Isolation, characterization and mass multiplication of entomopathogenic fungi: A review

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Abstract: An attractive alternative method to chemical pesticides is the microbial biocontrol (MBCAs) agents. They are the natural enemies devastating the pest population with no hazard effects on human health and the environment. Entomopathogenic fungi has an important position among all the biocontrol agents because of its route of pathogenicity, broad host range and its ability to control both sap sucking pests such as mosquitoes and aphids as well as pests with chewing mouthparts, yet they only cover a small percentage of the total insecticide market. Entomopathogenic fungi differ from other microorganisms in their infection process: they directly break the cuticle to enter the insect hemocoel, while other microorganisms enter by ingestion through mouth and then cause disease. Various attempts have been made to isolate and characterize native entomopathogenic fungi. Isolation of these fungi has done from dead insect larvae. Surface and mass culturing of these fungi has been done in Potato Dextrose Agar and Potato Dextrose Broth respectively. Different solid substrates i.e such as grains, vegetable wastes, maize, bran, cotton seed, rice husk, wheat and liquid media such as coconut water were evaluated at variable moisture content and yeast extract concentration for mass production of two entomopathogenic fungi: Beauveria bassiana (Bals.) Vuillemin and Metarhizium anisopliae. In the present review the isolation, mass multiplication and characterization of entomopathogenic fungi will be discussed.

Key words: Entomopathogenic fungi, Isolation, Surface culture, Mass multiplication

Introduction

The increased use of conventional chemical pesticides over the years has not only contributed to an increase in food production, but also has resulted in adverse effects on the environment and non-target organisms. In view of these side effects, the necessity for sustainable crop production through eco-friendly pest management technique is being largely felt in the recent times. Of the several microbial pathogens viz., bacteria, fungi, viruses, protozoans and entomopathogenic nematodes reported, only a few have been studied systematically for their usefulness. Entomopathogenic fungi have played a uniquely important role in the history of microbial control of insects. Historical evidence indicated that entomopathogenic fungi were the first to be recognized as disease causing microorganisms in insects. Agostino Bassi de Lodi wrote about a disease caused by a fungus, which was later, identified as Beauveria bassiana. (Ainsworth, 1956). Beauveria bassiana, commonly known as white muscardine fungus attacks a wide range of immature and adult insects. Metarhizium anisopliae a green muscardine fungus is reported to infect 200 species of insects and arthropods. Both of these entomopathogenic fungi are soil borne and widely distributed.

Entomogenous fungi are potentially the most versatile biological control agents, due to their wide host range that often results in natural epizootics. An attractive feature of these fungi is that infectivity is by contact and the action is through penetration (Nadeau et al., 1996). These fungi comprise a heterogenous group of over 100 genera with approximately 750 species, reported from different insects. Many of these offer a great potential in pest management. The most important fungal pathogens are Metarhizium spp., Beauveria spp., Nomuraea rileyi, Verticillium lecanii and Hirsutella spp. It is known to attack over 200 species of insects belonging to orders Coleoptera, Dermaptera, Homoptera, Lepidoptera and Orthoptera (Moore et al., 1996). The major issues involved in mass production and utilization of mycopathogens are selection of effective strains, development of cost effective methods for mass rearing, development of effective methods for storage and shipment and creation of effective formulation. Environmental factors like temperature, humidity and sunlight play a profound role on the field persistence of entomopathogenic fungi. One of the critical factors in the effective use of microbial agents as insecticides is their relatively short persistence on leaf surfaces. Keeping these points in mind an attempt has made to review the available literature on isolation, mass multiplication and characterization of entomopathogenic fungi.

Isolation and Characterization of Entomopathogenic Fungi

Entomogenous fungi comprise a heterogenous group of cover 100 genera with approximately 750 species, reported from different insects and living in diverse habitats including fresh water, soil surfaces and aerospaces (Hajek and Ledger, 1994), many of which offer greater potential in pest management (Maddox, 1994). They belong to zygomycota, ascomycota, basidiomycota and deutsermycota. The major taxa of entomopathogenic fungi are presented in Table 1. Several of these genera are principally or exclusively associated with a single family, genus or a few species of insect pests. A number of publications discuss insect mycosis caused by Entomophthora, Beauveria, Metarhizium and Aspergillus. Among the entomopathogenic fungi, Metarhizium spp. and Verticillium lecanii (Zimm) have wide host range. Metarhizium spp. infect almost all insect orders (almost 7200 insect species) inhabiting a variety of environmental niches like soil, soil surface, aerial location and fresh water. M. anisopliae has been reported to be effective in the suppression of soil borne pests like termites, crickets, locusts, brown plant hopper in rice, pyrilla, spittle bug in sugarcane and rootgrubs. The fungus as commercial product “metaquino” has
been in use in Brazil. It was also used against coffee berry borer in Brazil and coconut leaf beetle in Taiwan. Usage of entomopathogenic fungi in IPM of rhinoceros beetles paid good dividend in Samoa (Ferron et al., 1975). V. lecanii has been proved to be a potent fungal pathogen in IPM of sucking pests. Wettable powder formulation (mycotol) caused high mortality of aphids, glasshouse whiteflies, thrips on tomato and ornamental crops. The organism exhibited greater promise in the management of coffee green scale in peninsular India (Wilding, 1981). Other fungi pathogenic on insects include Coelomycetes, Nomuraea, Fusarium and Hirsutella.

The green muscardine fungus, *Metarhizium anisopliae* was described for the first time by Metschnikoff in 1879 as *Entomophthora anisopliae*. Tulloch (1976) changed the name to *Metarhizium anisopliae* and identified another species *Metarhizium flavoviride*. *Metarhizium anisopliae* has been recorded from over two hundred insect hosts belonging to Orthoptera, Hemiptera, Lepidoptera, Diptera, Hymenoptera and Coleoptera (Veen, 1968). The entomopathogenic fungus *N. rileyi* was described for the first time by Farlow in 1983 as *Botrytis rileyi*. Later, it was transferred to *Spicaria* (Beauveria rileyi) by Charles in 1936. Kish et al. (1974) changed the name of *Spicaria rileyi* (Farlow) Charles to *Nomuraea rileyi* (Farlow) Samson. The genus *Beauveria* was described in 1912 by Vuillemin Macleod. Dead larvae generally mummify due to fungal infection. The cadavers show an initial white mycelial growth on the insect surface (except on the head capsule) and dark green (*M. anisopliae*), white (*Verticillium lecanii*) due to formation of conidia either in localized patches or over the entire surface. However, difference in conidial colour was observed. Hence, for confirmation of the pathogen, microscopic examination (400x) was necessary. *N. Rileyi* conidiophores bear dense whorls of branches and phialides i.e. conidiogenous cells which are short necked, conidia are broadly ellipsoidal to cylindrical 3.5 – 4.5 x 2.3 μm. The conidiophores of *M. anisopliae* are arranged in compact to nearly stromatic patches, mostly, mononematous. Conidigenous cells phialides in whorls, arranged in a candle like fashion, clavate to cylindrical, conidia are single celled, smooth walled hyaline to slightly denticulate apical extension bearing one conidium. Conidia were produced dark herbage green or yellowish green, ovulaceous colonies after 7 days of incubation. Colonies were closely packed, highly branched with cylindrical conidiophores. Conidial chains were round, columnar and conidia were cylindrical to oval measuring 7.5 mm. Many authors have used Sabourauds Dextrose Agar (SDA) medium for the culture of fungi but in this study both the fungi grew very well in PDA medium. Goettel and Inglis (1997) also induced external sporulation by placing insect cadavers in higher relative humidity (97-100%). However, the growth of both the fungi was evident from cultures initiated from the homogenates of the cadavers.

**Surface culture of fungi:** Surface culture of fungus *Beauveria bassiana* yielded white or lightly coloured colonies after 10 days of incubation Conidiophores were single globose and conidia were small, round to oval measuring 5 mm. Surface culture of *Metarhizium anisopliae* produced dark herbage green or yellowish green, ovulaceous colonies after 7 days of incubation. Colonies were closely packed, highly branched with cylindrical conidiophores. Conidial chains were round, columnar and conidia were cylindrical to oval measuring 7.5 mm. Many authors have used Sabourauds Dextrose Agar (SDA) medium for the culture of fungi but in this study both the fungi grew very well in PDA medium. Goettel and Inglis (1997) also used Potato Dextrose Agar (PDA) for culture of entomopathogenic fungi. The morphological characters of both fungi are akin to the description of Humber (1997). He observed the colonies of *B. bassiana* as densely clustered conidiogenous cells, which had dentilicate apical extension bearing one conidium. Conidia were long and ovoid measuring 3.5 mm. He also described colonies of *Metarhizium* as broadly branched interwined conidiogenous cells which formed chains or cylindrical colonies with conidia being ovoid and light green measuring < 9 mm.

It was seen in this study that *M. Anisopliae* had a faster growth compared to *B. bassiana* under same incubation conditions. This is in contrast to the findings of Humber (1997), who reported *M. anisopliae* would develop relatively slow in slant cultures. This might be due to strain variation of fungi used in the study. **Nutritional requirements:** The growth requirements of most entomopathogenic fungi have been poorly defined despite the fact that this information is essential for mass production. The choice of
the nutrients will obviously be directly related to the nutritional requirements of the selected fungus. Entomopathogenic fungi require oxygen, water, an organic source of carbon and energy, a source of inorganic or organic nitrogen and additional components including minerals and growth factors. Many nutrient compounds including carbohydrates, organic acids, amino acids, and vitamins are important components of plants root exudates and soil. In addition to significant quantities of these substances exuded from healthy roots, more are released through cell lysis. The survival of fungi in a soil may be affected greatly by these compounds through their effects on mycelial growth, sporulation and conidial germination. Organic acids are important, not only because they are a source of readily available substrates for soil microorganisms, but also because of their secondary effects such as modification of pH in the rhizosphere and chelation of metals (Rovira and Wildermuth, 1981). Nutrients are also present on the surface of insects. Woods and Grula (1984) reported the presence of 17 amino acids, glucosamine, amines and peptides on the surface of Helicoverpa zea larvae and that these were sufficient to initiate germination and support the growth of Beauveria bassiana and Aspergillus niger. Mass production of B. bassiana on solid or liquid media under sterile or semi sterile conditions has been described (Samsinakova et al., 1981). Holdom et al. (1986) from their studies in Australia showed that N. rileyi could grow on an inexpensive protein based culture medium made of Brewer’s yeast, yeast hydrolysate, skim milk powder and whole milk powder. Various carbon sources such as soluble starch, corn starch or malt extract were additionally used with the protein base medium. However, neither the growth and conidial production were consistent. Carrot malt agar and oats malt agar were good for sporulation of N. rileyi (Balardin and Loch, 1989). The best combination of components for sporulation of the Londrina isolates was 340 ml carrot extract and 22 g malt meal per litre of water with 12 min sterilization. Dillon and Chamley (1990) found that soaking M. anisopliae conidia in distilled water could stimulate initial process in conidial germination, and that the conidia needed carbon sources for spherical growth and germ-tube formation.

Maltose and dextrose were found to be good carbon sources for sporulation of N. rileyi (Im et al., 1988; Vimaladevi, 1995). The fungus grew well on media with pH 5-9. Li and Holdom (1994) examined the effects of a range of carbon, nitrogen sources and vitamins on colony formation, mycelial growth and sporulation of two isolates (EF25 and EF55) and concluded that soluble starch was best among different carbon sources tested for growth of M. anisopliae. Interestingly, they found that nitrogen sources rich in amino acids showed stimulatory effect on growth and germination. Gopalkrishnan and Mohan (2000) tested 13 different synthetic media for sporulation of N. rileyi. Only SMAY, Carrot Agar Yeast (CAY), Corn Meal Agar Yeast (CMAY), Nutrient Agar Yeast (NAY) and Czepecks dox Agar Yeast (CAY) showed sporulation. According to them, enrichment of synthetic medium with yeast extract was a must for mycelial growth and sporulation. SMAY and CAY were found suitable for production and culturing of N. rileyi. Though the spore yield in both cases did not differ (0.56 g/100 ml of medium) cost wise, CAY was cheaper. James (2001) tested exogenous protein and sugar sources on conidial germination of two pathogens, Beauveria bassiana and Paecilomyces fumosorosus. In liquid culture, sugars stimulated only 5-27 per cent germination of B. bassiana and less than or equal to 11 per cent germination of P. fumosorosus, whereas yeast extract or peptone stimulated 95-100 per cent germination. Rath et al. (1995) examined the utilization of 49 carbohydrates for 134 isolates of M. anisopliae and concluded that carbohydrate utilization was a useful and biologically relevant taxonomic criteria for the separation of Metarhizium strains and other entomogenous fungi.

**Natural media**: Several attempts have been made to multiply the entomogenous fungus using semisynthetic media and solid substrates in order to cut down the cost of production. Simple and cost effective mass production technology is required to make it a highly acceptable bioagent. It is reported the most of the deuteromycetes sporulate readily on solid media under aerated conditions. Semi solid fermentation provide an alternate in which the fungi grown primarily on the wet surface of a solid material form of processed cereal grains to which nutritional adjuvants were added or media of low value such as agricultural wastes. Loose substrates were observed to yield more conidia than solid substrates like agar (Muller, 2000). For mass production of conidia of M. anisopliae, a number of naturally occurring carrier cum growth media have been evaluated. Mass culturing of B. bassiana and production of conidial masses have been achieved using substrates such as wheat bran, whole grains, hay, straw, potatoes etc. either in plastic bottles, flasks or trays. McCoy and Carver (1941) described a simple method for the mass production of conidia of B. bassiana using wheat bran as the carrier medium. Grain and bagasse were shown to support the multiplication of the mycopathogen in a two phase conidial. The yield was significantly reduced on synthetic media but cost was low (Holdom et al., 1986). Silva and Loch (1987) opined that the organism could easily be multiplied on polished rice grains. Boiling the rice grains before sterilization resulted in higher spore yields. The optimum temperature for growth was 25°C for sporulation and biomass production. Among various cereal grains tested as growth media, rice was the most suitable substrate. Among the agro-based material tested, puffed rice waste was found to be superior (Patel and Yadav, 1990).

A method for production of the entomogenous fungus Metarhizium anisopliae on coarse grain rice was described (Quintela and McCoy, 1997). The production of conidia on coarse grain was significantly greater than on whole grain. With coarse grain, there was reduction in the cost of production by four times and increase in the production of conidia by 30 per cent. Vimaladevi (1994) cultured the mycopathogen N. rileyi on crushed sorghum enriched with one per cent yeast extract. Sporulation was highest with maximum of 1.44 x 10^9 conidia/g of crushed sorghum after 8-9 days at 25°C. Lopez et al. (1995) grew the fungus N. rileyi on rice sorghum and soybean and stored it for three months at 40°C. Multiplication on barley and also a semisynthetic medium wherein maltose was replaced by cereal extract were promising for cost effective production of the mycopathogen. Kulkarni (1999) studied the suitability of different cereal grains for mass production of N. rileyi and found that sorghum and rice grains were the most efficient media with yield of 13.45 x 10^9 and 13.15 x 10^9 respectively.
Various agricultural products and by products such as grains, vegetable wastes, seeds, rice husk, saw dust and liquid media such as coconut water, rice and wheat washed water and rice cooked water were evaluated for mass production of three entomopathogenic fungi; Beauveria bassiana, (Bals.) Vuil. Paecilomyces fumosoroseus (Wize) Brown and Smith and Verticillium lecanii. (Zimm) Viegas. Among the grains, wheat supported maximum spore production for B. bassiana while sorghum recorded maximum spore production in P. fumosoroseus and V. lecanii. Similarly carrot, jack seeds and ladies finger also supported good growth and sporulation of all the three tested fungi. Coconut water supported maximum growth and sporulation (Sahayaraj and Namasiyavam, 2008), among the grains tested, B. bassiana spore production was significantly higher on wheat (11.76 x 10^8 spores/100 g). The spore count recorded at the respective temperatures on rice was slightly lesser than wheat. But the statistical composition between the wheat and rice were insignificant. In the case of P. fumosoroseus, sorghum recorded the highest spore count of 10.37 x 10^8/100 g. Sorghum was found to be ideal for the mass production of V. lecanii; it recorded 11.31 x 10^8 spores/100 g. Pearl millet was found to be the next best media for the spore production (10.17 x 10^8 spores/100 g). The lowest spore production was recorded in maize which was statistically insignificant (P > 0.05, P = 0.071) (Sahayaraj and Namasiyavam, 2008).

According to Ibrahim and Low (1993) and Sharma et al. (2002), rice was found to be the suitable media for the mass culture of B. bassiana. This cereal was also used for the mass production of other deuteromycete fungi. Gopalakrishnan et al. (1999) reported that sorghum was the ideal cereal for the mass production of Paecilomyces farinosus. In the case of V. lecanii, sorghum was found to be the ideal cereal for mass production, which is in confirmation with the findings of Lakshmi et al. (2001). Lowest spore production was recorded in maize at all temperatures. Seema et al. (2013) evaluated different solid substrates i.e such as grains, vegetable wastes, maize, bran, cotton seed, rice husk, wheat and liquid media such as coconut water at variable moisture content and yeast extract concentration for mass production of two entomopathogenic fungi: Beauveria bassiana (Bals.) Vuillemeu and Metarhizium anisopliae. In that study rice was used as a best solid substrate for spore production and their viability but fungus also grows equally well on maize or other grains. This gives an overview of the mass production of fungal spores using diphasic liquid-solid fermentation i.e LUBILSOA technique which is particularly well adapted to the production of EPF’s B. bassiana and M. anisioiae and provides explanations for the use of various techniques and procedures.

Sahayaraj and Namasiyavam (2008) reported among the liquid media, coconut water produced significantly higher spore production in all tested fungi. (5.27 x 10^8, 10.17 x 10^8 and 5.27 x 10^8 spores/100 ml) and 0.51, 0.71 and 0.51 of biomass production was recorded in B. bassiana, P fumosoroseus and V. lecanii respectively. Among the seeds, vegetables and solid wastes such as rice husk and saw dust tested, B. bassiana recorded the maximum spore production (10.76 x 10^8 spores/100 g with 0.73 g) on carrot followed by jack seeds (P < 0.005, P = 0.006). Carrot waste also supported maximum spore as well as biomass production of P. fumosoroseus. In V. lecanii, jack seeds produced significantly (4.11 x 10^8) higher spores (P < 0.05) followed by the ladies finger. Carrot also recorded 2.17 x 10^8 spores/100 g and 0.24 g of biomass production and 1.27 x 10^8 spores/100 g was recorded in rice husk. However, V. lecanii produced more and the least spores as well as biomass on jack seeds and sawdust, respectively (Sahayaraj and Namasiyavam, 2008).

Future Prospects

The application of entomopathogenic fungi in biological control is increasing largely because of greater environmental awareness, food safety concerns and the failure of conventional chemicals due to an increasing number of insecticide resistant species. In determining whether the use of entomopathogenic fungi has been successful in pest management, it is necessary to consider each case individually, and direct comparisons with chemical insecticides are usually inappropriate. Gelernter and Lomer (2000) concluded that for any microbial control agent to be successful, technical efficacy was essential but had to be combined with at least two other criteria from the following: practical efficacy (easy and cheap uptake), commercial viability (profitability), sustainability (long-term control) and/or public benefit (safety).

Mass production of entomopathogenic fungi are important steps in successful utilization of EPF’s as biocontrol agents. Fungi can be cost-effectively mass-produced on different solid substrates such as maize, bran, wheat, vegetable waste etc. but the findings comes through this study confirms that rice grain was found to be appropriate substrate for higher multiplication of spores of B. bassiana and M. anisioiae at the suitable temperature 28 °C. The diphasic liquid-solid fermentation i.e. LUBILSOA technique is an appropriate method for the mass production of mycotoxins of B. bassiana and M. anisioiae for the commercialization in the small market by many manufacturing companies. The study and optimization of the mass production process led to the adoption of quality standards for the produced spores, which could be imposed on those who wanted to produce the spores commercially under licence. Various aspects of quality control are important, including: levels of contamination (especially the absence of human pathogens), virulence to target pests, particle size spectrum and, not least, viable spore count. Extensive research was carried out on optimising storage of spores, which should be dry (<5% moisture content) and ideally be maintained under cool conditions.

References


