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Opinion Article

New perspectives on insect pathogens

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ABSTRACT

Recent research on entomopathogenic fungi suggests that many of them have a multifactorial influence on plant growth and soil ecology by also being endophytes, pathogens of protozoans, antagonists of plant pathogens and associates with the rhizosphere. There is very little data as to the ecological consequences of these interactions, but a deeper understanding of the mechanisms by which entomopathogens interact with other microbes, as well as with insects and plants could be used to develop the potential of these fungi as comprehensive plant symbionts. The genome sequences of the specific locust pathogen *Metarhizium acridum* and the broad host range *Metarhizium robertsii* have been used to investigate adaptations to insect parasitism and determine the identity, origin and evolution of traits needed for diverse lifestyles and host switching. Functional genomic approaches confirmed that *M. robertsii* up-regulates different genes in the presence of plants and insects, demonstrating that it has specialist genes for a bifunctional lifestyle. The more versatile life history pattern of *M. robertsii* is also reflected in a larger genome encoding more toxins and extracellular enzymes than *M. acridum*. However, secreted proteins are markedly more numerous in both *Metarhizium* spp. than in plant pathogens and non-pathogenic fungi, pointing to a greater complexity in the interactions between *Metarhizium* spp. and their environments. Commercial development of entomopathogenic fungi for pest control has been hindered by poor performance relative to chemical insecticides. We have demonstrated that the expression of genes encoding arthropod neurotoxins in *M. robertsii* can greatly improve virulence. We have also produced a *Metarhizium anisopliae* strain that expresses a single-chain antibody fragment that blocks transmission of malaria. Recombinant antibodies provide a vast array of potential anti-insect effectors that would allow construction of highly specific biopesticides with minimal additional negative environmental impact relative to parental wild type strains.

1. Introduction

At least 90 genera and more than 700 species of fungi are insect pathogens. These are distributed in virtually every major fungal taxonomic group except the higher basidiomycetes (Roberts and Humber, 1981). Fungi are the commonest insect pathogens and are particularly well suited for being developed as biopesticides because unlike bacteria and viruses they infect insects by direct penetration of the cuticle and so function as contact insecticides (Thomas and

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Read, 2007a). Biocontrol researchers have therefore made a tremendous effort to find naturally occurring fungal pathogens capable of controlling mosquitoes and other pest insects. This typically involved the selection of strains pathogenic to target insects without considering the mechanisms involved or the role of these fungi in their natural habitats. These deficiencies have hindered realization of the potential of these fungi as classical biocontrol agents that persist in the environment and recycle through pest populations (Hajek *et al.*, 2007). Most of the commercially produced fungi are species of *Beauveria*, *Metarhizium*, *Lecanicillium* and *Isaria* that are relatively easy to mass produce and can be used as inundative insecticides rather than in classical biocontrol.

Beauveria bassiana (Fig. 1) is named after Agostinio Bassi, who discovered it in 1835 as the cause of white muscardine disease of domesticated silk worm. It was instrumental in development of the germ theory of disease, and it is currently second only to yeast as a biological catalyst in industrial applications. In spite of this long history, and hundreds of publications and patents, its important role as a plant endophyte was only discovered in 1990 (Vega *et al.*, 2008). The genus *Metarhizium* (Fig. 1) includes the best studied entomopathogenic fungi at the molecular and biochemical levels. However, the principal habitat of some *Metarhizium* spp. may not be insects, but the root rhizosphere (the layer of soil influenced by root metabolism), which thus places sharp focus on the soil/root interphase as a site where plants, insects and pathogens will interact to determine fungal efficacy, cycling and survival (Hu and St. Leger, 2002). In retrospect, we realized that there was evidence in the literature before our study to indicate that *Metarhizium* spp were rhizosphere competent. Thus, general surveys have shown that while *Metarhizium* is ubiquitous, it is most abundant ($\sim 10^6$ propagules/g) in grass root soils (Milner, 1992). This abundance would have been very suggestive of rhizosphere competence to a soil microbiologist.

The failure to appreciate the relationship between *Metarhizium* and plants seems to be an example of scientists that belong to different scientific disciplines not being familiar with each other's work. Furthermore, as shown by their antagonism to plant pathogenic fungi (Kang *et al.*, 1996), and pathogenicity to soil amoebae (Bidochka *et al.*, 2010), at least some *Metarhizium* isolates have additional unpredicted flexibility in their trophic capabilities. *Metarhizium* spp. have not yet been reported as endophytes, but the genus is closely related to endophytic *Epichloe* spp. (Fig. 2). These systemically infect mostly grasses and have been shown to have negative effects on over 40 insect species in six orders (Clement *et al.*, 1994). The potential for interactions may need to be studied on a strain-by-strain basis. The genus *Metarhizium* contains biologically distinct subtypes with wide insect host ranges, e.g., *Metarhizium robertsii* [formerly known as *Metarhizium anisopliae* var. *anisopliae* (Bischoff *et al.*, 2009)], and subtypes that show specificity for certain locusts, beetles, crickets, hemipterans, etc (Bidochka *et al.*, 2001; Driver *et al.*, 2000) (Fig. 1). Different species/strains of *Metarhizium* also show differing

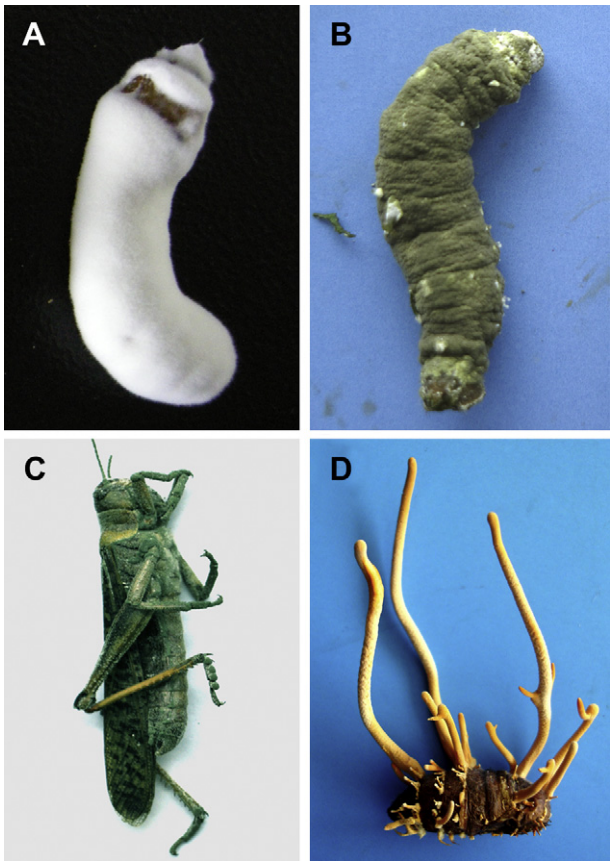

Fig. 1 – Insects killed by Ascomycete insect pathogens. A, *Beauveria bassiana* on a wax worm larvae; B, *Metarhizium robertsii* on a silk worm larvae; C, *Metarhizium acridum* on a locust, and D, *Cordyceps militaris* on a silk worm larvae (producing perithecia that contain the asci).

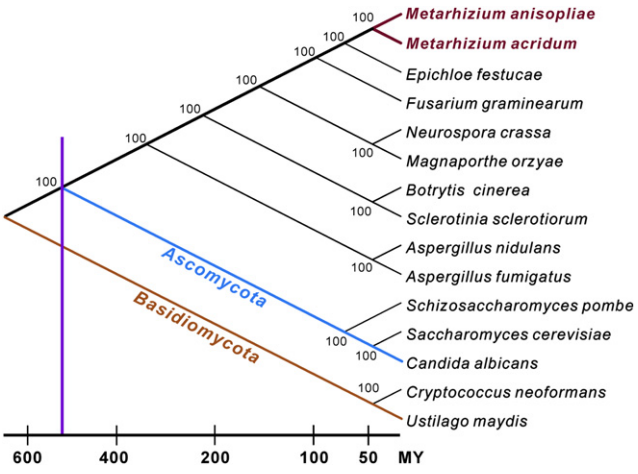

Fig. 2 – A neighbor-joining consensus tree constructed using the Poisson amino acid substitution model showing the evolutionary relationships of *Metarhizium* with other fungi. The divergence time is calibrated by the reassessed origin of the Ascomycota at *ca.* 560 million y (MY) ago (Lucking *et al.*, 2009). *Metarhizium anisopliae* and *M. acridum* lineages diverged about 33–43 million y (MY) ago and are most closely related to the mutualistic plant endophyte *Epichloe festucae* (divergence time 88–114 MY) and to the wheat head blight fungus *Fusarium graminearum* (divergence time 144–187 MY).

abilities to form associations with different plant species (Bidochka et al., 2001; Fisher et al., 2010). Overall, the effects of *Metarhizium* on plants are favorable as application of conidia to corn seeds significantly increased yields (Kabaluk and Ericsson, 2007), and the fertility of soils treated with some *Metarhizium* strains can be improved beyond insect control (unpublished data), but there is very little data as to the ecological consequences of these interactions. The fact that many genotypes of *Metarhizium* appear to be specialized to different plants (Fisher et al., 2010), suggests that the impact of rhizosphere competence by *Metarhizium* on plant ecology in general could be considerable with implicit co-evolutionary implications.

2. Genomic approaches

As the anamorphs of medicinally valued *Cordyceps* spp. (Liu et al. 2001), *Metarhizium* spp. are prolific producers of enzymes and diverse secondary metabolites with activities against insects, fungi, bacteria, viruses and cancer cells (Isaka et al., 2005; Kim et al., 2010). In addition, the enzymes from *Metarhizium* spp. are frequently exploited as industrial catalysts (Pereira et al., 2007; Silva et al., 2009). Comparative genomic approaches using the broad spectrum *M. robertsii* and the locust-specific *Metarhizium acridum* confirmed that secreted proteins are markedly more numerous in *Metarhizium* spp. than in plant pathogens and non-pathogens, pointing to a greater complexity and subtlety in the interactions between *Metarhizium* spp. and their environments (Gao et al., 2011). As expected, many of the secreted proteins are in families which could have roles in colonization of insect tissues, such as proteases. The trypsin family has the highest relative expansion among the proteases with 32 genes in *M. anisopliae*, almost twice as many as *M. acridum* and 6–10 times as many as any other fungal taxa. Overall, fewer genes were associated with plant utilization in *Metarhizium* than in plant pathogens, but almost all families of plant wall degrading enzymes were represented in the genome. Even necrotrophs such as *Trichoderma reesei* lack many families of plant cell wall degrading enzymes (Martinez et al., 2008), and the existence of such families in *Metarhizium* spp. implies that these species are able to utilize living plant tissues, which presumably could facilitate colonization of root surfaces. Consistent with their broad lifestyle options, *Metarhizium* spp. exhibit an extremely versatile metabolism, enabling growth under various environmental conditions, with sparse nutrients and in the presence of compounds lethal to other fungi (Roberts and St. Leger, 2004). As expected, both *Metarhizium* genomes contain a relatively large number of genes involved in detoxification, but the broad spectrum *M. robertsii* possesses a much greater potential for the production of secondary metabolites than *M. acridum* or most other fungi, even *Fusarium* spp. (Gao et al., 2011). Many of the additional virulence related genes in *M. robertsii* have resulted from unique gene duplication events, but comparative genomics using microarrays also revealed divergence and loss of virulence related genes in the genomes of *Metarhizium* species specialized to beetles and crickets (Wang et al., 2009).

We also addressed the mechanisms of plant–fungus–insect interactions by indexing the core-set of insect and rhizosphere-induced transcripts of *M. robertsii* strain 2575 using EST, microarray analyses and high throughput transcriptomics (Freimoser et al., 2005; Wang et al., 2005a; Wang et al., 2005b; Wang & St. Leger, 2005c; Wang et al., 2009; Gao et al., 2011). About 20 % of the genes most highly expressed by both *Metarhizium* species during early infection processes on their respective insect hosts show sequence similarities with experimentally verified pathogenicity, virulence and effector genes from other fungi, particularly related plant pathogens (Gao et al., 2011). These include many signal transduction components that provide *M. robertsii* and *M. acridum* with highly complicated finely-tuned molecular mechanisms for regulating cell differentiation in response to different insect hosts. *Metarhizium* spp. also resembled *Magnaporphe oryzae* (Oh et al., 2008) and the mycoparasite *Trichoderma harzianum* (Lorito et al., 2010) in up-regulating pathways associated with translation, post-translational modification, and amino acid and lipid metabolism. Formation of infection structures in all three species is associated with up-regulation of genes that respond to nitrogen deprivation and related stresses (Gao et al., 2011). This is probably because of basic similarities in the fungi involved and common characteristics of the hosts outer surfaces (hard and wax covered in plants and insects). Microarray studies confirmed that *M. robertsii* has the ability to produce a great variety of expression patterns, which allows it to adapt to many different environments (soil, water, root exudates, insects cuticles and hemolymph) (Wang et al., 2005b; Pava-Ripoll et al., 2011).

Construction of *M. robertsii* deletion strains for some of the highly expressed genes has identified their roles. Some of these genes encode regulators such as the protein kinase A that controls expression of many secreted virulence factors (Fang et al., 2009), an osmosensor that signals to penetrant hyphae that they have reached the hemocoel (Wang et al., 2008) and a perilipin protein (the first characterized in a fungus) that regulates lipolysis, osmotic pressure and formation of infection structures (Wang & St. Leger, 2007c). Some genes are highly adapted to the specific needs of *M. robertsii* e.g., Mcl1 (immune evasion) with its collagen domain is so far unique to *M. robertsii* (Wang and St. Leger, 2006). *M. robertsii* also has separate adhesins (Mad1 and Mad2) that allows it to stick to insect cuticle and plant epidermis, respectively (Wang and St. Leger, 2007b). This seems a critical point because *M. robertsii* up-regulates a specific plant adhesin in the presence of plants and a specific insect adhesin in the presence of insect cuticle, demonstrating that it has specialist genes for a bifunctional lifestyle. Other genes required to colonize the rhizosphere include a novel oligosaccharide transporter for root-derived nutrients, particularly raffinose, and an RNA binding protein that has important roles in both saprotrophy and pathogenicity (Fang and St. Leger, 2010a, b). Both the transporter and the RNA binding protein are the first of their kind characterized in fungi, and reveal new unsuspected stratagems of adaptations to soil living which may be relevant to all fungal biology.

3. Genetically engineering improved pathogens

Fungal pathogens of plant and insect pests have achieved moderate success as biocontrol agents in several developing

countries, particularly in niche markets or where control programs have been subsidized by governments or international agencies e.g., the use of *M. acridum* for locust control in Africa, Australia and China. This small market share is in part because of inconsistencies in performance and low virulence (slow kill and high inoculum load). Low efficacy may be inbuilt because an evolutionary balance may have developed between microorganisms and their hosts so that quick kill, even at high doses, is not adaptive for the pathogen (Gressel *et al*., 2007). Some of the knowledge provided by functional genomics is being used to improve the application of *Metarhizium* spp. as biocontrol agents. The genomic sequences will facilitate identifying candidate genes for manipulation to increase the benefits of applying *Metarhizium* not just as an insecticide but also potentially as a biofertilizer and competitor against plant pathogens. The range of exploitable fungal virulence genes is enormous as species-specific toxin-encoding genes and systems for evading host immunity have probably evolved independently in many insect pathogens. These could be used to create novel combinations of insect specificity and virulence by recombining them in other pathogens.

Genetic engineering has also provided a myriad of non-fungal choices to enhance the efficacy and hence cost effectiveness of insect pathogens. Arthropod neuropeptides are particularly attractive as they offer a high degree of biological activity, and rapidly degrade in the environment providing environmental safety (Edwards and Gatehouse, 2007). Expression of a scorpion neurotoxin (AaIT) in *M. robertsii* (Wang and St. Leger, 2007a) reduced the time to kill by 40 % and lethal spore dose by up to 22-fold in caterpillars, mosquitoes and beetles (Wang and St. Leger, 2007a; Pava-Ripoll *et al*., 2008; Lu *et al*., 2008). Although the arthropod toxins offer strict insect selectivity and AaIT has already passed regulatory hurdles for field release in the USA, it is possible that fear of hypervirulence genes could stifle this line of research in some countries. A possible "backlash" against arthropod toxins being used in *Metarhizium* was considered in two editorials regarding the Wang and St. Leger (2007a) *Nature Biotechnology paper*. A commentary by Matthew Thomas and Andrew Read lauded the environmental safety of the biopesticide, lack of side effects and the safeguard of using a promoter so that the toxin was not expressed until the fungal hyphae encountered hemolymph inside the target insect. Thus the toxin could not be expressed outside of the insect (Thomas and Read, 2007b). An article by Bernard Dixon used the inflammatory title, "Questionable experiments" (http://forms.asm.org/microbe/index.asp?bid=58054) but he was making the point that the words used to describe some avenues of research may be so emotive outside of science as to foster acute anxiety in the wider world. He also lauded the positive reasons for developing this technology, which was to improve biopesticides that are by nature selective and lack side effects, but more importantly, replace broad spectrum neurotoxic insecticides.

We recently produced a *Metarhizium* strain that expresses a single-chain antibody fragment that blocks transmission of malaria (Fang *et al*., 2011). Recombinant antibodies also provide a vast array of potential anti-insect effectors that could target, for example, insect hormone receptors. These would allow construction of very effective, highly specific, biopesticides with minimal additional negative environmental impact relative to parental wild type strains. There are many international crop pest and disease problems that are amenable to biotechnology solutions. Many of these problems could require transgenic technology for which there is only a beginning precedent being set. There is a willingness in the regulatory community to take on these issues, but what is most needed are clear and compelling needs, such as malaria control. To a large extent, we think the acceptability of the technology will be resolved by the development of fungi that can significantly reduce the occurrence of malaria, and concomitantly have no negative environmental impact. We think there is a high likelihood these fungi will be widely accepted by the people who live in areas where their health is impacted. In the end, it will be their choice.

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