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A REVIEW :

Entomopathogenic fungi as potential bio-control agents and mode of action against insect pests

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SUMMARY : The growing demand for reducing chemical inputs in agriculture and increased resistance to insecticides have provided great impetus to the development of alternative forms of insect-pest control. Biocontrol offers an attractive alternative to the use of chemical pesticides. Entomopathogenic fungi are naturally occurring organisms which are least damaging to the environment. Their mode of action appears little complex which makes it highly unlikely that resistance could be developed to the biopesticide. Past research has shown some promise of the use of fungi as a selective pesticide. The current article updates us about the recent progress in the field of entomopathogenic fungi in biocontrol of insect pests and their possible mechanism of action to further focus on research lacunae in developing them as effective biocontrol agents against insect pests.

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KEY WORDS :
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BACKGROUND AND OBJECTIVES

Entomopathogenic fungi are among the first organisms to be used for the biological control of pests. More than 700 species of these fungi from around 90 genera are pathogenic to insects. Most of them are found within the deuteromycetes and entomophthorales. Some entomopathogenic fungi have restricted host ranges, for example, Aschersonia aleyrodes infects only scale insects, and whiteflies, while other fungal species have a wide host range, with individual isolates being more specific to target pests. Entomopathogens such as *M. anisopliae* and *B. bassiana* are well characterized in respect to pathogenicity to several insects, and they have been used as agents for the biological control of agriculture pests worldwide. About 11 companies offer at least 16 products based on the entomopathogenic fungi *B. bassiana*. These products are not only used in the coffee crop but also in other crops such as bean, cabbage, corn, potato and tomato. The biopesticide has very complex mode of action unlike chemical pesticides, therefore resistance in pest could not be developed.

Entomopathogenic fungi are now known to be plant endophytes, plant disease antagonists, rhizosphere colonizers, and plant growth promoters (Lacey *et al.*, 2015). These newly understood attributes provide possibilities to use fungi in multiple roles. In addition to arthropod pest control, some fungal species could simultaneously suppress plant pathogens and plant parasitic nematodes as well as promote plant growth. A greater understanding of fungal ecology is needed to define their roles in nature and evaluate their limitations in biological control. More efficient mass production, formulation and delivery systems must be devised to supply an ever increasing market. More testing under field conditions is required to identify effects of biotic and abiotic factors on efficacy and persistence. Lastly, greater attention must be paid to their use within integrated pest management programms; in particular, strategies that incorporate fungi in combination with arthropod predators and parasitoids need to be defined to ensure compatibility and maximize efficacy.

Important genera of entomopathogenic fungi for insect pests :

The power of these entomogenous micro-organisms in bringing about a certain degree of natural or microbial control of insect pests is directly related to human welfare which has attracted the attention of microbiologist, molecular biologists, and entomologists for years. Several entomopathogens, when inundatively introduced into a variety of habitats, can provide effective short term to long-term control. The most propitious integration of pathogens, predators, insect's growth regulators, and conventional insecticides may provide us with long-term control of serious agricultural insect pests. Some of the potential candidates for biocontrol of insect pests are discussed here.

Beauveria :

B. bassiana, a filamentous fungus, belongs to a class of insect pathogenic deuteromycete also known as imperfect fungus. Strains of *Beauveria* are highly adapted to particular host insects. A broad range of *Beauveria* spp. has been isolated from a variety of insect worldwide which are of medicinal or agricultural importance. *Beauveria bassiana* is a fungus that grows naturally in soils throughout the world and acts as a pathogen on various insect species, causing white muscardine disease (Jain *et al*., 2008). An interesting feature of *Beauveria bassiana* is the high host specificity of many isolates. Hosts of medicinal importance include vectors of tropical infectious diseases such as tsetse fly *Glossina morsitans*, and sand fly *Phlebotomus* that transmits Leishmania and bugs of genera *Triatoma* and *Rhodnius*, the vectors of Chagas disease. Hosts of agricultural and forest significance include the Colorado potato beetle, the codling moth, and several genera of termites, American bollworm *Helicoverpa armigera* (Thakur and Sandhu, 2010) and *Eutectona machaeralis*. Furthermore, the high level of persistence in the host population and in the environment provides long-term effects of the entomopathogenic fungi on pest suppression, if an epizootic is caused. It is being used as a biological insecticide to control a number of pests such as termites, whitefly, and in malaria-transmitting mosquitoes (McNeil Donald, 2005) *B. bassiana* is the anamorph (asexually reproducing form) of *Cordyceps bassiana*. The latter teleomorph (the sexually reproducing form) has been collected only in eastern Asia (De Faria and Wraight, 2001) . Rehner and Buckley have shown that *B. bassiana* consists of many distinct lineages that should be recognized as distinct phylogenetic species. This ubiquitous fungus has long been known to be the most common causative agent of disease associated with dead and moribund insects in nature and has been scrutinized worldwide as a microbial control agent of hypogeous species. Many curculionid weevils with a sub-terranean larval stage are highly susceptible to the white muscardine disease. Like many species of entomogenous fungi, *B. bassiana* is composed of many genetically distinct variants associated with geographical location and host which differ substantially in their ability to produce pathogenesis. As an insecticide, the spores are sprayed on affected crops as an emulsified suspension or wettable powder. *B. bassiana* parasitizes a very wide range of arthropod hosts and therefore is considered as a nonselective biological insecticide. *B. bassiana* is also applied against the European corn borer *Ostrinia nubilalis*, pine caterpillars *Dendrolimus* spp. and rice green leafhoppers *Nephotettix* spp.

Verticillium :

Verticillium lecanii is a widely distributed fungus, which can cause large epizootic in tropical and subtropical regions, as well as in warm and humid environments (Nunez *et al*., 2008). It was reported by Kim *et al.* (2002) that *V. lecanii* was an effective biological control agent against *Trialeurodes vaporariorum* in South Korean greenhouses. This fungus attacks nymphs and adults and stucks to the leaf underside by means of a filamentous mycelium (Nunez *et al*., 2008). In 1970s, *V. lecanii* was developed to control whitefly and several aphids species, including the green peach aphids (*Myzus persicae*) for use in the greenhouse chrysanthemums. *V. lecanii* was considered as a major parasite which caused a massive decline of cereal-cyst nematode populations in monocultures of susceptible crops. *V.* chlamydosporium has a wide host range amongst cyst and root-knot nematodes but it is very variable and only some isolates may have potential as commercial biological control agents.

Metarhizium :

Metarhizium anisopliae is also very potential pathogen on insect pests and is explored for mycobiocontrol of notorious insect pests. A complete bioactivity of *M. anisopliae* has been tested on teak skeletonizer *Eutectona machaeralis* and found *M. anisopliae* to be a potential myco-biocontrol agent of teak pest (Sandhu *et al*., 2000). Hasan *et al*. 2002 have tested spore production of *M. anisopliae* by solid state fermentation.

Nomuraea :

Nomuraea rileyi another potential entomopathogenic fungi is a dimorphic hyphomycete that can cause epizootic death in various insects. Many insect species belonging to Lepidoptera including *Spodoptera litura* and some belonging to Coleoptera are susceptible to *N. rileyi*. The host specificity of *N. rileyi* and its ecofriendly nature encourage its use in insect pest management. Although, its mode of infection and development have been reported for several insect hosts such as *Trichoplusia ni*, *Heliothis zea*, *Plathypena scabra*, *Bombyx mori*, *Pseudoplusia ni* includes, and *Anticarsia gemmatalis*. Another insect *Spilosoma* was found to be severely attacked by *Nomuraea rileyi*, hence studied in detail for its biocontrol. Similarly an epizootic of *Nomuraea rileyi* was observed on Junonia orithya (Rajak *et al*., 1991) which was proved to be the best alternative to manage the hedge plant eater *Junonia orithya*.

Paecilomyces :

Paecilomyces is a genus of nematophagous fungus which kills harmful nematodes by pathogenesis, causing disease in the nematodes. Thus, the fungus can be used as a bionematicide to control nematodes by applying to soil. *P. lilacinus* principally infects and assimilates eggs of root-knot and cyst nematodes. The fungus has been the subject of considerable biological control research following its discovery as a biological control agent in 1979. *P. fumosoroseus* is one of the most important natural enemies of whiteflies worldwide, and causes the sickness called "Yellow Muscardine". Strong epizootic potential against *Bemisia* and *Trialeurodes* spp. in both greenhouse and open field environments has been reported. *P. lilacinus* has been considered to have the greatest potential for application as a biocontrol agent in subtropical and tropical agricultural soils. The ability of this fungus to grow extensively over the leaf surface under humid conditions is a characteristic that certainly enhances its ability to spread rapidly through whitefly populations.

Natural epizootics of these fungi suppress *B. tabaci* populations. Epizootics caused by *P. fumosoroseus* also leads to substantially reductions in *B. tabaci* populations during or immediately following rainy seasons or even prolonged periods of cool, humid conditions in the field or greenhouse. However, in general, epizootics of naturally occurring fungi cannot be relied upon for control. Only a few species of fungi have the capacity to cause high level of mortality, and development of natural epizootics which is not only dependent on the environmental conditions, but also influenced by various crop production practices. Also, epizootics often occur after intense injury has already been inflicted by whiteflies (Li *et al*., 2001). Kim *et al*. (2002) reported that *P. fumosoroseus* is best for controlling the nymphs of whitefly. These fungi cover the whitefly's body with mycelial threads and stick them to the underside of the leaves. The nymphs show a "feathery" aspect and are surrounded by mycelia and conidia. *P. furiosus* is also used to control mosquito sp. *Culex pipiens* .

Mode of action of entomopathogenic fungus :

Entomopathogenic fungi constitute the largest single group of insect pathogens among micro-organisms. Such insect killing fungi are very fast Micro-organisms to be recognized as disease causing agent in insects. Entomogenous fungi are promising biocontrol agent for a number of crop pests. Several insect species belonging to order Lepidoptera, Coleoptera, Homoptera, Hymenoptera and Diptera are susceptible to various fungal infections. Entomopathogenic fungi have a great potential as biocontrol agents, as they constitute a group with over 750 species that, when dispersed in the environment, provoke fungal infections in insect populations.

The infection process :

Fungi have a unique mode of infection; they reach the haemocoel through the cuticle or possibly through the mouth parts. Ingested fungal spores do not germinate in the gut and are voided in the faeces. The death of the insect results from a combination of factors: mechanical damage resulting from tissue invasion, depletion of nutrient resources and toxicosis, and production of toxin in the body of insect.

Conidial attachment with the cuticle :

For most of the entomopathogenic fungi host location is a random event and attachment is a passive process with the aid of wind or water. Attachment of a fungal spore to the cuticle surface of a susceptible host represents the initial event in the establishment of mycosis. It was observed that dry spores *of B. bassiana* possess an outer layer composed of interwoven fascicles of hydrophobic rodlets. This rodlet layer appears to be special to the conidial stage and has not been reported on the vegetative cells. The adhesion of dry spores to the cuticle was suggested to be due to nonspecific hydrophobic forces imposed by the rodlets. Some of these moieties like lectins, a kind of carbohydrate binding glycoproteins, have also been detected on the conidial surface of *B. bassiana*. It was also observed that lectins could be involved in binding between conidia and the insect cuticle. The exact mechanisms responsible for the interaction between fungal spores and the cuticle remain to be determined. When the pathogen reaches and adheres to the host surface, it proceeds with rapid germination and growth which are profoundly influenced by the availability of water, nutrients, oxygen as well as pH, and temperature, and by the effects of toxic host-surface compound. Fungi with a broad host range germinate in culture in response to a wide range of nonspecific carbon and nitrogen sources (Sandhu, 1995). Entomopathogenic fungi with restricted host range appear to have more specific requirements for germination.

Formation of an infection structure :

Entomopathogenic fungi invade their hosts by infection process: penetration of the host cuticle or put pressure on cuticle by making appressorium and then penetrate by penetration peg (Sandhu, 1995). The cuticle has two layers: the outer epicuticle and the procuticle. The epicuticle is a very complex thin structure that lacks chitin but contains phenol-stabilized proteins and is covered by a waxy layer containing fatty acids, lipids and sterols. The procuticle forms the majority of the cuticle and contains chitin fibrils embedded into a protein matrix together with lipids and quinones (Neville, 1984). Protein may account for upto 70% of the cuticle. In many areas of the cuticle, the chitin is organized helically giving rise to a laminate structure. Entomopathogenic fungi, *B. bassiana* conidia germinate on the host surface and differentiate an infection structure termed appressorium. The appressorium represents an adaptation for concentrating physical and chemical energy over a very small area so that access may be achieved efficiently. Thus, formation of the appressorium plays a pivotal role in establishing a pathogenic interaction with the host. Appressorium formation may be influenced by host surface topography, and biochemical investigations indicate the involvement of the intracellular second messengers Ca^{2+} and cyclic AMP (cAMP) in appressorium formation Leger *et al.* (1991) or in general when the cuticle in hard.

Penetration of the cuticle :

Entomopathogenic fungi need to penetrate through the cuticle into the insect body to obtain nutrients for their growth and reproduction. Entry into the host involves both enzymic degradation and mechanical pressure as evidenced by the physical separation of lamellae by penetrated hyphae. A range of extracellular enzymes that can degrade the major components of insect cuticle, including chitinases, lipases, esterases and at least four different classes of proteases, have been suggested to function during the fungal pathogenesis. Although the complex structure of the insect cuticle suggests that penetration would require the synergistic action of several different enzymes, much of the attention has focused on the cuticle-active endoprotease as a key factor in the process. The production of cuticle-degrading enzymes by *M. anisopliae* during infection structure formation on Calliphora vomitoria and Manduca sexta has been investigated by biochemical and histochemical analyses both *in vivo* and *in vitro*. Among the first enzymes produced on the cuticle are endoproteases (termed PR1 and PR2) and aminopeptidases, coincident with the formation of appressoria. NAcetylglucosaminidase is produced at a slow rate as compared to the proteolytic enzymes. These fungi begin their infective process when spores are retained on the integument surface, where the formation of the germinative tube initiates, the fungi starts excreting enzymes such as proteases, chitinases, quitobiases, lipases, and lipoxygenases. *V. lecanii* is capable of penetrating the insect cuticle only with its germ tube while *M. anisopliae* and *B. bassiana* produce specific infection hyphae originating at appressoria. After the successful penetration, the fungus is then distributed into the haemolymph by formation of blastospores (Bhattacharyya *et al*., 2004).

Different works are going on all over the world to distinguish the various enzymes which are required for the mechanism of entomopathogenic *Metarhizium anisopliae*, *M. flaviviridae, Paecilomyces farinosus, Beauveria bassiana* and *B. brongniartii*. Host specificity may be associated with the physiological state of the host system (*i.e.*, insect maturation and host plant), the properties of the insect's integument with the nutritional requirements of the fungus, and the cellular defense of the host. In contrast to bacteria and viruses that pass through the gut wall from contaminated food, fungi have a unique mode of infection. They reach the haemocoel through the cuticle.

Production of toxins :

A plethora of work with circumstantial evidence is available from deuteromycete pathogens for the involvement of fungal toxins in host death. The action of cytotoxins is suggested by cellular disruption prior to hyphae penetration. Behavioural symptoms such as partial or general paralysis, sluggishness, and decreased irritability in mycosed insects are consistent with the action of neuromuscular toxins. *B. bassiana* and *M. anisopliae* produced significant amounts of toxic compounds within their hosts. For example, the toxins Beauvericin, Bassianolide, Isarolides, and Beauverolides have been isolated from *B. bassiana* infected hosts , toxins Destruxins (DTXs) and Cytochalasins have been isolated from *M. anisopliae* infected hosts. The toxins have shown to have diverse effects on various insect tissues. DTX depolarizes the lepidopteran muscle membrane by activating calcium channels. In addition, function of insect hemocytes can be inhibited by DTX (Bradfisch and Harmer, 1990). Presumably, there are still many toxins that remain to be isolated from parasitized insects and except DTXs, their relevance in the process of pathogenicity remains to be studied in detail.

Genetic engineering studies of entomopathogenic fungi :

A more widespread use of fungi for biocontrol depends on improvements of wild-type strains by combining characteristics of different strains and mutants. Two types of improvements could be considered: (i) improving the efficacy of the insecticide, by reducing the dose necessary to kill the insects, by reducing the time to kill the pest or decreasing crop damage caused by the pest by reducing the feeding time; (ii) expanding the host range. Essential for the development of a hyper virulent strain is a complete understanding of the remarkable pathology of fungal infections. Molecular biology provides the necessary tools for dissecting the mechanisms of pathogenesis and in the longer term for producing recombinant organisms with new and relevant characteristics. Initial development towards these goals has occurred with *M. anisopliae* and to a much lesser extent with *B. bassiana*. Genetic transformation systems, which are an essential part of modern fungal research, and are necessary for the experimental manipulation of virulence genes *in vitro* and *in vivo*, have been established (Sandhu and Vikrant, 2006). The success of utilizing these procedures depends on the availability of selectable transformation markers. Transformation techniques have been used to isolate specific pathogenic genes, investigate virulence determinants of *M. anisopliae* and *B. bassiana*, and to produce a strain with enhanced virulence. Unraveling the molecular mechanisms of fungal pathogenesis in insects will provide the basis for the genetic engineering of entomopathogenic fungi.

Advantages of entomopathogenic fungi :

The advantages of using fungi as biocontrol agents are as follows (1) Their high degree of specificity for pest control. Fungi can be used to control harmful insect pests without affecting beneficial insect predators and nonharmful parasites. (2) The absence of effects on mammals and thus the reduction of the hazards normally encountered with insecticide applications, such as pollution of the environment. (3) The lack of problems caused to insect resistance and prolonged pest control. (4) A high potential for further development by biotechnological research. (5) High persistence in the environment provides long-term effects of entomopathogenic fungi on pest suppression.

However, there are also a number of constraints on the use of fungi as insecticides: (1) 2-3 weeks are required to kill the insects whereas chemical insecticides may need only 2-3 hours. (2) Application needs to coincide with high relative humidity, low pest numbers, and a fungicide free period. (3) Due to the high specificity additional control agents are needed for other pests. (4) Their production is relatively expensive and the short shelf life of spores necessitates cold storage. (5) The persistence and efficacy of entomopathogenic fungi in the host population varies among different insects species, thus insect-specific application techniques need to be optimised to retain long-term impacts. (6) A potential risk to immunosuppressive people.

Entomopathogenic fungi are important as they are virulent, infect by contact, and persist in environment for a long period of time. These can be mass produced in liquid or solid media. Most of the entomopathogenic fungi are facultative parasites which exist as saprotrophs and therefore can be grown apart from living hosts. Few groups are obligate parasites which must be reared in living hosts. Introduction of fungal pathogens into the host population initiates epizootic and prevents or reduces damage by the pest. The initiation of artificial epizootics has been accomplished for long-term control especially in areas where high humidity condition prevails. There are several defense mechanisms in insect which prevents the penetration and the growth of the fungus. The most common is the melanisation of the cuticle at infection site. Entomopathogenic fungi can display either a very broad host spectrum like *M. anisopliae*, *B. bassiana* or have a very narrow host range like *Aschersonia* spp. (Bhattacharyya *et al*., 2004).

Conclusion :

Modern techniques in genetic engineering and biotechnology are extremely helpful in manipulating the desired traits in entomopathogenic fungi which can further improve its bioactivity. Numerous advantages one can foresee of using these fungal pathogens as pest control agents. They are host specific having a wide host range and more importantly being less toxic to animals. Biological control agents have shown a lot of promise in terms of activity, though its efficacy is affected by many factors such as biotic and non biotic factors, host plant, and at the level of nematode infestation. There is a strong urge to elucidate the essence of these factors to improve the overall efficacy of these control agents along with developing novel methods to deliver sufficient inoculum at the target sites. Modern techniques in biotechnology has the potential to manipulate desirable traits of these entomopathogenic fungi to improve the overall field activity.

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