

INTEGRATED PEST MANAGEMENT AND ENTOMOPATHOGENIC FUNGAL BIOTECHNOLOGY IN THE LATIN AMERICAS: II. Key Research and Development Prerequisites

by

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Resumen

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En una primera entrega (Valencia & Khachatourians, 1998), se plantearon las posibilidades de los hongos entomopatógenos. Aunque existen muchas posibilidades en el manejo integrado de plagas, aún restan obstáculos para su aplicación. Existen sólo dos especies, *Beauveria bassiana* y *Metarhizium anisopliae*, en las cuales se dispone del conocimiento necesario para su desarrollo industrial. Se requiere promover el uso comercial de los hongos entomopatógenos en Latinoamérica, de manera que se logre su aceptación a nivel general. En este trabajo se evalúan los puntos clave necesarios para avanzar en la investigación y el desarrollo industrial.

Palabras clave: Biotecnología, manejo integrado de plagas, hongos entomopatógenos, Latinoamérica.

Abstract

In the first part of this review article (Valencia & Khachatourians, 1998) we presented the special opportunity that entomopathogenic fungi (EPF) offer for integrated pest management (IPM) in the Latin Americas. As expected, along with the opportunities, there are challenges for the use of EPF. First that there are only two fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, for which some prerequisite knowledge of basic and applied mycology for industrial research and development (R & D) are in place. Because of precedent setting leadership in the development of certain EPF, e.g., *B. bassiana* in IPM, Latin America stands to contribute to and gain from future

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discovery research coupled to a strong and dependable industrialization effort. There is also need to develop the framework for public acceptance, newer commercialization potential and widespread use of EPF in IPM. Here we examine issues that are prerequisites for industrial R & D.

Key words: Biotechnology, integrated pest management, entomopathogenic fungi, Latin Americas.

1. Introduction and scope

The most important prerequisite for industrial production and use of EPF is the full understanding of fungal physiology, applied genetics and applied mycology. The field performance and ultimate commercial success of EPF in the practice of IPM hinge on this prerequisite knowledge. To date, only two fungi, *Beauveria bassiana* and *Metarhizium anisopliae* have been reasonably studied at the molecular level (Khachatourians, 1996). However, molecular dissection of several properties including growth, sporulation, virulence and environmental stress responses for either fungus remains a major challenge (Frankland, Magan & Gadd, 1996). Virulence depends on a complex interaction between physiological and genetic factors which determine a pathogen's ability to cause morbidity and mortality. Utilization of the tools of molecular sciences therefore should be the basis for a rational approach to exploitation of virulence and other particular traits of EPF in the context of rational design of pest insect biocontrol (RADBIO, Valencia & Khachatourians, 1998). Given the state of current knowledge of applied mycology and biotechnology there should be significant and improved understanding of the complex traits and properties of EPF and cross-disciplinary use of such knowledge in their production, formulation, and hence performance.

2. Fungal maintenance and culturing

For a vast majority of species of EPF considered for commercialization we still need information on culturing and growth of particular isolates (Khachatourians, 1996). Additional areas for further discovery research include methods of preparation and maintenance of fungal stocks, inoculum preparation, large scale growth and substrate utilization, the accumulation of reserve carbohydrates, extracellular hydrolytic enzymes production, and process technologies to maximize spore yield, separation, drying and milling, spore compatibility with formulation ingredients, and shelf-life conditions for storage of dry or liquid formulae. The impact for industry in the market place are significantly connected with the basic elements of applied fungal physiology and bioprocess engineering.

General considerations and criteria for terrestrial EPF formulations requires determination of a reasonable shelf-life under particular storage conditions for efficacy in an agricultural field. Further the control of target pests within distinct habitats may require different methods of application. Therefore, formulations should satisfy the particular modes of product delivery. Data obtained from on ground or aerial applicators would also need statistical considerations which are connected to commercialization. After these steps, product formulation and performance must be re-evaluated as they could further enhance product design. The compatibility of ingredients used for a formulation ideally should not compromise infectivity, shelf-life, high viability, and virulence. Fungal agents with an inherent limited shelf life can benefit from new formulation technologies such as micro-encapsulation and expedited transportation to delivery points.

3. Biophysical properties of spore surfaces

At the field level, EPF spores initiate their infective action through contact. Biophysical studies have indicated that there are nonspecific- followed by specific-, modes of associations, requiring hydrophobic and electrostatic attractions between spores of EPF and insect exoskeleton. Spores then initiate the second and more specific attachment, frequently mediated by lectin-like associations between spore surface sugars and the insect cuticle. The hydrophobicity of EPF spores is in part determined by proteins called hydrophobins (Wessels, 1997). A particular characteristic of insect epicuticle fatty acid and waxes is relevant to the hydrophobic or hydrophilic elements of the spore, favoring or impairing attachment. For example, a heavily waxy cuticle found in some homopterans is unlikely to be a target of EPF species with highly hydrophilic spores. Characterization of spore hydrophobicity by phase partition assay (Jefferies & Khachatourians, 1997) has been cumbersome. As a result, the employment of hydrophobicity tests a key initial step of selection of potentially superior pathogenic EPF has lagged.

A new salt-mediated aggregation and sedimentation assay (SAS) for the determination of hydrophobicity with corroborating correlation to occurrence of specific

proteins among EPF isolates has been developed in our laboratories (Jeffs & Khachatourians, 1997; Jeffs, Xavier, Mattai & Khachatourians, 1999). The SAS hydrophobicity assay could be readily done under laboratory conditions for a large number of strains to aid determination of the spore surface types and to improve the performance of the commercially exploitable fungal strains (Valencia, Cruz & Khachatourians, unpublished data). Hydrophobicity and its optimization could facilitate effective commercial formulation by determining the type and amount of final product ingredients such as emulsifiers and adherents required for particular environmental performance condition and the costs of production. Additionally, lectins found on the spores of EPF present another level of specificity for external interactions (attachment to insect surfaces) and internal interactions within the hemolymph (immune response) (Pendland & Boucias, 1986). Biophysical properties are particularly important from the environmental and ecotoxicological points of view. A high level of specificity of attachment to the cuticle of the pest insect vs. non-target organisms (NTO) could ensure a first order of interaction for pest insects, thus in favor of beneficial and NTOs in the agroecosystem.

4. Enzymes, toxins and pigments and pathogenicity or virulence

The entry into the host and successful growth of EPF needs production of extra-cellular hydrolytic enzymes such as proteases, chitinases and lipases, adhesive mucilaginous substance(s) and appressoria to aid penetration of a peg and spread of hyphal bodies. Also, production of toxins, whether peptides/ proteins or secondary metabolites is important in the disease process. In spite of the arsenals of toxins and hydrolytic enzymes, the speed or time with which EPF kill target pests is slow and unlike those of the synthetic chemicals. Chemical pesticides that work by contact mode of action work quickly and visibly in a matter of hours or a day. The performance of the microbial agents in general falls short of the chemicals and has generated a negative perception.

As long as EPF survive, spores should be able to germinate after contact with the host cuticle even if it occurs through the tarsi of the target insect as it moves on treated plant's or other surfaces in a field environment. The knowledge of the timing of the EPF life cycle should allow the selection of those isolates with fast germination rates (Bidochka, Pfeifer & Khachatourians, 1987). Early stages of infection events are critical to further successful performance particularly when these events

occur under extremes of environmental temperature and solar radiation. The comparative study of the germination and infectivity process of a number of industrial strains has offered interesting scenarios for selection of choice isolates for *in vitro* production (Valencia & Khachatourians, 1999).

Therefore there is need for added pre-requisite knowledge of the germination process if we wish to aid the manipulation of specific genes which signal germination of EPF either under mass culturing or preferential killing of target pest insects. Some of the germination specific signals affecting hyphal growth of *Beauveria* spp. reside in their response to fatty acids or waxes (Bidochka & Khachatourians, 1992; Leucona, Clement, Riba, Joulie & Juarez, 1997) and others may reside in the lesser understood outcome of enhance virulence after multiple host passages (Hayden, Bidochka & Khachatourians, 1992). Our recent observations of the presence of particular groups of genetic sequences in response to fungal growth in particular environments is offering exciting departures from conventional thinking in that a combination of genetic and epigenetic interrelations are suspected (Valencia, unpublished experiments).

After crossing the host surface layers, the invading EPF spreads within the host in spite of the surveillance mechanisms of the host immune system (Pendland, Hung & Boucias, 1993). Because EPF surface sugars and lectins are elicitors of the immune response, pathogenic strains could be identified among isolates with varying lectin-binding profiles. This strategy could minimize inconsistencies in insect biocontrol due to i) heterogeneity of immune responses of the pest, ii) presence of different stages of insect's development, or iii) their occurrence in a wide diversity of unrelated crops, exhibiting differential plant injury capacities, like the case of the tobacco bud worm *Heliothis virescens* (Lagunes, 1974).

Toxins, pigments and other secondary metabolites that could affect immune cell function and even singularly kill some insects, could be detoxified within certain insects (Dowd, 1988). For example, cyclic depsipeptides and linear peptides lower the immune system of the host (Boucias, Mazet, Pendland & Hung, 1995). In fact, toxic metabolites from *B. bassiana* affect filopodia formation and hemocytic activation (Pendland, Hung & Boucias, 1993). Because the genetic basis for the expression of such peptides is understood, the overproduction of linear or cyclic toxic peptides by fungal mutants in order to enhance pathogenicity should be within the realm of possibility (Pannacione & Annis, 2000).

Overall, the end point for EPF infections and their multi-step and complex processes are disease and death. There are new insights on the prerequisites for the killing time. Bioassay of a collection of *B. bassiana* strains isolated from around the world produced LT_{50} values ranging from 2 to 12 days (Khachatourians, 1992). Through the use of temperature sensitive mutants of *B. bassiana*, we were able to measure the 'critical milestone events' (CME) responsible for mortality in grasshoppers (Hegedus & Khachatourians, 1996c; 1996d). It appears as though CME determine the minimum time required for the insect kill. Although the widest practical application of CME is related to the search for, or engineering of isolates with a faster kill remains to be explored, the concept of a CME suggests that there is a minimum time which is required between the initiation of an insect disease and death. However, there needs to be a systematic testing of CME in terms of behavioral consequences of EPF infected insects for feeding, mobility, behavioral fever response, horizontal and vertical migration pattern changes.

5. General and molecular genetics

Three of the elements of the RADBIO concept, depend on the knowledge and application of genetics (Valencia & Khachatourians, 1998). Entomopathogenic fungi have been difficult subjects for genetic studies. Therefore, an understanding of the molecular basis for growth, development and pathogenicity is not fully understood. The most complete genetically studied EPF are *B. bassiana* and *M. anisopliae* (Khachatourians, 1996). Compatible fungal strains having complementary desirable characteristics (i.e. high enzymatic activity for one vs. a fast germination for the other), can be hybridized (Pfeifer & Khachatourians, 1992) to attain particular improvements of polygenic traits. This point is the third element of the RADBIO concept (Valencia & Khachatourians, 1998). Once the individual peptide or protein limiting infection of a fungus has been identified, its modification and improvement based on other genetic manipulative technologies could begin. For novel commercial use there are conditional lethal mutants of *B. bassiana* (Hegedus & Khachatourians, 1994; Chelico & Khachatourians, 1999). These mutants are non-viable at certain low or high temperatures and therefore self-limit environmental survival and spread some time after spray as mycoinsecticides. Such traits can be an advantage for pest control cold sensitive mutants in temperate and heat sensitive mutants in tropical zones if the introduction of new pathogens for the management of exotic pests is required.

However, in spite of the need, in other EPF such as *Verticillium lecanii*, *Paecilomyces farinosus*, or *M. anisopliae* newer information about molecular genetics is slow to be gathered. For example, electrophoretic karyotyping of the chromosome(s) for the development of physical genomic maps and localization of genes involved in pathogenic and virulence properties is still outstanding. Information about molecular genetics is being collected through localization of genes in pathogenic and virulence properties, restriction fragment length polymorphisms, random amplified polymorphic DNA (RAPD), transposons, internal transcript spacer of ribosomal DNA and PCR using consensus tRNA gene primers (Kosir, MacPherson & Khachatourians, 1991; Tiagano-Milani, Honeycutt, Lacey, Assis, McClelland & Sobral, 1995; Rohel, Couteaudier, Paperok, Cavalier & Dedryver, 1997; Maurer, Rejasse, Capy, Langin & Riba, 1997; Hegedus & Khachatourians, 1993a; 1993b).

The prerequisite knowledge of molecular genetics of EPF also represents advantages not only for taxonomy and biosystematics, but also for commercial product R & D. DNA probes can identify genera, species, varieties and even mutants (Hegedus & Khachatourians, 1993a; 1993b Hegedus & Khachatourians, 1996a; 1996b). Using systems such as DNA probes to differentiate between isolates, PCR amplification and allelic polymorphism, one can identify base changes within hundreds of nucleotides. RAPD analysis in conjunction with protein fingerprints can be effective in monitoring of the biological and genetic stability of isolates (Hegedus & Khachatourians, 1996a; 1996b; 1996c).

After a build-up of optimistically hopeful scenarios, a word of caution is necessary. Because of the multigenic nature of virulence and the complexity of the EPF-pest interactions, the development of "improved strains" based only on recombinant DNA technologies may not necessarily enhance commercial deployment of EPF for their biocontrol potential. This is because in some jurisdictions there will be the concomitant and additional impediment of registration as the "genetically engineered" component of rDNA techniques, which would remove the "natural status" of wild type isolates, thus creating the need for more tests for environmental impacts. However, rDNA techniques are powerful in the case of monogenic traits involved with the pathogenic process which is not a general feature of EPF as insect biocontrol agents. In this sense, it would also be useful to consider the pace of acceptance of transgenic food plants, before one jumps to the conclusion. This simple strategy might determine the future outcomes for those

companies who subscribe to the use of transgenic plants, for example *Bacillus thuringiensis* based plants, as there is a continued shift in the public acceptance in certain regards (Epstein, 1999).

6. Ecological and regulatory considerations.

Ideally, it is in part the ecological axioms that should be the foundation of a regulatory framework for approval of EPF as insect biocontrol agents. It is unlikely that EPF can suppress the target pest with a single application. As in nature, deliberately released EPF could not persist at numbers higher than that which occur within natural populations. Repeat applications when needed unfortunately could add to the public belief that similar to the chemical pest control agents, EPF will impact negatively on the ecosystem. This belief would be contrary to a large body of literature of EPF in the larger context of the ecosystem. In the ecosystem, EPF success depends on how it approaches the pest, transmits and disperses itself. Questions of epizootics and enzootics are complex and depend on particular seasonal, pest generation, weather condition and regional characteristics. After the death of diseased insects, fungal outgrowth, the production and dispersal of spores to new hosts and the environment occurs. These factors are not only important for ecological studies but also in public understanding of EPF commercialization.

Is there real or perceived hazard associated with EPF to warrant delay in further environmental experimentation? Regulatory agencies dealing with the ever changing state of the knowledge and public perception have been flexible in granting permission for testing. However, instead of continued tightening of the rules on the regulation for 'biological' products for release into the environment, decisions should be based on *a priori* scientific and validated proof and not on fear. Without compromising debate, there is an urgent need to move forward with reasonable experimental use permits and continue acquisition of data on ecological ramifications of EPF, as many are naturally occurring microorganisms within their niche specific environments.

7. Commercialization, formulation and application.

Commercialization of mycoinsecticides is a serious and ultimate component of total R & D efforts in many parts of the world. This endeavor has been more visible in Latin Americas and China (Feng, Poprawski & Khachatourians, 1994), specifically with *B. bassiana*. With a worldwide resurgence of interest in the use of

EPF significant advance in development and manufacturing can be expected.

In terms of specific commercial niches, there is a large inventory of insect biocontrol needs that can be satisfied with EPF. *Beauveria bassiana* affects over 400 insect species, representing a commercial market equal to or larger than that covered by bacteria-based products.

Discovery and applied research of EPF is half of the equation for commercialization. The other half is associated with questions of post-formulation and pre- and post- application issues; bulk transportation, storage, customer perception of performance and pricing. With the issue of use, EPF do not pose much danger, however, the end user education is important. The user needs to know that they are dealing with a microbiological agent and due care must be taken with instructions from handling to spraying. Although safe at final spray dosage, accidental human exposure to concentrates of EPF can occur and must be prevented through special label warnings and education, on their application and use.

8. Business challenges and opportunities in EPF in Latin America

Latin American countries present an average of annual population growth of 1.8%, which is still one of the lower rates among world continental regions. Additionally, only 15% of the potentially arable land has been brought to technologically advanced agricultural production (Stubler & Kern, 1996).

A proportion of these lands could be progressively lost to urbanization, industrialization, erosion and salinization (Stubler & Kern, 1996). However, low technology (slash and burn) agriculture is the major threat to the preservation of arable lands. Urgent actions should be taken to wisely utilize the generally favorable conditions of agricultural and peri-agricultural zones in Latin America. These conditions should put this region in a very competitive position.

The "lower degree of development" in parts of Latin Americas can represent extraordinary opportunities for designing and attaining an adequate model of sustainability and better development with utilization of EPF through the RADBIO concept (Valencia & Khachatourians, 1998). In our opinion RADBIO can be compatible with the needs, opportunities and limitations of the region, in the context of the production process and economics. One opportunity involves enhancing the sustainability of farming lands and the future of the

remaining natural ecosystems in Latin America. This could significantly depend on our ability to demonstrate the advantages of the combination of conventional and innovative concepts of planting for food production. Trends in applied mycology and fungal biotechnology project a significant chance for the adoption and utilization of EPF in pest management schemes. Already, farmers who had to deal with outbreaks of *la broca*, or the coffee bean borer (CBB, *Hyphtenemus hampei*) are finding that an IPM program using *B. bassiana* in combination with certain synthetic chemical insecticides (at much lower administration rates per hectare per year), can control the insect pest. As CBB is not susceptible to *B. thuringiensis* or Baculoviruses, the choice for EPF for the IPM of this insect pest remains in the long term very attractive. In Colombia efficiencies in the management of both coffee plants and CBB have reduced the pest control costs and have increased product quality and profitability, raising the minimum international coffee price in 1998 to US \$1.75 and in Brazil, a figure of US\$0.70-80 a pound (Thompson, 1998). Colombia has 16% share of the world coffee market and a reasonably optimistic future for this commodity (Thompson, 1998).

The particular conditions of biodiversity, in terms of pest complexes and beneficial arthropods in most of the agricultural ecosystems in Latin America, gives EPF a significant potential in crop protection strategies. The practical advantages of EPF alone or in combined applications as in IPM are powerful enough to ensure the development of innovative and cost-effective strategies of crop protection for Latin America.

The sustainability of farming lands and the future of the remaining natural ecosystems in Latin America significantly depends on the capacity and diligence of people to develop and introduce the practice of new concepts such as RADBIO, and use of IPM-EPF alternatives. What needs to be done for the next twenty years, has to start now. The momentum for agricultural production is high and the technological tools are in place. There will be a global demand for new biocontrol agents, as many governments are taking the political decision in favor of sustainable agricultural systems (Matteson, 1995). These decisions are formally recognizing IPM and application of bioinsecticides including EPF because there are many emerging pests resistant to the new generation of transgenic plants bearing *B. thuringiensis* toxins and recent data indicates that the refugia based models for management will not succeed or may be difficult to meet in practice (Dove, 1999). The reason behind is that Bt resistance is a dominant trait.

The combination of fungal biotechnology and innovative IPM modeling approaches, appears very promising in the context of a long term sustainable agriculture. Furthermore, such approaches serve the purpose of a low input sustainable agriculture (LISA), which is adaptable to newer technologies and compatible with current crop protection and crop production strategies. Companies providing systems of integrated crop production will be in a strong position (Stubler & Kern, 1996). A combination of market pull, science push and consumer in the driver's seat should be ideal for growth of LISA in agriculture of Latin Americas. The challenges and the opportunities that lie ahead could be a benefit to all.

A positive outcome can be forecasted, if the prerequisite R & D work, industrial base, expenditure in scientific research of highest impact is adopted by the appropriate sectors (Macilwain, 1999). Many Latin American governments are beginning to address the problem of lack of resources and timeliness of access to materials. Ministries of science are adopting a stronger push for institutional and collaborative basic and applied research to increase national profiles of countries such as Brazil, Argentina, Venezuela, Chile and Colombia both in R & D expenditures and its outcomes. Having said that, it is equally important to translate the discoveries from Latin America to intellectual property rights. Brazil and Korea both produce 1% of the science published in international journals. Yet, whereas Korea generates 1% of patents filed at the United States patent office, Brazil record is only 0.2% (Macilwain, 1999). Clearly there is more that can be done and certainly as we offered in our article, some of these come from the use of applied mycology and biotechnology in the arena of IPM through appropriate R & D initiatives.

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