drug discovery (Gibson et al. 2014). There are also examples of endophytes capable of synthesizing pharmaceutically active metabolites when associated with plants. For example, taxol, is an anticancer drug produced by endophytic fungi when associated with yew trees (Taxus family) (Garyali and Reddy 2013). Hence, more focus on metabolite production analysis during plant root colonization is needed, as the specific interaction between fungus and plant may yield commercially relevant bioactive metabolites.

Conclusions

Traditional research on insect pathogenic fungi has focused almost exclusively on insect virulence, and on increasing virulence, focusing on a faster acting biological control agent. Recent advances, however, have indicated that a number of insect biocontrol agents, such as *Beauveria* and *Metarhizium*, are also plant colonizers, and may play a larger role in the ecosystem than previously realized. These ubiquitous fungi may be critical to processes such as soil nutrient cycling. It has also been suggested that a number of other insect pathogenic fungi have a plant colonization life cycle, and therefore, the complete role of these fungi must be further elucidated in order to develop strong, industry applicable, biological control agents and pharmaceuticals. Future research into EIPF as biological control agents requires consideration of their role in the ecosystem, in order to exploit the genetic history of these fungi, leading to more powerful research and industrial applications that could potentially be exploited in a myriad of positive ways.

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