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Exploitation of *Nomuraea rileyi* and *Beauveria bassiana* for the management of lepidopteran pests

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ABSTRACT

Pest management in agriculture started leaning towards biocontrol options for increasing agricultural production, sustaining the health of farmers and environment. Entomopathogenic fungi are potential biocontrol agents due to their infectivity by contact unlike bacteria or viruses which infect after ingestion. *Nomuraea rileyi* (Farlow) Samson and *Beauveria bassiana* (Balsamo) Vuilemin are potentially versatile fungi because of their wide spread occurrence, broad host ranges, ability to infect at different stages of their hosts and cause natural epizootics on major lepidopteran pests. Recent advances in large-scale *in vitro* production, formulation and application technology have resulted in commercial availability of several mycoinsecticide products based on *B. bassiana* and *N. rileyi*. These formulations provide good control of pests and are being used as biocontrol agents in various crop ecosystems with minimal effects on non-targets. Combination formulation of *B. bassiana* and *Bacillus thuringiensis* lowered the dose requirement and improved the speed of kill. Improving our understanding on the mode of action of the fungi at cellular and molecular level, exploiting secondary metabolites of the fungi to develop natural insecticides, refinement of formulation and application technology, and use of nanotechnology for improved delivery and persistence will enable us to utilize these fungi more effectively as well as economically under varied agro-climatic conditions. This chapter brings together the information generated on various aspects of *N. rileyi* and *B. bassiana*, their biocontrol potential in insect pest management as mycoinsecticides, registration requirements and regulatory approaches for commercial exploitation of these entomopathogenic fungi.

Keywords: Fungi; *Nomuraea rileyi; Beauveria bassiana;* lepidopteran; pests.

INTRODUCTION

Agricultural research has been oriented more and more towards developing biointensive integrated pest management (BIPM) or ecology-based pest management techniques for plant protection that use compounds which are non-toxic to man, wildlife and the environment. This rekindled the interest on the search for biotic agents that can control important insect pests of crops. Some of the most significant developments in recent years have come from studies of insect pathogenic organisms, particularly those of entomopathogenic fungi. These fungi are known to constitute the single largest phylum of insect pathogens with more than 700 species of fungi from around 90 genera pathogenic to insects (Khachatourians and Sohail, 2008). Most insect species are attacked by one or other fungal species under specific conditions. Entomopathogenic fungi have been described from terrestrial and aquatic insects in tropical, subtropical, temperate and even desert regions. Entomopathogenic fungi are potentially the most versatile biocontrol agents, because they have wide host ranges, infect at different ages and stages of their hosts and often cause natural epizootics. A striking feature of these fungi is their infectivity by contact and active penetration (Nadeau *et al*., 1996) thereby not necessitating ingestion to initiate infection. However most other entomopathogens such as bacteria, viruses

and protozoa have to be necessarily eaten, to be infective. Similarly, the entomopathogenic fungi are important among all the biological control agents due to easy delivery, improved formulations, availability of a vast number of pathogenic strains, easy engineering techniques and over-expression of endogenous proteins or exogenous toxins (St. Leger et al., 1996; Butt et al., 2001; Wang and St. Leger, 2007; Federici et al., 2008; St. Leger and Wang, 2010).

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Several reviews have been published on the importance of entomopathogenic fungi as biological control agents (Ferron, 1978; Roberts and Humber 1981; Ferron, 1981; Ignoffo, 1981; Hall and Papierok 1982; Zimmerman 1986; McCoy et. al. 1988; McCoy, 1990; Ferron et al 1991; Roberts 1989; Roberts and Hajek, 1992; Tanada and Kaya, 1993; Hajek and St. Leger, 1994; Monsour *et al*., 1994; Butt and Goettel, 2000; Ambethgar, 2001; Butt, 2002, Copping L.G., 2004, Goettel et al, 2005, Khan et al., 2012). The objective of this chapter is therefore to summarize works on two important entomopathogenic fungi *Nomuraea rileyi* (Farlow) Samson and *Beauveria bassiana* (Balsamo) Vuilemin and their biocontrol potential in insect pest management as microbial insecticides.

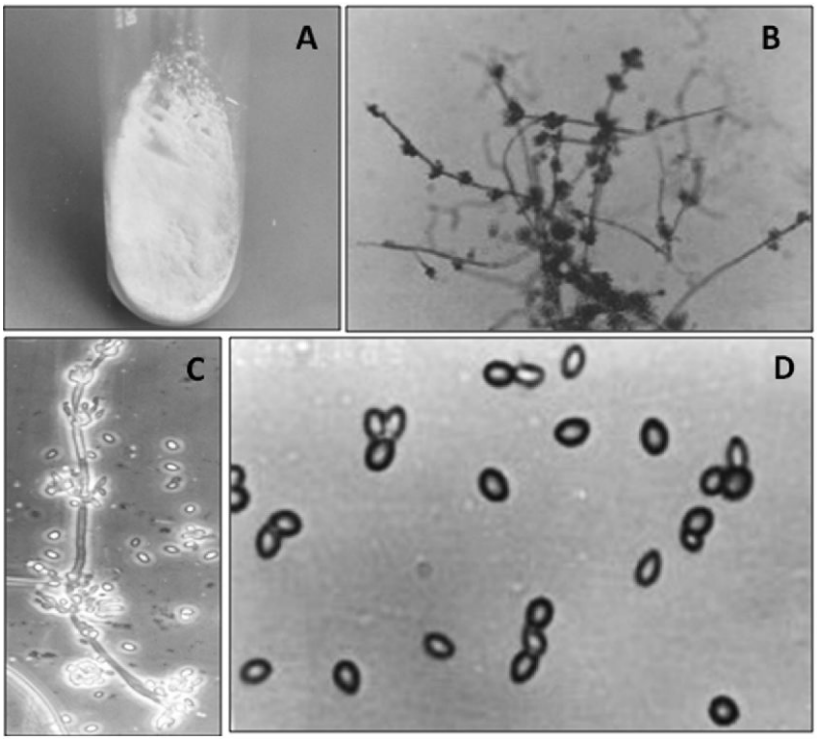

Fig. 1. Identification of Nomuraea rileyi. A. Sporulation on SMAY slope B. Mycelium C. Conidiophore. D. Conidia

CLASSIFICATION AND IDENTIFICATION

Most common entomopathogenic fungal species are from the fungal divisions Ascomycota and Zygomycota. Many genera belonging to Ascomycota *viz*., *Beauveria*, *Nomuraea*, *Metarhizium*, *Verticillium*, *Hirsutella*, *Paecilomyces*, *etc*., offer great potential in insect pest management. But later on, cultural and molecular studies have demonstrated that some of these "imperfect fungi" (formally class Hyphomycetes in the Deuteromycota) were in fact anamorphs (asexual forms) of the Ascomycota within the order Hypocreales, and Clavicipitaceae family (Fukatzu, 1997; Hodge, 2003; Krasnoff, 1995). *Beauveria bassiana* and *Nomuraea rileyi* are currently placed within the Division Ascomycota; Class Sordariomycetes; Order: Hypocreales; Family: Clavicipitaceae (Humber, 2005; Roy *et al*., 2006).

A detailed description of the entomopathogenic fungi and a key to their identification can be found in earlier reviews (Ignoffo 1981, Samson 1981, Samson *et al*., 1988, Humber, 2005). Preliminary identification of *N. rileyi* is possible by looking for malachite-green colouration on the insect surface. When viewed under the microscope, conidiophores are seen bearing dense whorls of phialides i.e., conidiogenous cells that are short necked. Conidia of *N. rileyi* are broadly ellipsoidal to cylindrical with a size of 3.5-4.5 x 2.0-3.1 µm (Humber, 1998) (Fig. 1).

B. bassiana, the white muscardine fungus was the first disease in animals shown to be caused by a fungus or any microorganism. Bassi de Lodi demonstrated the contagious and pathogenic nature of the fungus (Ainsworth, 1956). In his honour, Balsamo described and named the fungus, *Botrytis bassiana.* Vuillemin (1912) created the genus *Beauveria* and selected *bassiana* as the type species (Humber, 1998). Recent phylogenetic studies have demonstrated that the genus *Beauveria* (so far known to be only anamorphic fungus) is monophyletic within the *Cordycipitaceae* (*Hypocreales*), and has been linked developmentally and phylogenetically to *Cordyceps* species (Rehner, 2005; Rehner and Buckley, 2005; Rehner *et al*., 2011) (Fig. 2).

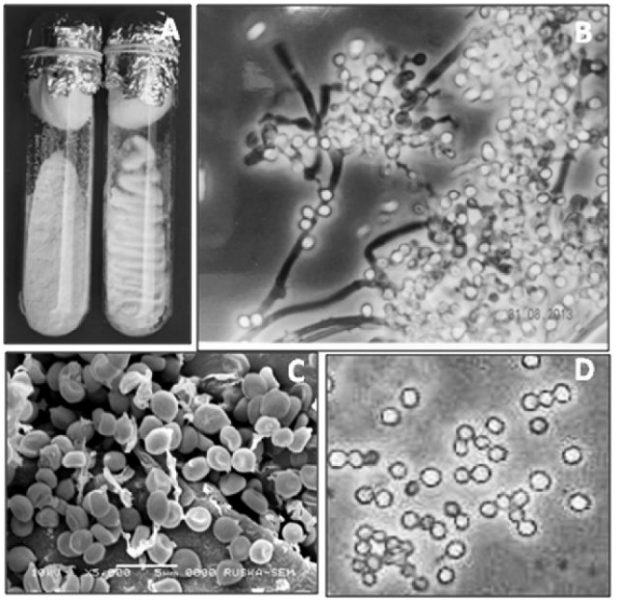

Fig. 2. Identification of Beauveria bassiana. A. Multiplication on PDA slope B & C. Conidiophore D. Conidia

HOST RANGE

Nomuraea rileyi is of cosmopolitan occurrence, primarily infecting Lepidoptera and particularly the economically important, polyphagous noctuid pests. The natural occurrence of the fungus reported on a variety of insect pests is summarized in Table 1.

Beauveriq bassiana is the causative agent of the white muscardine disease of many insect species (Tanada and Kaya, 1993), and has been extensively used for the control of many important pests of various crops around the world (Keller and Zimmermann, 1989; Varma and Morales, 1996). Insect pests susceptible to this promising pathogen both under natural ecosystem and experimental studies are presented in Table 2.

Table 1. Insect pests susceptible to *Nomuraea rileyi* under natural ecosystem and experimental studies

Insect Species	Food plant	Occurrence	Country	Reference
Achaea janata	Castor	Laboratory	India	Phadke and Rao, 1978 Kamat *et al.,* 1978
Acontia graellsii	Soybean	Natural	India	Gopalakrishnan and Narayanan, 1988
Acrocercops tenera	Forest	Natural	India	Sandhu *et al.,* 1993
Agrotis ipsilon, Amsacta moore *Hypocala rostrata,* *Lamprosema indicate, Mocis u* *Plusia orichalcea*	Various crops & Fore nurseries	Natural	India	Agarwal and Rajak, 1985
Anticarsia gemmatalis	Velvet bean, Soybean	Natural/ Laboratory	Brazil, Pureto Rico, US Ecuador	Stansly *et al*., 1990
Atteva fabriciella	Forest	Natural	India	Sandhu *et al*., 1993
Chrysodeixis acuta	Soybean	Natural	India	Singh and Singh, 1987
Diacrisia obliqua	Soybean	Natural	India	Singh and Gangrade, 1975
Eutectona machaeralis	Forest, Teak	Natural	*India*	Sandhu *et al.* 1993
Glypodes phyloalis	Mulberry	Natural	Japan	Kawakami *et al*., 1969
Helicoverpa armigera	Cotton, Tomato, Pigeonpea, Field bean Sorghum, Potato	Natural	India, Africa, Taiwan, Indonesia and Korea	Vimala Devi and Prasad, 1998; Gopalakrishanan and Nara 1988, 1989; Vandamme and Angelini, 1966; Tang and Ho Nurindah and Indrayani, 1989; Lu and Yin, 1998; Lingappa 2000; Manjula *et al*. 2003, 2004
Helicoverpa virescens	Cotton, Soybean	Natural	USA	Ignoffo, 1981
Helicoverpa zea	Alfalfa, Corn, Soybea	Natural	USA	Ignoffo, 1981
Hyblea puera	Forest, teak	Natural	India	Sandhu *et al*. 1993
Hypera punctata	Clover	Natural	USA	Ignoffo, 1981
Junonia orthyia	*Justicia gendarussa*	Natural		Razak *et al.,* 1991
Mythimna (Pseudaletia) unipun	Rice	Natural	USA, Japan, India	Ambethgar, 1991; Ambethgar and Kumaran, 1998
Ostrinia nubilalis	Corn	Natural	USA	Ignoffo, 1981
Ostrinia furnacalis	Maize	Natural	Japan, Venezuela	Agudelo and Silva, 1986
Panolis flammea	Pine	Natural	UK	Hicks and Watt, 2000
Panoquina sp.	Rice	Natural	Columbia	Vargas and Sachez, 1983
Pieries rapae	Cabbage	Natural	USA	Ignoffo, 1981
Plusia spp.	Soybean	Natural	Brazil	Ignoffo, 1981
Pseudaplusia includes	Cotton, Soybean	Natural	USA	Carner *et al.*1975
Spodoptera exigua	Soybean, Gram, Millet	Natural	India	Phadke *et al.,* 1978; Goh *et al.* 1992
Spodoptera exigua	Soybean	Natural	USA	Ignoffo, 1981
Spodoptera frugiperda	Maize	Natural	Argentina, Brazil, Colombia, USA, Venezuela	Ignoffo, 1981; Wheeler *et al*. 1989; Ríos-Velasco *et al*, 2010
Spodoptera litoralis	Cotton	Natural	Israel	Ignoffo, 1981
Spodoptera litura	Tobacco, Castor Grou Blackgram, Tomato	Natural / Laboratory	India, Japan	Rao and Phadke, 1977, Vimala Devi *et al.,* 1996; Asayama Ohoishi, 1980; Patil *et al.* 2003; Manjula et al.,2003, 2004; Shanthakumar *et al*, 2010
Trichoplusia ni	Cabbage, Soybean, C	Natural/ Laboratory	USA, Taiwan, Brazil, Indonesia	Ignoffo, 1981

Table 2. Insect pests susceptible to *Beauveria bassiana* under natural ecosystem and experimental studies

Insect Species	Food plant	Occurrence	Country	Reference
Agrotis segetum	Laboratory	Crucifers	UK	Butt *et al.*, 1994
Aphis craccivora	Laboratory	Groundnut	India	Kamala Jayanthi and Padmavadamma, 1996
Atteva fabriciella	Natural/ Laboratory	Teak, Softwood Trees	India	Ali and Varma, 1994
Chilo suppressalis	Natural/ Laboratory	Rice	India	Padmanaban, 1993
Cnaphalocrocis medinalis	Natural/ Laboratory	Rice	India	Padmanaban, 1993
Cosmopolites sordidus	Natural/ Laboratory	Banana	Kenya	Kaaya *et a*l, 1993
Dendrolimus pini	Natural	*Pinus silvestris*	Poland	Sierpinska, 1998
Dendrolimus punctatus	Natural	*Masson pine*	China	Ge *et al*., 2009
Dicladispa armigera	Natural/ Laboratory	Rice	India	Puzari And Hazarika, 1994
Diuraphis noxia	Natural/ Laboratory	Wheat	Ithaca	Vandenberg, 1996
Empoasca kerii	Natural/ Laboratory	Groundnut	India	Kamala Jayanthi and Padmavadamma, 1996
Eutectona machaeralis	Natural/ Laboratory	Teak	India	Ali And Varma, 1994
Fiorinia externa	Natural	*Tsuga canadensis*	USA	Marcelino *et al*., 2009
Helicoverpa armigera	Natural/ Laboratory	Chickpea, Pigeonpea	India	Gopalakrishnan And Narayanan, 1980
Hyblaea puera	Natural	*Tectona grandis*	India	Gowda and Naik, 2008
Hypothenemus hampei	Natural/ Laboratory	Coffee	Colombia	Varma and Morales, 1996
Leptinotarsa decemlineata	Natural /Laboratory	-	USA	Butt *et al.,* 1994
Leucoma salicis	Natural	*Populus nigra*	Poland	Ziemmnicka, 2008
Marasmia patnalis	Natural	Rice	India	Ambethgar, 1997
Marasmia ruralis	Natural	Rice	India	Ambethgar, 1997
Melanoplus saanguinipes	Natural/ Laboratory	Alfalfa, Wheat	Canada	Douglas *et al*., 1995
Melanitis ismene	Natural	Rice	India	Padmanaban, 1993
Nymphula depunctalis	Natural	Rice	India	Padmanaban, 1993
Ostrinia nubilalis	Natural	Corn	Iowa	Pingel and Lewis, 1996
Panolis flammea	Natural	*Pinus contorta*	Scotland	Hicks *et al*., 2001
Pelopidas methias	Natural	Rice	India	Padmanaban, 1993
Sesamia inference	Natural/ Laboratory	Rice	India	Padmanaban, 1993
Spodoptera exigua	Natural/	-	Iowa	Pingel and Lewis, 1996
	Laboratory			
Spodoptera litura	Natural/ Laboratory	-	Iowa	Pingel and Lewis, 1996
Syntypistis punctatella	Natural	*Fagus crenata*	Japan	Kamata, 2000
Tryporyza incertulas	Natural	Rice	India	Padmanaban, 1993

MODE OF INFECTION

Entomopathogenic fungi infect host insects through the cuticle unlike bacteria or viruses which infect after ingestion. The first step in the infection process is the adhesion of conidia to the insect cuticle which for most species is a non-specific event (Brobyn and Wilding 1977, Zacharak 1970, Boucias *et al* 1988). The conidia germinate on the insect cuticle by producing germ tubes which penetrate the body wall. This penetration is both mechanical (pressure exerted by germ tube) and enzymatic through the action of proteinase, lipase and chitinase on the cuticle. Growth of the fungus after it reaches the haemocoel is by budding which produces hyphal bodies (Fig. 3). The ability to convert to the yeast-like phase may be a prerequisite for pathogenicity (Srisukchayakul et al., 2005; Khan *et al*. 2012). These are transported throughout the haemocoel and give rise to localized concentration of mycelia. Only one report on insecticidal toxin production by *N. rileyi* exists (Ye *et al* 1993). *B. bassiana* produce toxins *viz*., Beauvericin, Bassianolide, Beauverolides, Tenellin and Bassianin which act as poisons for the insects (Ignoffo, 1981). Several studies have revealed a positive correlation between RH/rainfall with rate of fungal

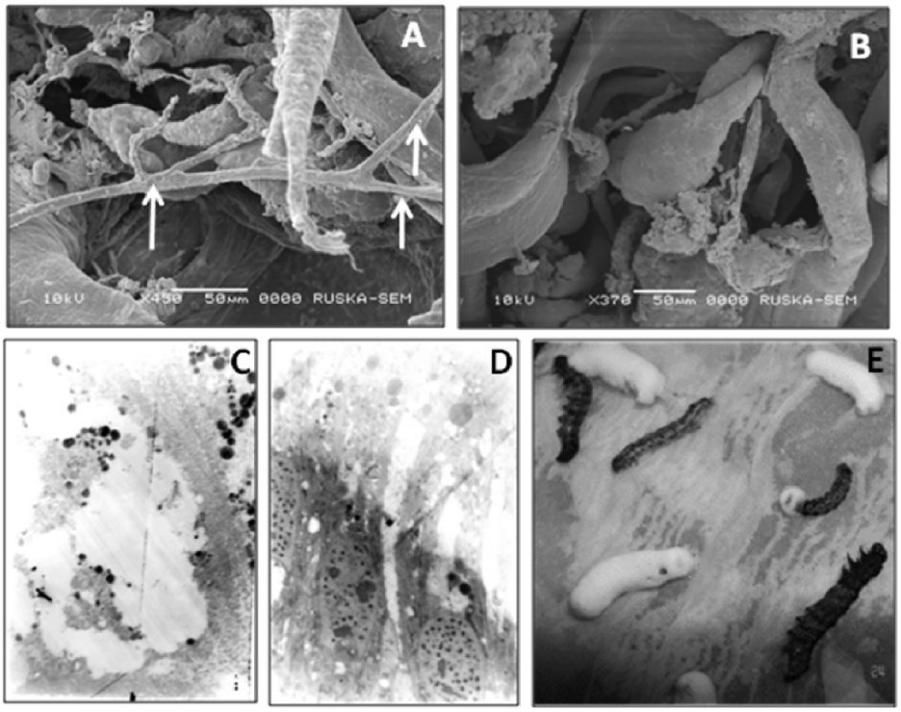

Fig. 4. Mode of action of combination formulation of Bt and B. bassiana. A. Scanning electron micrograph (SEM) of the inner surface of larval cuticle of combination formulation treated larvae 48h after treatment. Arrow indicates the mycelium of B. bassiana. B. SEM of inner surface of cuticle of larva treated with B. bassiana conidia 48h after treatment - no mycelial growth C. Heavy vacuolation and damage to cell organelles with complete loss of cellular details (Transmission electron micrograph of the midgut of combination formulation treated larvae) D. Intact columnar epithelial cells lining midgut of untreated larvae E. Dead larvae showing symptoms of Bt and fungal infection or both

infection thus indicating that fungal survival and spread are assured under higher rainfall and humidity conditions. Insects can also spread the fungus through mating (Long et al. 2000).

ISOLATION AND CULTURE MAINTENANCE

Details on initial handling and diagnosis of diseased insects have been reviewed (Butt and Goettel, 2000; Goettel and Inglis, 1997; Lacey and Brooks, 1997). *N. rileyi* is a fastidious fungus with specific growth and sporulation requirements limiting its prospects of mass production and commercialization. The standard medium used for isolation and culturing of *N. rileyi* is Saboraud's Maltose agar medium fortified with 1% yeast extract (SMAY). Optimum temperature for *in vitro* growth and sporulation is usually around 20-25^{0}C. Light has no effect on either growth or sporulation of *N. rileyi* on SMAY (Bell, 1975). However, Glare (1987) reported severe inhibition of *in vitro* sporulation of *N. rileyi* by darkness and concluded that light is essential to produce conidia on diseased cadavers of *Heliothis* sp. We routinely culture *N. rileyi* on SMAY slants incubated in darkness with apparently no effect on sporulation. Possibly, isolate differences exist. In general, cultures can be held for many months between subcultures on slopes at 4^{0}C. *N. rileyi* isolates stored under sterilized mineral oil at 3^{0}C showed no changes in viability or pathogenicity even after 6 months (Balardin and Loch 1988). Several attempts have been made to multiply *N. rileyi* using semi-synthetic media and solid substrates in order to cut down the cost of production (Holdom and Klashorst 1986, Silva and Loch 1987, Balardin and Loch 1989, Im *et al* 1988, Vimala Devi 1994). Median germination time on solid artificial media varies with isolates (Tigano-Milani *et al* 1995). Growth of *N. rileyi* and the cost of production have been studied on complex media (Bell *et al* 1982), on basal salts, and on media containing a number of inorganic and organic compounds including addition of cuticular extract. Growth and germination requirements of various *N. rileyi* pathotypes were affected by the presence of sterols, phospholipids, and some cuticular lipids of insects (Boucias and Pendland 1984). Holdom and Klashorst (1986) showed that *N. rileyi* could grow on an inexpensive culture medium made of brewer's yeast, yeast hydrolysate, skim milk powder, and whole milk powder with tap water and solidified with 1.5% agar. However, the results were not consistent. Various carbon sources such as soluble starch, corn starch, or malt extracts were additionally used with a protein base medium. *N. rileyi* was also multiplied on SMAY. In all cases SMAY was generally superior to all other media, giving rise to good yields of spores. In addition, both grain and bagasse were shown to support the fungus multiplication in a two-phase conidial production process. The fungus could be multiplied on

polished rice grains (Silva and Loch 1987), crushed sorghum (Vimala Devi 1994) and barley (Vimala Devi et al. 2001). Maltose released by the action of starch hydrolyzing enzymes secreted by the fungus induces sporulation (Preez *et al* 1985)

B. bassiana can grow in a variety of synthetic and defined media in solid, liquid and semisolid submerged culture conditions (Campbell *et al*, 1983 and Kaya et al., 1993; Gupta and Mukerjii, 2000; Soper and Ward 1981). Sabouraud's dextrose yeast agar (SDAY) containing 0.01-0.03% chloramphenicol to prevent bacterial contamination conditions (Goettel and Inglis 1997; Zayed, 2003) and water agar medium containing 15 g of purified grade agar mixed in 1 litre deionized water (Zoberi and Grace, 1990) have been used for isolation. Growth and sporulation of *B. bassiana* is optimum at 25±1°C, under a 16:8 h (L:D) photoperiod and 60±5% RH. *B. bassiana* could be cultured on crushed moist sorghum, rice, maize grains, wheat bran and used effectively against *Sylepta derogata*, *Nilaparvatha lugens*, *Helicoverpa armigera* and *Holotrichia consongunia* (Sharma *et al*., 1999, Vimala Devi and Hari, 2009).

MAINTENANCE AND PRESERVATION

For long-term preservation of entomopathogenic fungi without genetic change, it is recommended that cultures be stored under 10% glycerol in liquid nitrogen (-196^{0}C) (Couch, 1982; Samson, 1982). Slopes of *B. bassiana* and *N. rileyi* overlaid with sterile mineral oil can be held at 25^{0}C for 6-12 months without loss of viability. More details on isolation and storage of entomopathogenic fungi are given by several researchers (Goettel and Inglis 1997; Humber 1997, Kervin and Petersen, 1997; Morrow et al 1989 and Papierok and Hajeck, 1997).

EFFECTIVENESS AGAINST PESTS

Many attempts have been made to exploit *N. rileyi* and *B. bassiana* to control crop pests through classical and mycoinsecticide approaches (Upadhyay *et al*, 1997; Vimala Devi, 1995; Vimala Devi, 1998; Lingappa and Patil, 2002; Vimala Devi *et al*., 2002; Vimala Devi *et al*., 2003, Vimala Devi and Hari, 2009). The effectiveness of *N. rileyi* and *B. bassiana* against insect pests are discussed below:

Nomuraea rileyi

Field applications of *N. rileyi* have generally been as foliar sprays of conidia, soil application of conidia along with the solid substrate, dusting of dry formulations and field distribution of diseased cadavers. Laboratory and field effectiveness of *N. rileyi* against insect pests have been reported in various crop ecosystems (Vimala Devi, 1994, 1995, Vimaladevi and Prasad, 1996, Lingappa and Patil, 2002, Vimala Devi and Prasad, 2001, Sreenivas *et al*. 2006). Soil treatment of conidia along with the solid substrate or foliar spray of *N. rileyi* has shown promise for the control of *S. litura* in groundnut (Vimala Devi, 1995 and Patil, 2000), castor (Vimala Devi, 1994), cabbage (Gopalakrishnan and Mohan, 2000) and potato (Hegde, 2001). In soybean, *N. rileyi* @1.2 x 10^{12} conidia/l caused more than 50 per cent reduction in *S. litura* larvae and proved superior to SlNPV and Bt after 14 days of application (Kulkarni, 1999). The fungus was found effective against soybean caterpillar (Ignoffo *et al*., 1976), *H. virescens* (Ignoffo *et al*., 1978) and *Anticarsia gemmatalis* (Melo, 1990) in soybean ecosystem. Three applications of *N. rileyi* @ 1.6x10^{12} recorded significantly lower ear damage due to *H. zea* in maize (Mohamed *et al.,* 1982). The fungus @ 2x10^8conidia/l applied twice at 35 and 45 days after sowing resulted greater reduction in larval population of *Mythimna separata* in sorghum and yielded higher grain yield (Anonymous, 2001). An artificial infection of *T. ni* on cabbage @ 1.2 x 10^{12} conidia per 0.4 ha killed 67 per cent of the larvae (Getzin, 1961). Application of *N. rileyi* in cotton (@1.2x10^{12} conidia/l), pigeonpea (@10^8-10^{12} conidia/l), chickpea (@ 2x10^8 conidia/l), tomato (@3.2x10^8conidia/l), corn (10^8 conidia per ml) found effective against *H. armigera* (Kulkarni, 1999; Gundannavar, 2001; Hegde, 2001; Sreenivas *et al*. 2006; Anonymous, 1991; Tang and Hou, 2002).

Wettable Powder formulations of *N. rileyi* conidia along with bentonite and sucrose powder (1:7:7) recorded lower LC_{50} values of 168 conidia per larva of *S. litura* as compared to LC_{50} of 797 conidia per larva with fresh conidia (Wiwat, 2004). Oil based formulations recorded lower LC_{50} and LT_{50} values compared to WP formulations. Among oil formulations, safflower oil recorded lowest LC_{50} (1.42×10^4 conidia/ml) values followed by groundnut and sunflower oils (Ramegowda, 2005). The fungus as an oil in water emulsion spray has recorded 65.9 per cent mortality in post-rainy season groundnut and 62.8 per cent mortality in rainy season castor against *S. litura* coupled with 26.9 and 12.4 per cent parastization due to the larval parasitoid *Apanteles* spp. respectively (Vimaladevi *et al.*, 2002). *N. rileyi* in sunflower oil formulation along with Triton-X-100 showed 83.9 per cent mortality of 7 to 8 days old *S. litura* larvae. Oil based (diesel:sunflower oil 7:3) formulations of conidia of *N. rileyi* were found effective in controlling *H. armigera* (Nahar *et al.*, 2003). Oil formulation (sunflower oil + Tween-80 (0.02%), wettable powder (talc) and crude formulation of *N. rileyi* caused cumulative mortality of 95.0, 83.1 and 77.0 per cent in third instar larvae of *S. litura*, respectively (Nagaraja, 2005). The wettable powder formulations of *N. rileyi viz*.., bentonite + sucrose (7: 1) and talc + sucrose (7: 1) recorded 87.00 and 75.00 percent mortality in *S. litura*, 88.0 and 80.0 percent in *H. armigera* respectively. Tank mixes of *N. rileyi* conidia with sunflower oil and groundnut oil recorded 90.0 and 87.0 percent mortality in *S. litura*, 89.00 and 87.00 percent in *H. armigera*, respectively (Mallikarjuna, 2006). Seven weekly

applications of dust formulation of conidia @ 5.6 x 10^{13} per 0.4 ha gave significant control of *T. ni* in spring and fall cabbage (Bell, 1975). Oil formulation of *N. rileyi* was found effective against *H. armigera* in chickpea (Nagaraja, 2005; Mallikarjuna, 2006) and pigeonpea (Nahar *et al.,* 2004).

IPM modules tested for cotton bollworm management involving *Nomuraea rileyi* as a component (seed treatment with imidacloprid@10g/kg, intercropping with bhendi to attract shoot weevil and destruction of infested plants, monitoring of bollworm through pheromone traps @10 traps/ha, release of *T. chilonis* thrice at weekly intervals starting from 45 DAS @ 2.5 lakh/ha, three sprays of *N. rileyi* @ 2x10^8 conidia/l at 60-70, 70-80 and 90-100 days after sowing and need based application of insecticides after110 DAS) resulted in benefit cost ratio of 3.77 as against 3.92 in the adoptable IPM (Ramegowda, 2005). IPM module in tomato comprising of *N. rileyi* (2.0 x 10^{11} conidia/ha) registered incremental benefit of Rs. 11.55 to 11.83 and additional net profit of Rs. 19329 to Rs.20349/ha over untreated control (Karabhantanal *et al*., 2005).

Beauveria bassiana

Beauveria bassiana has been extensively studied as a common natural enemy causing important epizootics of various agricultural pests (Bonnie et al., 2010; Goettel et al., 2008; Milner, 1997; Li and Sheng, 2007; Khan *et al*, 2012). *B. bassiana* was found infecting nearly 95% of migratory alate aphids, especially *M. persicae* (Chen *et al*., 2008).

Bing and Lewis (1991) applied the fungus to maize whorls by foliar application of granular formulation of conidia and injection of a conidial suspension and found effective against European corn borer, *O. nubilalis*. Prasad *et al* (1989 and 1990) reported the susceptibility of *Spodoptera litura* and *H. armigera* to *B. bassiana* under laboratory conditions. *B. bassiana* @ 2.68x10^7 spores/ml against *H. armigera* reduced the pod damage (6.8%) compared to control (16.3%) and increased the yield in chickpea (Saxena and Ahmad, 1998). Burdeos and Villacarlos (1989) reported the susceptibility of adult sweet potato weevil, *C. formicarius* to *B. bassiana*. The cotton boll weevil, *Anthonomas grandis* was reported susceptible to *B. bassiana* with mortalities of 43.3, 46.7, 53.3 and 60.0% for spray suspensions of 10^5, 10^6 and 10^7 spores/ml respectively (Camargo *et al*., 1985). Gopalakrishnan and Narayanan (1990) reported that *B. bassiana* was pathogenic to all stages of *H. armigera* causing 60-100% mortality. *B. bassiana* (10^6 spores/ml) was found on par with monocrotophos 0.07% in controlling the rice hispa, *Dicladispa armigera* and leading to increase in yield (Hazarika and Puzari, 1997). A liquid and a wettable powder formulation of *B. bassiana* were effective against field population of *P. xylostella* on cauliflower (Sairabanu, 2000). The fungus was also found effective against banana weevil, *Cosmopolites sordidus* (Omukoko, 2010).

Wettable powder formulations of *B. bassiana* were found effective against *Plutella xylostella* (Sood *et al.* 2001, Vandenberg *et al.*, 1998), *Ostrinia furnacalii* (Zhang *et al.* 1992), whitefly (Ota *et al.* 1999) and *Plectodera scalator* (Forschler and Nordin, 1989). Oil palm kernel cake based formulations of conidia and conidial powder were found effective against adult banana weevils (Godonou *et al.*, 2000). *B. bassiana* maize flour formulated wettable powder @ 2×10^6 conidia per ha proved most effective in reducing the weevil population by 65.72 per cent (Nankinga and Moore, 2000). Wettable powder formulations containing pressmud as carrier caused higher mortality of *Holotrichia serrata* (Fab.) over formulation containing lignite as carrier (Easwaramoorthy *et al.,* 2003). Commercial W.P. formulations of *B. bassiana* (Mycotrol and Botanigard) were found effective against whitefly (Murphy *et al.* 1998, Orozco *et al.* 2000) while Biopower – a talc based formulation *was* found effective against diamondback moth, *P. xylostella* on cauliflower (Ramarethinam *et al.*, 2002).

Oil formulation of *B. bassiana* (10^9conidia/ml) caused 100 per cent mortality in larvae of *Rhipicephalus appendiarlatus* and *Amblyomma variegates* (Kaaya and Hassan, 2000). Coconut oil based formulation of *B. bassiana* proved effective against cocoa weevil adults *Pantorhytes plutus* by recording lower LD_{50} of 1.18x10^3 conidia per ml as compared to water + 0.01% Tween–80 which showed LD50 of 4.29x10^4 conidia per ml (Prior *et al.*, 1988). Corn oil formulation *B. bassiana* @ 1×10^8 conidia per ml against larvae of *Diabrotica speciosa* (Germal) (Coleoptera : Chrysomelidae) showed 65 per cent mortality (Consolo *et al.* 2003). Groundnut oil formulation of *B. bassiana* recorded 100 per cent mortality of adults of *Bemisia tabaci* which was followed by coconut oil (97.8%), sunflower (85.6%) and castor oil formulation (64.4%) (Manjula *et al.*, 2003). Emulsifiable suspension formulation of *B. bassiana* was found effective against whitefly on tomato (Ota *et al.* 1999). *B. bassiana* formulated as a Suspension Concentrate (SC) using mineral oil as carrier found effective against 5-day-old *H. armigera* larvae with LC_{50} value of 61.22 mg l^{-1} at 3 days after treatment. The formulation at 200 mg l^{-1} found effective against *H. armigera* on sunflower and there was no phytotoxic effect on the crop. No change in the consistency of the SC formulation was observed even at the end of 24-month storage period (Vimala Devi and Hari, 2009a).

SAFETY TO PARASITES, PREDATORS AND BENEFICIAL ORGANISMS

The spectrum of *N. rileyi* is primarily limited to Lepidoptera. Only two species of Coleoptera are

susceptible (Ignoffo, 1981). The fungus, *N. rileyi* is reportedly safe to several parasitoids and predators *viz*., *Chrysopa carnea, Apanteles* sp., *Campoletis* sp., *Telenomus proditor, Coccinella* sp. and *Microplitis croceipes* (Ignoffo 1981). The egg parasitod, *T. proditor* was not susceptible when exposed at rates ca. 25 x higher than that used in field experiments to induce epizootics (Ignoffo *et al* 1976, Phadke and Rao 1978). *N. rileyi* is safe to the predatory beetle *Hippodamia convergens* (James and Lighthart 1994). Extent of parasitization of *S. litura* (25-30%) by *Apanteles* sp. was similar in fungus sprayed and unsprayed castor and groundnut crops. Natural parasitization levels of larval parasitoids on *S. litura viz*., *Cotesia* sp. and *Apanteles* sp. were found similar in the N. *rileyi* treated and untreated control plot in groundnut (Vimala Devi *et al*., 2002). In field studies with *N. rileyi* on post-rainy groundnut crop in the coastal belt of Andhra Pradesh, the predatory beetle *Coccinella* sp. was found in abundance in fungus treated fields, a majority of parasites and predators belong to the Orders Hymenoptera, Diptera and Coleoptera (Vimala Devi and Prasad, 2000). Hence, most beneficial insects were not adversely affected by applications of *N. rileyi.*

Based on the natural occurrence of *B. bassiana* and the low toxicity profile demonstrated by ecotoxicology studies conducted with commercial formulation of *B. bassiana* (Botanigard®), the ecological risk due to exposure to this microorganism is expected to be minimal (US EPA, 2006). No adverse effects of *B. bassiana* was noticed on predators *viz*., carabidae (Hicks et al., 2001), *Amblyseius cucumeris* (Jacobson et al., 2001), *Coleomegilla maculate* (Pingel and Lewis, 1996). No significant impacts on parasitoids *Lysiphlebus testaceipe* and *Aphidius colmani* was noticed under field conditions (Murphy et al., 1999). No detectable effects due to *B. bassiana* were noticed on nontarget arthropods of predators, parasitoids and pollinators (Parker *et al*., 1997 and Ivie *et al*., 2002). Temperature, starvation and nutrition stresses significantly affected the susceptibility; nutrition stress caused the most increase in adult and larval mortality in *Chrysoperla carnea* (Donegan and Lighthart, 1989). Shipp *et al.* (2003) reported that the predatory mites release is not recommended during application of *B. bassiana*. Dietary and contact studies with commercial formulation of *B. bassiana* (Naturalis-L, Bio-Power) found no significant effect on *Apis mellifera* (Copping, 2004) and no effect on hatching rate of cocoons of earthworms, *Aporrectodea caliginosa* (Nuutinen et al., 1991). *B. bassiana* was able to infect bumblebees (*Bombus terrestris*) and it reported that there are no risks if the fungus is incorporated into soil or sprayed onto plants that are not attractive to bumblebees (Hokkanen *et al*., 2003). Suspension Concentrate formulation of *B. bassiana* was found safe to spiders, which are natural enemies of *H. armigera* in sunflower ecosystem (Vimala Devi and Hari, 2009a). Suspension Concentrate formulations of *B. bassiana* (employing groundnut and mineral oils) @ 2.5 x 10^{10} conidia L^{-1} (*i.e.,* 2.5 times higher than the dose effective against *H. armigera*) found safe to the eggparasitoid, *Trichogramma chilonis* (Vimala Devi and Hari, 2009b).

MASS PRODUCTION

The entomopathogenic fungus, *B. bassiana* is easy to grow on standard agar media and can be commercially produced as mycoinsecticides (Kaaya et al., 1993) while *N. rileyi* is a fastidious fungus with specific growth requirements (Vimala Devi, 1994). For small scale inoculum production where economics are not a primary concern, relatively expensive media such as saboraud dextrose agar and potato dextrose agar have been used successfully to induce sporulation (Ambethgar, 1991; Bullard *et al*., 1993). However, mass production of mycoinsecticide on a larger scale such as in pilot test studies and by industry, requires that the candidate mycoinsecticide be produced as cost-effectively as possible while the quality and quantity of the final product is retained (Gupta and Mukerjii, 2000; Ambethgar, 2001).

Submerged fermentation

In submerged or deep-tank fermentation the fungi are grown in a fully liquid system which has

the advantage of control over the process parameters such as temperature, pH, aeration and dispersion for efficient growth and yield of the infective propagules. The method is most suitable for scale-up. In submerged fermentation, blastospores are readily produced but lose viability relatively quickly during storage (Rombach 1989, Kleespies and Zimmerman 1992) as they are thin walled and unstable during the drying process after fermentation and have a very low field persistence. This has led to a spurt of interest in addressing the possibility of producing submerged conidia (Jenkins and Prior 1993 and Hegedus *et al* 1990) that are similar to aerial conidia in biological characteristics. However, it is believed that submerged conidiation is likely to depend on the nature of the isolate and particular physiological conditions used. Still submerged conidia are hydrophilic and not easily formulated in oils. Despite the advantages of submerged fermentation some fungi will not yield a satisfactory product by this technique. *N. rileyi* multiplication by submerged fermentation is not feasible since it does not sporulate in liquid medium.

Solid state fermentation

Nomuraea rileyi culturing on solid media is promising. Aerial conidia are produced on solid media and, in morphology and infectivity, are indistinguishable from those produced on the surface of insect cadavers as is the case with *Beauveria* and *Metarhizium* spp. An addition of 1% yeast extract to crushed sorghum was found to be an ideal substrate with a maximum yield of 1.4

x 10^9 conidia/g substrate after 8-9 days at 25^{0}C (Vimala Devi 1994). *N. rileyi* could be multiplied on polished rice grains (Silva and Loch 1987), agro wastes like refuse raw bananas and refuse potato chips (Thakre et al., 2011). Boiling the rice grains before sterilization resulted in higher spore yields. Cost-effective multiplication on crushed barley and also a semi-synthetic medium wherein maltose has been replaced with barley extract is also possible (Vimala Devi et al., 2001). Conidiation of *N. rileyi* occurs readily on semi-synthetic media in general. However, only a few isolates sporulate on cereal grains although mycelial growth occurs readily.

Diphasic production

Mass production of aerial conidia by a diphasic fermentation involves production of vegetative mycelia by liquid culture followed by transfer of the mycelia on a nutrient or inert carrier for surface conidiation (Soper and Ward 1981) and is commonly practised although labour-intensive. The method has been adopted for the production of *Beauveria bassaina* (Rombach *et al* 1988, Maniania 1993). *N. rileyi* produces blastospores in liquid cultures but not conidia. Blastospores were not infective against *H. zea* larvae (Bell, 1975). One feature of fungal growth in liquid media is the tendency of some isolates to grow as discrete mycelial pellets. Unfortunately these are not suitable for use as inoculum of a solid substrate as they do not permit even coverage.

On insects

Nomuraea rileyi can also be multiplied without much difficulty on 7-8 days old *S. litura* larvae on castor leaves treated with *N. rileyi* conidia. Larval death results in 6-7 days after exposure. Mycelial growth on mummified cadavers occurs within 24 h followed by sporulation 1-3 days later. Each larva yields about 2 - 4 x 10^{10} conidia (Vimala Devi and Prasad, 1997). Sporulating cadavers can be dispersed in the field to increase the inoculum before the peak incidence of the pest. The feasibility of multiplication needs to be explored.

The solid substrates commonly used for mass production of entomopathogenic fungi are various cereal grains (sorghum, rice, wheat, millets, maize, oats, barley) and agricultural byproducts (rice bran, rice husk, puffed rice waste, rice flakes, wheat bran, maize meal, maize cobs, soya flour, soya bean mash, groundnut hull meal, bengal gram husk, sugarcane bagasse, potato, carrot). The liquid media used for mass production of the entomopathogenic fungi are molasses yeast medium, carrot yeast medium, coconut waste water (Zimmermann, 1993; Puzari *et al*., 1998; Leite *et al*. 2005; Kassa *et al*. 2008; Sahayaraj *et al*. 2008; Machado *et al*. 2010; Kim *et al*. 2010; Sonai Rajan and Muthukrishnan, 2010). A solid-culture process was patented to mass-produce aerial conidia of *B. bassiana* (Bradley *et al*. 1992). An addition of 1% yeast extract to crushed sorghum was found to be an ideal substrate for culturing *N. rileyi* and yielded a maximum of 1.4x10^9 conidia/g after 8-9 days at 25 C (Vimala Devi, 1994). Cost effective and rapid multiplication of *N. rileyi* on semi-synthetic media with 2% barley extract and 1% soybean extract is reported by Vimala Devi *et al.* (2000) with spore yields in the semi-synthetic media comparable to or significantly higher than the standard medium.

FORMULATION

The type of formulation and selection of additives for a given formulation are critical to their stability. The basic components of most formulations include, in addition to the active ingredient (fungal spore), one or more of the following: a carrier, diluent, binder, dispersant, UV protectants and virulence-enhancing factors (Moore and Caudwell, 1997, Burges, 1998). The most widely used carriers are talc, kaolin etc. in wettable powders and oil in liquid formulations. Because of their hydrophobic and lipophilic nature, conidia of *B. bassiana* and *N. rileyi* readily suspend in oils. In formulation, priority is the retention of viability and virulence of the infective units during storage and application. Several extensive reviews have been published concerning the most important factor to be considered in formulating insect pathogens (Angus and Luthy, 1971; Soper and Ward, 1981). Biological and physical properties of the formulation must remain stable for at least one year, but preferably for more than 18 months for commercialization to take place (Couch and Ignoffo, 1981). The mycopathogen could be stored as dried mycelium or as blastospore or conidia. In terms of stability and virulence, conidial storage is preferable.

Information on formulations of *N. rileyi* is scanty. Formulating entomofungal pathogens in oils increases their effectiveness (Prior *et al* 1988) probably by preventing conidial dessication, increasing adhesion, spreading the inoculum over the host's body even into crevices, and possibly by interfering with the defensive nature of the cuticle. Conidia of *N. rileyi* are hydrophobic and lipophilic in nature. It is therefore desirable to develop oil formulations to improve shelf-life as well as to increase their field persistence. The fungus has been formulated as an oil-emulsion and a wettable powder and results from field tests are encouraging. *N. rileyi* conidia formulated as an oil-emulsion and applied as a high volume spray was superior in terms of higher kill (Vimala Devi *et al*., 2002). Wiwat (2004) evaluated water based, oil based and WP formulations of *N. rile*yi for their shelf life. Among water based formulations, 30 per cent glycerol, 20 per cent KCl + 10 per cent glycerol solution, 20 per cent KCl + 10 per cent sucrose solution were found superior as they recorded >8 weeks of shelf life with 80 per cent viability of conidia at 4ºC, whereas, at 30ºC they are able to be viable for less than one week period. After storage

period of 17 weeks, at 4ºC, the oils *viz.,* shellsol T, paraffin oil, castor oil were found to be superior by recording germination of conidia up to 72.20, 76.27 and 79.64 per cent, respectively. In case of WP formulations, aluminium silicate was able to maintain 80 per cent viability upto 43 weeks at 4ºC, wherein it is less than 1 week at 30ºC. Bentonite clay and its combination with sugars at the ratio of 7:1 were found effective by recording conidial germination of *N. rileyi* after 23 weeks of storage at 4ºC as they recorded more than 80 per cent of conidial germination. Mallikarjuna (2006) reported the higher conidial germination in wettable powder formulation bentonite + glucose (7:1) under ambient room temperature up to 180 days of storage. A granular formulation of the *N. rileyi* consisting 1mm particles of defatted corn germ containing 10^7conidia/g protected the conidia against UV radiation and killed 80% of *S. frugiperda* larvae (Pavone *et al*., 2009).

Studdert *et al.* (1990) coated dry *B. bassiana* conidia with a bentonite clay by mixing 1:3 by weight, moistening a thin layer tightly with a water mist and drying. The clay increased the half-life of conidia in both sandy loam and peat from 2-7 weeks to 7-12 weeks at 30°C and from 12-44 weeks to 20-64 weeks at 10°C, at different combinations of soil and moisture content. Fargues *et al.* (1983) prolonged survival of *B. bassiana* blastopores by clay coating. Hidalgo et al. (1998) reported the viability of the formulations of conidia of *B. bassiana* as fat pellets, dispersible powder (with talc) and oil suspension (in shell sol). Inglis *et al.* (1993) found that *Beauveria bassiana* conidia dispersed better in oil (Mycotech 9209) than in 0.05 per cent aqueous Tween-80, while substantial clumping occurred in a 5 per cent emulsion of the oil in water even after homogenization. An emulsifiable suspension formulation of *B. bassiana* showed better ability to withstand rain in comparison to wettable powder formulation (Kovach and English-Loeb, 1997). Wettable powder and emulsifiable suspension formulations of *B. bassiana* were found effective against *Plutella xylostella* (Vandenberg *et al*., 1998). The commercial formulation of *B. bassiana* "Mycotrol" was found stable for more than 12 months of storage at 25º C (Wraight *et al*., 2001). For some pest species, a baited formulation may be most effective (Bextine and Thorvilson 2002). Bentonite based liquid formulation of *B. bassiana* was observed to be most effective as determined by measuring *H. armigera* larval mortality as well as viability of fungal spores and ease of applicability (Ritu *et al*., 2012).

Commercial Formulations

Commercial products of *N. rileyi* (Ago-Biocontrol Nomuraea 50) were registered for control of Lepidoptera in ornamentals and vegetables in Columbia (Shah and Goettel, 1999; Iqtiat *et al*., 2009). Some *B. basssiana* mycoinsecticides produced commercially and experimentally are given below (Table 3) (Butt *et al*., 2001;

Table 3. *Beauveria basssiana* mycoinsecticides produced commercially and experimentally

Commercial Nam	Target pest	Manufacture
Boverin	Colorado potato beetle	Glavmikrobioprom/USSR
Biotrol	European corn borer	Nutrilite Products Inc./USA
FBB	Pine caterpillar	Nutrilite Products Inc./USA
ABG 6178	White grub	Abbott Laboratories /USA
Boverol	Colorado beetle	Fytovita, Czech Republic
BotaniGard ES; Botani-Gard 22WP	Whitefly, aphids, thrips, mealybugs Whitefly, aphids, thrips, psyllids, weevils, and mealybugs	Laverlam International (formerly Emerald BioAgriculture),
Mycotrol ES; Mycotrol-O	Whitefly, aphids, thrips, mealybugs, leathoppers, weevils, and leaf-feedir insects	Laverlam International (formerly Emerald BioAgriculture),
Naturalis	Aphids, spittle bug, mites, thrips, whitefly, aphids, caterpillars, mealybu fungus gnats, and shoreflies	Intrachem, Italy
Naturalis-L Andermatt Bi	Cotton pests including bollworms	Troy Biosciences USA Switzerland
CornGuard	European corn borer	mycotech, usa
Ostrinil	European Corn Borer, Asiatic corn borer	Arysta (formerly NPP, Calliope), France
Bio-Power	Mite and Coffee green bug	Stanes, India
BioGuard Rich	Coloptera, Hemiptera, Lepidoptera, Thysanoptera	Plantrich Chemicals & Biofertilizers Ltd.,India
Racer BB	*Helicoverpa*, leaf folder and other caterpiller pests	SOM Phytopharma; Agri Life, India
Proecol		Probioagro, Venezuela
Trichobass-L; Trichobass-	Coleoptera , Lepidoptera, Hemiptera, Thysanoptera and Acari	AMC Chemical/Trichodex, Spain
Conidia	Coleoptera (Curculionidae)	Hoechst Schering AgrEvo, Columbia

Wraight *et al*., 2001; Copping, 2004; Faria and Wraight, 2007; Kabaluk and Gazdik, 2005; Zimmermann, 2007; Khachatourians, 1986, Khan *et al*, 2012).

TOXINS PRODUCED BY ENTOMOPATHOGENIC FUNGI

Life cycles of entomopathogenic fungi are associated with the synthesis and secretion of several toxic metabolites including extracellular enzymes, proteins, and low molecular weight compounds such as toxins (Bandani, 2005). Diverse toxic metabolites have been described in several fungal biological control agents including *Beauveria, Metarhizium and Paecilomyces* (Vey *et al.,* 2001). Some of these metabolites have been found to display antibiotic, fungicidal or insecticidal properties against insect pests and diseases (Vey *et al*., 2001, Ross, 2005, Parine *et al*., 2010). Species of the genus *Beauveria* have been reported to produce the secondary metabolites bassianin, bassiacridin, beauvericin, bassianolide, beauverolides, tenellin, dipicolinic acid and oosporein (Strasser *et al.*, 2000; Vey *et al.*, 2001; Quesada-Moraga and Vey, 2004). Sixteen different mycotoxins have been analyzed of which the cyclodepsipeptidic mycotoxin, beauvericin produced by *B. bassiana* has been documented to be most effective for its larvicidal properties. Growth of the *B. bassiana* in the hemolymph of the host is associated with the secretion of metabolites, especially those originating from proteins (Bandani et al. 2000; Sowjanya Sree and Padmaja, 2008, Bandani 2005). These peptides, such as destruxins and efrapeptins, are indicated as secondary metabolites to differentiate them from the cuticle-degrading protease that favors the invasion of the pathogen. The secondary metabolites are considered to be important pathogenicity determinants (Bandani *et al*. 2000; Bandani 2005; Zibaee *et al*. 2009; Zibaee *et al*. 2011). Only one report on insecticidal toxin production by *N. rileyi* exists (Ye *et al* 1993). To know which toxins are produced and why they are toxic to the insects is important for a better understanding of the mode of action of entomopathogenic fungi at both the cellular and molecular level. It is also important to note that the discovery of a certain metabolite during liquid cultivation of a specific strain cannot be extrapolated to all strains of the species. There are no reports of metabolites entering the food chain or accumulating in the environment as a result of such natural or artificial epizootics or natural metabolite secretion (Vey *et al.*, 2001).

EFFICACY OF COMBINATION OF ENTOMOPATHOGENIC FUNGI AND ENTOMOPATHOGENS

Co-occurrence of two fungi in epizootics is rare though not impossible (Humber, pers. comm.; Aoki 1974; Uma Devi *et al*., 2003; Wang et al., 2002). However, very few studies have reported the combined application of two or more entomopathogenic fungi with other pathogens or fungal 'cocktails' with the aim of increasing efficacy (Lecuona & Alves 1988; Glare 1994; Inglis *et al.* 1997, 1999, 2001).

Inglis *et al.* (1997, 1999) tested the efficacy of simultaneous treatment of the grasshopper, *Melanoplus sanguinipes* with *B. bassiana* and *Metarhizium anisopliae* to determine if efficacy over different temperatures could be increased. Two co-formulated strains of *M. anisopliae* were assayed against the mustard beetle, *Phaedon cochleariae* (Leal-Bertioli *et al*. 2000). Manipulation of the concentration of the components in the combination concoctions resulted in synergistic effect in *Heliothis zea* (Fuxa, 1979). Inglis *et al*. (1997, 1999) also reported that there was only a marginal advantage (depending on temperature) in combination treatment of grasshoppers with *M. flavoviridae* and *B. bassiana*. Wang *et al*. (2002) and Leal-Bertioli *et al*. (2000) observed that in combination treatments, the dose of the effective isolate is diluted due to co-formulation and hence, synergistic effect is not possible; instead, the time to death of the insect may be extended. Combination treatment with *Beauveria bassiana* and *Nomuraea rileyi* on *Spodoptera litura* did not have a synergistic effect on insect mortality (Rao *et al*., 2005). In treatments involving two insect pathogens, temperature (Inglis *et al*. 1997, 1999), virulence of the pathogens (Wang *et al*. 2002; Leal-Bertioli *et al*., 2000) and time interval between the inoculation of the two pathogens (Malakar *et al*., 1999) were found to affect the infection process and the subsequent expression of either or both of the pathogens.

Synergistic effects have been reported in treatments involving a combination treatment with *B. bassiana* and *Bacillus thuringiensis* (Bt) on Colorado potato beetle (Lacey *et al*. 1999) and European corn borer (Lewis *et al*. 1996) and in combination treatments with multiple entomopathogens (Ferron 1978; Fuxa 1979). The combination formulation of two microbials with different modes of actions *viz*., Bt and *B. bassiana* can not only lower the dose requirement and improve the spread of kill but also can kill older larvae of *H. armigera* effectively (DOR, 2006, 2007, 2008, 2011, 2012, 2013). Mycelial growth and sporulation was observed from more than 75% of the cadavers. Scanning electron microscopy of combination formulation treated larvae of *H. armigera* revealed mycelial growth on inner surface of cuticle within 48h after treatment but absent in larvae treated with *B. bassiana* alone. Transmission electron microscopic studies of midgut of combination formulation treated larvae revealed complete vacuolation of the columnar epithelial cells within 3h after treatment while it took 12-24h in larvae treated with Bt alone and the studies confirm that the double pronged attack hastened the kill (DOR, 2008, 2009) (Fig. 4). The combination formulation prepared in mineral oil was found effective against 6 and 10 day-old larvae of *H. armigera* resulting in a cumulative mortality higher than the Bt or *B. bassiana* treated singly. No change in

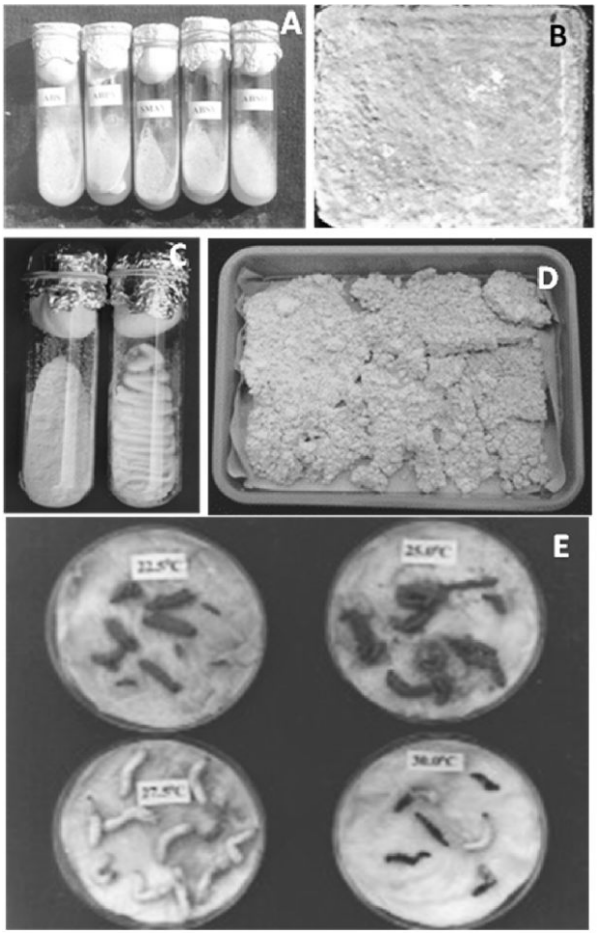

Fig. 3. Multiplication of Entomopathogenic Fungi. A. Multiplication of N. rileyi on synthetic medium B. Multiplication of N. rileyi on Solid Substrate C. Multiplication of B. bassiana on synthetic medium D. Multiplication of B. bassiana on Solid Substrate
E. Multiplication of the entomopathogenic fungi on insect

CFU of *B. bassiana*, heat viable spore count of Bt, contaminants and efficacy against *H. armigera* was observed in the combination formulation stored up to 24 months storage period in HDPE bottles at room temperature (DOR, 2009). Field testing of the combination formulation was found effective *H. armigera* on sunflower and on par with conventional insecticides at one week after spraying. High incidence of natural enemies and no phytotoxicity was observed in the combination formulation sprayed plots (DOR, 2013).

REGISTRATION AND QUALITY CONTROL OF FORMULATIONS

Rogolf (1982) has reviewed the development of guidelines for the registration of microbial pesticides in various countries. Betz *et al*. 1989 summarized the requirement under the US law for full as well as experimental use permit. The data requirements were Tier I. Toxicology Tests; Tier II. Environmental Fate; Tier III. Ecological effects and Tier IV. Simulated and/or actual terrestrial or aquatic field studies

The import, manufacture, sale, transport, distribution and use of microbial pesticides are regulated in India under the Insecticides Act, 1968 and rules framed there under (Vimala Devi, 2010; Vimala Devi et al., 2012).

Registration of microbial pesticides for commercial purposes has been made mandatory in India since 2006. It warrants data generation on toxicological data against mammals as well as eco-toxicity data non- targets such as (fishes, birds, earthworms, honey bees and silkworm). The data is to be generated with technical and formulation of every strain intended for commercialization. It is also mandatory to generate data on safety of the formulations to natural enemies along with data on bio-efficacy as well as data on phyto-toxicity to the crop. The guidelines have thus been framed so as to address the various concerns. For instance If the microbial pesticide under consideration is taxonomically similar to a clinically or agriculturally-significant micro-organism, its properties and effects should be examined in greater detail than suggested by the tests generally required.

Any microbial pesticide that needs to be registered for the first time in India can be provisionally registered under section 9(3b), wherein the required data need to be submitted except for long-term studies. A conditional registration will be granted under this special approval for even commercializing the product. A time span of two years will be given at the time of registration before which the entire data pending henceforth should be generated and submitted for permanent registration under section 9(3).

The data requirement for registration of entomopathogenic fungi for pest control has been approved by the CIB & RC as per the approval of the government in its notification No. 7-5/91-CR.II dtd. 7.10.91 of the Secretary, CIB&RC. Accordingly the manufacturer is required to submit the data for registration of entomopathogenic fungi under section 9(3B) and 9(3) of the Insecticide Act, 1968 in the following categories (CIB, 2011)

I. Standard of formulations: Colony Forming Unit (CFU) count on selective medium should be minimum of 1 x 10^8 per ml or gm for entomopathogenic fungi. Pathogenic contaminants such as gram negative bacteria *Salmonella*, *Shigella*, *Vibrio* and such other microbials should not be present. Other microbial contaminants should not exceed 1 x 10^4 count per ml or per g of formulation. Chemical/ botanical pesticide contaminants should not be present. Stability of CFU counts at 30°C and 65% RH should be provided.

II. Registration requirements: The data on Biological Characteristics and Chemistry (systematic name - genus, species and strain, common name, source of origin, natural

occurrence of the organism and morphological description, composition of the product, CFU/g of the product, percent content of the biocontrol organism in the formulation & nature of biomass, percentage of carrier/ filler, wetting/ dispending agent, stabilizers/ emulsifiers, contaminants/ impurities etc., moisture content, specification of the product, manufacturing process including type of fermentation and biological end products, pathogenicity test on insect, bioassay procedure, CFU on selective medium, pathogenic contaminants such as *Salmonella, Shigella, Vibrio* and such other microbials, other microbial contaminants, chemical and botanical pesticide contaminants, shelf life claims of not less than 6 months, data on storage stability as per shelf life claims and 100g sample for verification), Bio-efficacy (bio-effectiveness in field and laboratory tests and effect on non-target organism, natural parasites/ predators), Toxicity [toxicity data on mother culture *viz*., single dose oral - rat and mouse, single dose pulmonary, single dose dermal, single dose intra-peritoneal, human safety records; toxicity data on formulation *viz*., single dose oral- rat & mouse, single dose pulmonary, primary skin irritation, primary eye irritation and human safety records; mammalian toxicity testing viz., single dose oral (rat & mouse) - toxicity/infectivity/pathogenicity, single dose pulmonary-toxicity/infectivity/pathogenicity (intratracheal preferred), single dose dermal-infectivity, single dose intraperitoneal – infectivity, primary skin irritation, primary eye irritation, human safety records - effect/lack of effects are required for formulated product to be directly manufactured; environmental safety testing on non-target vertebrates and non-target invertebrates for formulation], Packaging & Labelling (details of manufacturing process/process of formulation, packaging and labels and leaflets)

The commercialization of biological control based on entomopathogenic fungi require proper control of the biological properties, physical and chemical made by the CIB & RC under Indian standards of entomopathogenic fungi specifications (CIB, 2011). Some microbiological tests recommended are (i) Concentration and germination of spores: Estimating the initial colony forming units (CFU) and storing the formulation in milky white polythene bags in the laboratory at room temperature for further studies on shelf life. Recording colony forming units (CFU) at an interval of 30 days up to 180 days. For counting CFU of in a formulation, 1g is added in 100 ml sterile water and mix thoroughly by using shaker, from this stock solution prepare 10^{-5}-10^{-6} serial dilutions for recording colony-forming units on SMAY medium and the colonies developed after 5 days of incubation at 25 ± 2^0C are considered for estimating the CFU per gram of formulation; (ii) Purity: It reveals the proportion of the biological agent in the formulation and identifies the microbial contaminants. Testing for human pathogens *E. coli*, *Vibrio* spp., *Salmonella* and *Shigella* spp. are required in order to improve the quality of production process and formulation of entomopathogenic fungi. Furthermore, it is necessary to make some physical tests according to the type of formulation (CIB, 2011).

CONCLUSION

With the ever-increasing awareness of the harmful effects of the synthetic chemical insecticides on man and his environment, the immediate need for sustainable, eco-friendly pest management has been felt very strongly providing an impetus to research and development of microbial insecticide. The National Farmer Policy 2007 has strongly recommended the promotion of microbial insecticides for increasing agricultural production, sustaining the health of farmers and environment. Entomopathogenic fungi are regular components of the natural agents that affect insect populations and known to produce rapid and spectacular epizootics when host densities are high. *N. rileyi* and *B. bassiana* are promising because of their wide spread occurrence, relative abundance and wide host range which includes all of the major lepidopteran pests. They have been successfully exploited for pest management. However improvement in viability of formulations for extended shelf-life and scale-up process for the fungi is the immediate need of the hour. In future, studies on genetic/molecular diversity of the fungal isolates, mode of action of the fungi at cellular and molecular level, molecular basis of toxicity, exploiting secondary metabolites of the fungi to develop natural insecticides, refinement of formulation and application technology, development of combination formulations of the fungi with other pathogens and use of nanotechnology for better delivery and persistence will enable us to employ these fungi more effectively as well as economically under varied agro-climatic conditions.

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