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Review

Metarhizium anisopliae enzymes and toxins☆

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ABSTRACT

Entomopathogenic fungi are both a feasible system for the control of insect pests in agriculture with a growing market and an important model for studies of host-pathogen interaction. In the last ten years the actual use of fungi, mainly *Metarhizium anisopliae* and *Beauveria bassiana*, is increasing reaching commercial scale in Countries like Brazil, China and Mexico among others. At the same time important progress has occurred in the understanding of the molecular aspects of the pathogenesis and in the development of tools to validate putative virulence factors by the construction of over-expressing and knock-out strains. This wealth of knowledge is helping to access more efficient strains from the biodiversity and to optimize formulation for large scale use of this efficient, economic and environmental safer form of insect plague control. Here we focus some of the progress accumulated specially in *M. anisopliae* and give an overview of the host infection process.

1. Introduction

The use of microorganisms for the biological control of pest and disease vector insects was firstly proposed in the midst of the 19th century, however only recently the full potential and the many advantages of this practice reached application on a commercial scale. While only a small percentage of arthropods are classified as pest species, they nevertheless cause major devastation of crops, destroying around 18% of the world annual crop production (Oerke and Dehne, 2004), contributing to the loss of nearly 20% of stored food grains (Bergvinson and Garcia-Lara, 2004), and causing around US$100 billion damage each year (Carlini and Grossi-de-Sa, 2002). Disease vectors such as mosquitoes, ticks, fleas, lice and triatomid bugs are of public health importance and are of increasing concern to the general population, particularly in third world countries. Therefore, they pose a threat to the productivity, health, and well being of humans, livestock, companion animals and wild life, urging for a proper and costless control (Nicholson, 2007).

Probably, the reasons in the delay to adopt biocontrol strategies are laborious work of isolating and identifying potential biocontrol agents and the developing of suitable formulations to maximize the efficiency of the bio-products. After the realization of all the problems associated to the massive use of chemical pesticides and the mounting knowledge of the many "entomopathogenic" microorganisms the biological control is now a reality.

Amongst the virus, bacteria and fungi applicable in pest control the latest are well studied and widely used. The reasons are their efficiency in killing the hosts, the great biodiversity of the fungi that represent the many possibilities of finding the most appropriated isolates to develop competitive biological control agents and their relative environmental safety (Thomas and Read, 2007). In particular, the mode of infection of the filamentous fungi is the subject of intensive research devoted to two main objectives: the characterization of virulence factors that can improve the infection process and the mechanisms of host

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Table 1
List of different strains of *Metarhizium anisopliae* and their sources.

ITS based specie identification strains	GenBank accession numbers	Host or source geographic origin
Metarhizium anisopliae var. *anisopliae* AL	EF051702	*Mahanarva posticata* Alagoas – BR
Metarhizium anisopliae var. *anisopliae* E6	EF051705	*Deois flavopicta* Espírito Santo – BR
Metarhizium anisopliae var. *anisopliae* E9	EF051706	*Deois incompleta* Espírito Santo – BR
Metarhizium anisopliae var. *anisopliae* M5	EF051703	*Deois* sp. Pernambuco – BR
Metarhizium anisopliae var. *anisopliae* MT	EF051704	*Deois* sp. Mato Grosso – BR
Metarhizium anisopliae var. *anisopliae* RJd	EF051707	Spontaneous mutant CBiot/UFRGS/RS/BR
Metarhizium anisopliae var. *anisopliae* Nordeste	EF051708	*Mahanarva postigata* Pernambuco – BR
Metarhizium anisopliae var. *anisopliae* CARO7	EF051709	*Spodoptera frugiperda* Mexico
Metarhizium anisopliae var. *anisopliae* CARO11	EF051710	*Phyllophaga* sp. Mexico
Metarhizium anisopliae var. *anisopliae* CARO12	EF051711	*Schistocerca piceifrons* Mexico
Metarhizium anisopliae var. *anisopliae* CARO14	EF051712	*Aeneolamia* sp. Mexico
Metarhizium anisopliae var. *anisopliae* CARO19	EF051713	*Plutella xylostella* Mexico
Metarhizium anisopliae var. *majus* CG27	EF051714	Monosporic culture Brazil
Metarhizium anisopliae var. *anisopliae* CG30	EF051715	*Deois flavopicta* Espírito Santo – BR
Metarhizium anisopliae var. *anisopliae* CG31	EF051716	*Deois flavopicta* Rio de Janeiro – BR
Metarhizium anisopliae var. *anisopliae* CG33	EF051717	*Deois flavopicta* Mato Grosso – BR
Metarhizium anisopliae var. *anisopliae* CG46	EF051718	*Deois incompleta* Espírito Santo – BR
Metarhizium anisopliae var. *anisopliae* CG47 (ARSEF552)	EF051719	*Galactica* sp. Minas Gerais – BR
Metarhizium anisopliae var. *anisopliae* CG87	EF051720	*Mahanarva posticata* Alagoas – BR
Metarhizium anisopliae var. *anisopliae* CG97	EF051721	Coleoptera Brasília – BR
Metarhizium anisopliae var. *anisopliae* CG125	EF051722	*Monalonion annulipes* Pará – BR
Metarhizium anisopliae var. *anisopliae* CG144	EF051723	*Piezodorus guildinii* Goiás – BR
Metarhizium anisopliae var. *anisopliae* CG291 (ARSEF 324)	EF051724	*Austracnis guttulosa* Australia
Metarhizium anisopliae var. *majus* CG320 (ARSEF 297)	EF051725	*Xyloryctes jamaicensis* Western Samoa
Metarhizium anisopliae var. *anisopliae* CG343 (ARSEF 23)	EF051726	*Curculio caryae* South Carolina – USA
Metarhizium anisopliae var. majus CG374 (ATCC 26471)	EF051727	Coleoptera Islands of the South Pacific
Metarhizium anisopliae var. *anisopliae* CG423	EF051728	*Schistocerca pallens* Rio Grande do Norte – BR
Metarhizium anisopliae var. *anisopliae* CG491	EF051729	*Deois* sp. Paraná – BR

Abbreviations are: CG: Entomopathogenic Fungus Collection, EMBRAPA, CENARGEN Recursos Genéticos e Biotecnologia, Brazil; ARSEF: Culture Collection of Entomopathogenic Fungal Cultures, USA. EF designates GenBank accession numbers. Some strains are registered with different designations in more than one collection. In this case, the equivalent identification in showed in parenthesis.

specificity, one of the most important advantages of the biological control.

Generally, conidia (asexual spores) are the infective form and share the same environment with the potential hosts. If conidia encounter the cuticle of a susceptible host, the infection process starts with attachment and germ-tube formation. Both entomopathogen and the host trigger the expression of genes related to infection structures and hydrolytic enzymes by the fungus or to defence mechanisms by the host. Normally the infection will succeed by the breach of the cuticle and as the haemolymph is reached by the growing hyphae the host body colonization occurs. Interestingly the system is well conserved in nature and the invasion of insect tissues resembles the process that occurs with fungal plant pathogens, including the formation of germ tubes, appresoria and penetration pegs (Shah and Pell, 2003).

The ascomycetes *Metarhizium anisopliae* and *Beauveria bassiana* are the best characterized entomopathogenic fungi and the most widely used in biological control programs amongst several other species described. About 13 species or sub-species of both fungi have been formulated and registered as mycoinsecticides or mycoacaricides (Faria and Wraight, 2007).

Metarhizium is wide spread in nature and is found in soil, at the rhizosphere of plants or arthropod cadavers as saprophyte and parasitizes a broad range of insects and ticks. The taxonomy of the genus *Metarhizium* has recently been revised (Bischoff et al., 2009) and the present review is mainly focused on recent studies on *M. anisopliae* strains (Table 1 and Fig. 1) originally isolated from infected insects. Fig. 1 shows the diversity in size and spore pigmentation of these strains. Strains CG291 and CG423 were previously identified by Magalhães et al. (1997) as *Metarhizium flavoviride* or *M. anisopliae* var. *acridum* according to Driver et al. (2000); however, in our laboratory after sequencing data from ITS1-5.8S-ITS2 region both strains were considered *M. anisopliae* var. *anisopliae*. Our observations are also consistent with the original classification based on the morphological characteristics of these strains (CG291 and CG423) as being *M. anisopliae* and not *M. flavoviride* (Fig. 1). Strain CG374 was identified before as *M. anisopliae* (ATCC 26471), and not as *M. anisopliae* var. *anisopliae*. Our sequence data analyses revealed that the isolate has 98% sequence similarity with *M. anisopliae* var. *majus*. In agreement with the molecular analyses of ITS region, the SEM analyses showed that the conidial shape and size of this isolate is very similar to that reported for var. *majus* (Fig. 1).

Metarhizium grows vegetatively as hyphae producing mycelia and conidia that are the infective propagules on arthropod hosts and in culture media. Although the overall host range of *M. anisopliae* is broad, individual strains can target only particular hosts. In bioassays, strains E6, CARO14, CG47 and CG97 (Table 1) were highly virulent to the tick *Rhipicephalus* (*Boophilus*) *microplus* considered to be the most important tick parasite of livestock in the world, and caused 90–100% mortality within 4 days of treatment; strains Nordeste, CARO11, CARO12, CG27, CG30, CG33, CG87, CG125, CG320 and CG374 (Table 1) were not

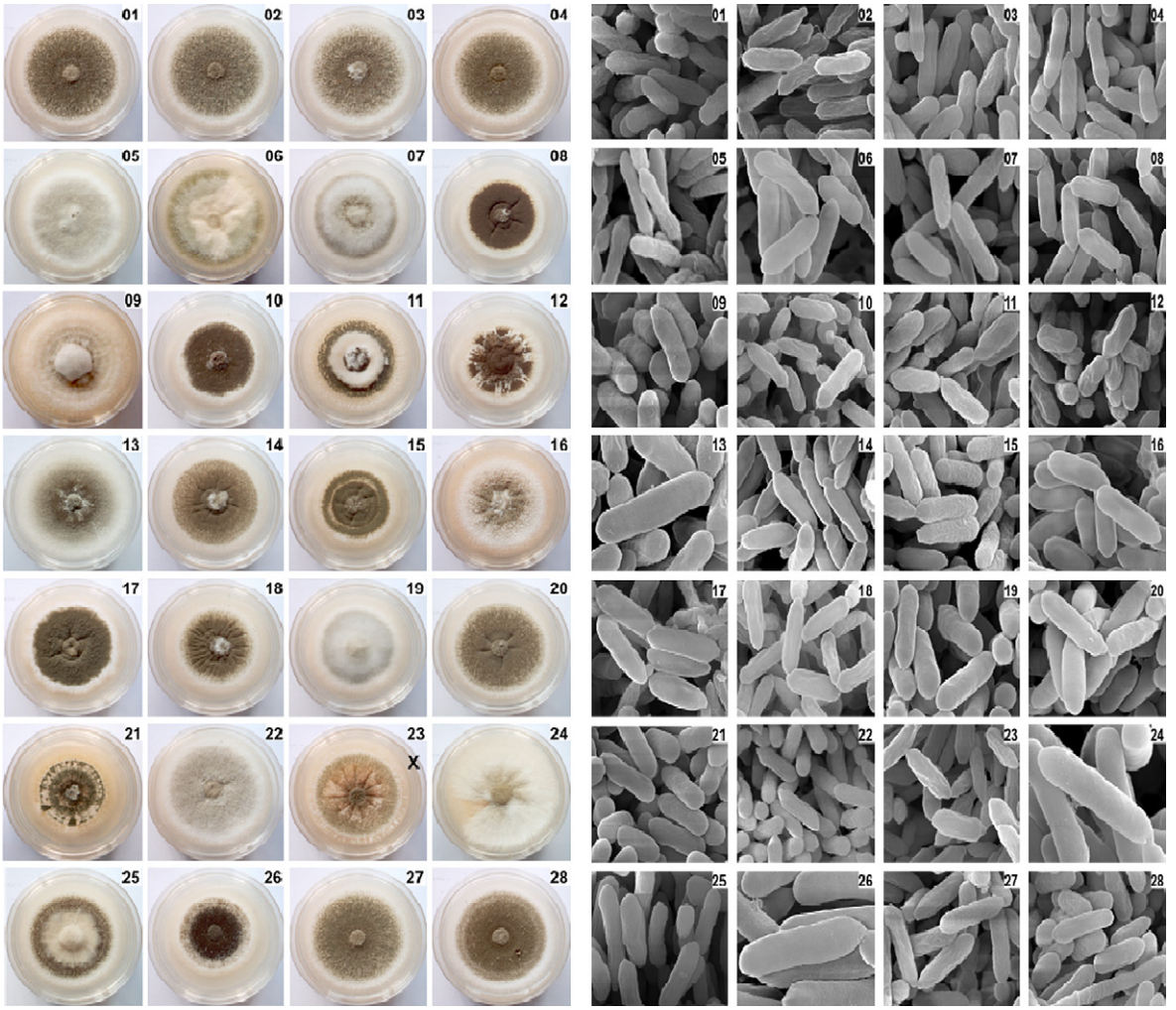

Fig. 1. Aspects of the biodiversity of different strains of *Metarhizium anisopliae*. Left panel – colony morphology. Right panel – SEM of conidiospores. The SEM is in the same scale and represents real size differences among the spores. Strains are: 1) AL; 2) M5; 3) MT; 4) E6; 5) E9; 6) RJd; 7) Nordeste; 8) CARO7; 9) CARO11; 10) CARO12; 11) CARO14; 12) CARO19; 13) CG27; 14) CG30; 15) CG31; 16) CG33; 17) CG46; 18) CG47; 19) CG87; 20) CG97; 21) CG125; 22) CG144; 23) CG291; 24) CG320; 25) CG343; 26) CG374; 27) CG423; 28) CG491. For details see Table 1.

virulent to *R. microplus* (Lubeck et al., 2008). Some strains of *M. anisopliae* presented in Table 1 are also effective against the cotton stainer bug *Dysdercus peruvianus*, especially females. Strains E6, CG47 and CG343 killed half of the females within 3.3, 3.6 and 3.66 days, respectively. Strain CG125 was the most efficient at infecting males, with a TL50 of 7.63 days, while strain Nordeste was the least infective (Lubeck et al., 2008).

M. anisopliae infects susceptible hosts via direct penetration through the cuticle. For description purposes the infection process can be divided as: (1) conidia adherence to the host cuticle through hydrophobic interactions and thin mucilaginous material; (2) conidia germination and development; (3) germ-tube differentiation into apressoria; (4) cuticle penetration; (5) hyphae differentiation into blastospores/hyphal bodies in the haemolymph; (6) host colonization; (7) extrusion to the host cadaver surface and (8) conidiophores formation and conidia production (Fig. 2).

2. Invasion steps and virulence factors

The infection initiates with the deposition of conidia on the host cuticle followed by adhesion and, under proper conditions of humidity and temperature, spore germination. Adhesion is pivotal to the initiation of the infection and involves hydrophobic interactions between the spore surface proteins (as hydrophobins, Fang et al., 2007) and the lipid layer that covers arthropod cuticle. Lipids degradation was recently related with pre-penetration growth of *M. anisopliae* on the host cuticle (Jarrold et al., 2007). These molecules are present in the epicuticle, the first barrier against arthropod pathogenic microorganism, reinforcing the importance of lipolytic enzymes in initial infection stages. Lipases produced by *M. anisopliae* are suggested to take part in this process (Silva et al., 2005). Moreover lipases could be identified in the conidia surface. In fact the degradation of the lipid layer of the host could be

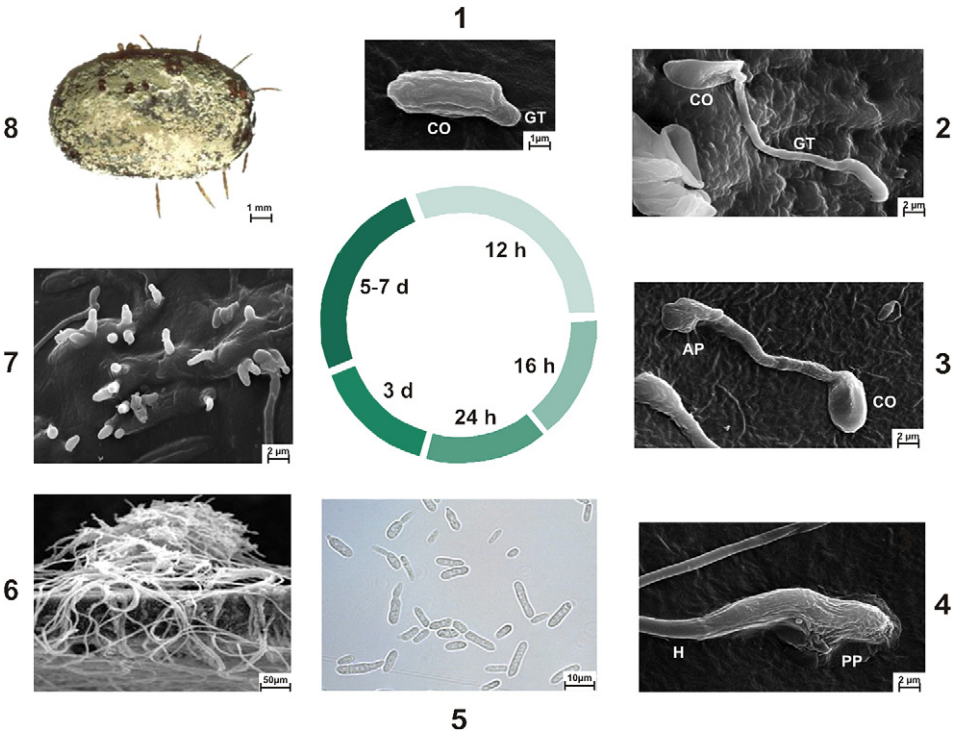

Fig. 2. *Metarhizium anisopliae* host infection stages. The events of the infection process are depicted using a tick as a host. (1) SEM of conidia adherence to the host cuticle; (2) SEM of conidia germination; (3) SEM of germ-tube differentiation into apressoria; (4) SEM of cuticle penetration; (5) optical microscopy of hyphae differentiation into blastospores – hyphae bodies; (6) SEM of host colonization; (7) SEM of extrusion to the host cadaver surface and (8) picture of conidiophores and conidia production on the tick cadaver. CO – conidia; GT – germ tube; AP – appressorium; H – hyphae; h – hours; d – days; SEM – scanning electron microscopy.

important for the recognition of a susceptible host as well as for the production of the very first nutrient molecules to support the start of the germination of the conidia (Silva et al., 2009). Adhesin protein MAD1 was shown to be important for the attachment of the swelling conidia to the host surface. It was suggested that MAD1 would replace the hydrophobins that are degraded as the cell wall of the conidia is altered Wang and St Leger, 2007.

To transpose the host cuticle the entomopathogenic fungi secrete proteases, chitinases and lipases that degrade their major constituents (proteins, chitin and lipids) and allow hyphal penetration (Frazzon et al., 2000; Wang et al., 2002; Barreto et al., 2004; Da Silva et al., 2005; Arruda et al., 2005; Lubeck et al., 2008; Boldo et al., 2009).

M. anisopliae produces several proteases as subtilisin (Pr1), trypsin (Pr2), cysteine proteases (Pr4) and metalloproteases. It has been reported that at least 14 protease isoforms are detected during growth in insects (St. Leger et al., 1987). There are 11 Pr1 subtilisin genes in *M. anisopliae* genome (Pr1A–Pr1K; Bagga et al., 2004). In particular, protease Pr1A has been implicated in entomopathogenicity. The overexpression of Pr1A increases pathogenicity against insects (St. Leger et al., 1996b). In addition, spontaneous mutants for this specific protease are less virulent, depending on the infected host. This suggests a possible host dependent regulation in protease secretion (Wang et al., 2002; Bagga et al., 2004).

The mechanisms of recognition of the susceptible host are still not elucidated. A bulk of differentially expressed proteins is observed when *M. anisopliae* is cultivated *in vitro* in the presence of cuticles of different arthropods, as *D. peruvianus*, *Anticarsia gemmatalis*, *R.* (*Boophilus*) *microplus* (Fig. 3). The analysis of the 2D gels carried out using the ImageMaster™ 2D Platinum 6.01 (*GeneralEletrics*) revealed 71 spots when *M. anisopliae* was grown in the presence of *D. peruvianus* cuticles, 79 spots in crystalline chitin, 130 spots in *B. microplus* cuticles, and 212 spots in *A. gemmatalis* cuticles. This suggests that the host cuticle recognition is complex and that differences in cuticle composition activate different sets of genes.

When the fungus breaches the cuticle, the first line of defence, successful infection can only result if the fungus can overcome the innate immune response of the host. Most arthropods respond in both a cellular and humoral manner to fungal infection, with immune activation occurring as early as the start of cuticle degradation at the penetration point. Fungi have two main strategies for overcoming host defence responses; the development of cryptic growth forms that are effectively masked from the arthropod defence responses, and the production of immunomodulating substances that suppress the host defence system. The production of a collagen-like protein (MCL1) by *Metarhizium* is suggested to be part of an evasion mechanism as a response to insect immune system (Wang and St Leger, 2006).

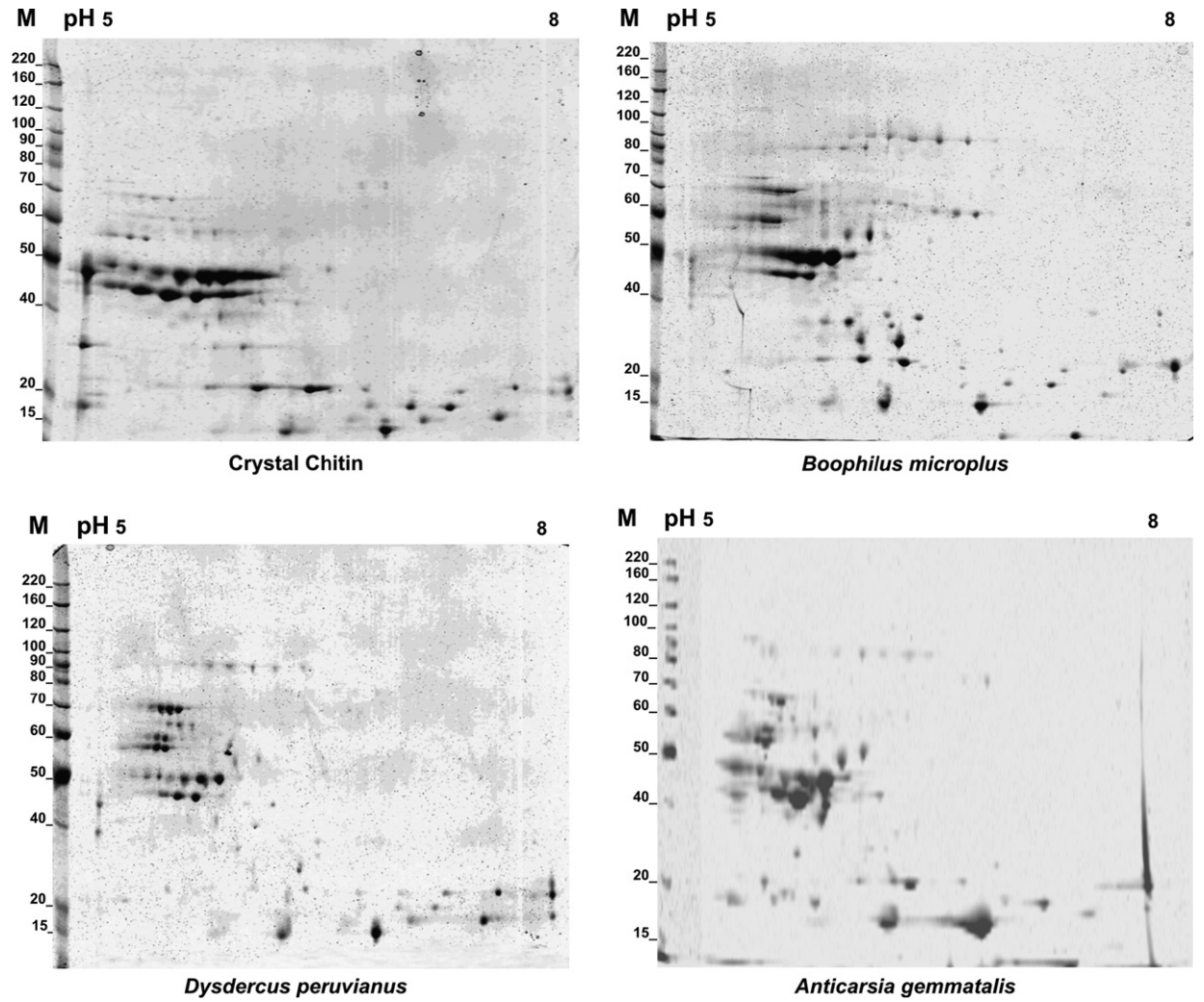

Fig. 3. Electrophoretic profile in 2D gels of secreted proteins of *Metarhizium anisopliae* E6 cultured 72 h in media containing crystal chitin, or cuticles from *Boophilus microplus*, *Dysdercus peruvianus* or *Anticarsia gemmatalis*. M: molecular mass marker (kDa). The following volumes for each culture supernatant were TCA precipitated (20% TCA in a ratio of 1:1) to obtain a final protein concentration of 400 μg. Crystalline chitin, 59.1 mL; *Boophilus microplus*, 49.23 mL; *Dysdercus peruvianus*, 29.89 mL and *Anticarsia gemmatalis*, 32.77 mL.

Metarhizium secretes large quantities of acid trehalase in insect haemolymph. As trehalose is the main sugar found in the haemolymph, the production of the enzyme to degrade it probably represents an adaptation to the host environment. The fungus may use the sugar for nutrition and at the same time depletes trehalose reducing the availability of the sugar for the host nutrition. Zhao et al. (2006) have shown that the trehalase gene *ntl-1* from *Metarhizium* is up-regulated during the infection of the locust *Locusta migratória manilensis*. After penetration, the hyphae in the haemolymph differentiate to blastospores that are unicellular yeast-like cells. These round shaped cells facilitate the spreading of the fungus to cause a generalized infection (Zhang and Xia, 2009).

As the host colonization proceed the nutrients become exhausted and the fungus produces hyphae that will emerge and produce conidia on the surface of the dead host. It was suggested that the increased expression of the subtilisin Pr1 facilitates this process (Small and Bidochka, 2005).

3. Chitinases

It is inferred that the production of enzymes that disrupt the integrity of the hosts will have a strong selective advantage for pathogens. Nevertheless, it is necessary to investigate the diversity of these enzymes and to determine which of the multiple activities is likely to be of most relevance to pathogenicity.

The enzymes that can fully degrade chitin into *N*-acetylglucosamine monomers are divided into *N*-acetylglucosaminidases [EC3.2.1.52, Glycoside hydrolase (GH) family 20] and chitinases (EC3.2.1.14, GH family 18 and19).

N-acetylglucosaminidases catalyze the release of GlcNAc monomers from GlcNAc dimers or from the non-reducing terminal end of GlcNAc multimers. Chitinases can be classified into two major classes: endochitinases that cleave the chitin polymer at any point inside the fiber and exochitinases that cleave from the non-reducing end of the polymer and release *N*-acetylglucosamine (GlcNAc) dimers. All fungal chitinases analyzed so far (including all of the available fungal genomes) belong to GH family 18. Current classification divides GH family 18 chitinases into three subgroups. Subgroup A (former class V) chitinases contain catalytic domain, but no carbohydrate-binding domain (CBM), and have molecular mass ranging from 40 to 50 kDa. Most of these chitinases possesses N-terminal signal peptide and are targeted to the secretory pathway, with some exceptions that have intracellular localization. Chitinases from subgroup B (former class III) also possesses N-terminal signal peptide and vary greatly in size, varying from 30 to 90 kDa. Smaller subgroup B chitinases contain a CBM, traditionally described as cellulose-binding domain, which has a broader polysaccharide binding spectrum and can also bind chitin. Larger subgroup B chitinases usually have serine/threonine rich domains and/or GPI-anchoring signal so that the mature proteins are bound to the plasma membrane. Subgroup C is composed by large chitinases that have a molecular mass of 140–170 kDa. They have N-terminal signal peptide and several features that distinguish them from other subgroups, like the peptidoglycan binding regions.

To date, six different chitinases (30, 33, 43.5, 45, 60 and 110 kDa) have already been characterized in *M. anisopliae* (St. Leger et al., 1991, 1996a; Pinto et al., 1997; Kang et al., 1999) however, only one *M. anisopliae* chitinase has been experimentally shown to participate in the infection process (Da Silva et al., 2005). A 43.5 kDa chitinase secreted from *M. anisopliae* strain E6 induced by the *R.* (*Boophilus*) *microplus* cuticle was also detected by immunoproteomics. IgG anti-spore surface proteins were used for searching for proteins possibly involved in early stages of fungus versus tick infection followed by LC–MS/MS (Santi et al., 2009). Considering the number of chitinases secreted by *Metarhizium*, it is expected that these enzymes act directly and synergistically along with other hydrolases throughout the infection process in order to solubilize the host cuticle, providing nutrition and allowing fungal penetration, to colonize the entire host.

Three different chitinases coding genes were isolated; the *chit1* gene encodes a 42-kDa endochitinase (Bogo et al., 1998; Baratto et al., 2003); the *chi2* gene also encodes a 42-kDa endochitinase (Baratto et al., 2006; Boldo et al., 2009); and the *chi3* gene encodes a 30-kDa exo/endo-acting chitinase (Pinto et al., 1997; Freimoser et al., 2003; Da Silva et al., 2005). The *chit1* gene was further characterized. It has a 1521-bp ORF that encodes a 423 amino acids pre-pro-protein, with a stretch of 35 amino acid residues displaying characteristics of signal peptide. The deduced mature protein has a predicted molecular mass of 42 kDa (Bogo et al., 1998) that is in close agreement with the 45 kDa determined for the *M. anisopliae* chitinase identified earlier (St. Leger et al., 1996a).

The *chi2* gene is up-regulated by chitin cuticle or the chitin monomer GlcNAc (Baratto et al., 2006). By using Agrotransformation (Duarte et al., 2007; Staats et al., 2007) and vectors based on homologous promoters (Nakazato et al., 2006) *Metarhizium* over-expressing and knock-out were constructed for the CHI2 chitinase and shown to influence insect (*D. peruvianus*) pathogenicity. The over-expression reduced the time to kill the insects whilst the knock-out of the single *chi2* gene reduced infection efficiency, suggesting an important role for this chitinase in the entomopathogenicity (Boldo et al., 2009).

The characterization of the whole chitinolytic system of *M. anisopliae* at the gene level is important in ascertaining the function of each enzyme in growth/morphogenesis and in pathogenicity.

4. Toxins

Destruxins (Dxs) are a class of insecticidal, anti-viral, and phytotoxic cyclic depsipeptides that are also studied for their toxicity to cancer cells. They are the most prevalent of the secondary metabolites produced by *M. anisopliae* in fermentation and, by far, the most exhaustively researched toxins of the entomopathogenic fungi characterized as important virulence factors accelerating the deaths of infected insects. There are 38 Dxs or Dx analogs divided chemically into five basically groups labeled A through E (Pedras et al., 2002; Hu et al., 2006). Some Dxs, especially destruxin A, E and B (DA, DE, DB) showed insecticidal activities (Cavelier et al., 1998; Thomsen and Eilenberg, 2000). DB and desmethyl-DB were phytotoxic to the plants of Brassica (Pedras et al., 2000; Saharan et al., 2003). DB also had suppressive effects on hepatitis B virus surface antigen gene expression in human hepatoma cells (Chen et al., 1997).

These toxins play an important role to weaken the host immune defences, damage the muscular system and the Malpighian tubules, affecting excretion and leading to feeding and mobility difficulties (Kershaw et al., 1999; Pal et al., 2007). Infected insects usually seek places with higher temperature in order to increase body temperature and so inhibit the development of the infecting microorganism (Elliot et al., 2002). Therefore, the action of the destruxins reducing host mobility would also impair this comportamental defence mechanism. Indeed *Metarhizium* isolates that produce higher quantities of destruxins are more virulent (Sree et al., 2008).

5. Strategies to increase infection efficiency

Microbial control has evolved as we have developed a better understanding of the pathogens, their hosts, and their environments. Several studies have assessed entomopathogenic fungi such as *M. anisopliae* for tick control (Frazzon et al., 2000; Kaaya and Hassan, 2000; Bahiense et al., 2006; Leemon and Jonsson, 2008). A *M. anisopliae* commercial formulation is already being used to control sugar cane pests in Brazil where the area treated with *M. anisopliae* for spittlebug control alone is estimated to be approximately one million hectares in 2008 (Faria and Wraight, 2007) and termites in Australia (Milner, 2000). Field experiments with acari have been carried out by Alonso-Dıaz et al. (2007) in the Mexican tropics. However, one obstacle in exploiting fungi for arthropod control is that they may kill their host too slowly. Even highly virulent

isolates take 2–5 days to kill an insect, and infected hosts can survive much longer, depending on dose and environmental conditions. Wang and St Leger (2007) have developed a method to accelerate the killing speed modifying *M. anisopliae* to express a neurotoxin from the scorpion *Androctonus australis*. The toxin dramatically increased pathogenicity and virulence and the modified fungus achieved the same mortality rates, compared to the wild type, in tobacco hornworm at 22-fold lower doses, and, at certain concentrations, reduced survival times of infected mosquitoes by >40%. Boldo et al. (2009) have also accelerate the killing speed superexpressing the CHI2 chitinase of *M. anisopliae*. The wild-type strain had an LT50 of 156 h and an LT90 of 209 h. In contrast, the CHI2 overexpression construct T33 had an LT50 of 125 h and an LT90 of 154 h. This represents a drop of over 20% in the time required to kill the host, suggesting that that overexpression of CHI2 increased the efficiency of killing *D. peruvianus*.

6. Conclusions and perspectives

The literature is rich in examples of promising microbial agents as *M. anisopliae*, and candidate biopesticide technologies, but, as evidenced by the limited penetration of the chemical-pesticide market, little of this potential is realized. The longer period of time between application and target killing still favor the chemical pesticides. It is expected that this will dramatically change in the next decade due to both: the far better knowledge of the molecular bases of the host infection and the increasing concern of society about the deleterious effects of the use of the pesticides. The possibility of construction of more efficient strains for biological control is already feasible but has still to overcome regulatory difficulties and safe practices. Nevertheless, the knowledge accumulated on the infection process can be readily applied to select more efficient strains from entomopathogen collections. Mainly the discovery of the molecular basis for host recognition is the research challenge for the next decade.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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