DEVELOPMENT OF BIOCONTROL AGENTS FOR THE CONTROL OF PESTS IN AGRICULTURE USING CHITIN METABOLISM AS TARGET

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DEVELOPMENT OF BIOCONTROL AGENTS FOR THE CONTROL OF PESTS IN AGRICULTURE USING CHITIN METABOLISM AS TARGET

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Affectionately dedicated to my beloved parents
and sisters

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Declaration

Certified that the work incorporated in the thesis entitled **"Development of biocontrol agents for the control of pests in agriculture using chitin metabolism as target"** submitted by Ms. Pallavi Balaram Nahar was carried out under my supervision. Such material as has been obtained from other resources has been acknowledged in the thesis.

(M. V. Deshpande)
Research guide

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National Chemical Laboratory
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List of abbreviations

µg	Microgram
µl	Microliter
β-ME	β- Mercaptoethanol
µmole	Micromole
GlcNAc	*N*-Acetylglucosamine
h	Hour
LM	Light microscope
min	Minute
Tris	Tris (hydroxymethyl) aminomethane
w/v	Weight by volume

Abstract

With every major outbreak of plant pathogen, the concept of plant pathogen has been revolutionized. Disaster in Ireland caused by fungal attack on potatoes in 1840s necessitated the development of control agents as one of the means for sustainable agriculture. While in 1960s, the insect pest on cotton crop in Mexico when became tolerant to organophosphate insecticides triggered the process of rethinking about the use of chemicals for pest control. Now the cost of global insect control has been raised by almost 40%.

The agricultural pests and pathogens known so far include 2,000 species of insects and 800 fungi. Among the insect pests *Helicoverpa armigera*, a lepidopteran insect causes more than 50% loss in yield of important crops such as cotton, pulses, vegetables and sunflower in India. In recent years, agricultural production of pulses repeatedly have suffered average yield loss of about 67% due to high levels of insecticide resistance in *H. armigera*. Additionally, legumes such as groundnut, cowpea, etc. are prone to attacks by soil-borne plant pathogenic fungi such as *Sclerotium rolfsii*, *Fusarium oxysporum*, *Rhizoctonia solani* and others. The development of resistance to chemical pesticides and negative impact of pesticides on the environment has prompted several researchers to evaluate alternative methods for the control of important agricultural pests. Thus, there is a need to develop biopesticides that are effective, biodegradable and do not leave any harmful effect on the environment.

Viruses, bacteria and fungi can act as biocontrol agents against insects and fungi. The viral and the bacterial control agents infect insects via their digestive tract while fungi make entry into the host through the cuticle that is the outermost covering in insect

and fungal cell wall. Insect-pathogenic fungi that act by contact and with no records of resistance developed so far could be a viable alternative. Louis Pasteur first recognized the potential of fungi for insect control. Over 400 species of fungi have been known to parasitize living insects. Most fungi that attack insects first make contact with the host in the form of conidia. Once the conidium attaches to the host, the fungus penetrates the insect cuticle with the help of hyphae produced from conidia. The fungal enzymes such as chitinases, proteases, lipases and others that weaken the cuticle accelerate the physical process of penetration. The cause of the insect's death is extensive fungal growth and production of different toxins in the haemolymph.

In case of the control of plant-pathogenic fungi, organisms such as *Trichoderma*, *Gliocladium* sp. are important and efficient mycoparasites that occur in soil worldwide. The involvement of extracellular metabolites produced by the mycoparasite in the process of parasitism is well documented. Cell wall lysis is a common feature of such interactions that may be mediated through the action of enzymes like chitinase, ß-1, 3 glucanase, and protease, popularly known as mycolytic enzyme complex.

The protective covers, that is, cuticle in insects and cell wall in fungi share common structural components. The insect cuticle is composed of wax, lipids, protein and chitin. The main component being chitin, its share is 25-50% of the dry weight of the insect cuticle. Fungal cell wall components are divided into two groups, such as skeletal and matrix components. Chitin and R-glucans are the main structural components while S-glucans and mannoproteins are the matrix components. Thus, insects and fungi share a key structural polymer, chitin, a β- 1,4-linked *N*-acetylglucosamine polymer. Therefore, it is advantageous to develop a common agent against both these groups. Furthermore

chitin is absent in plants and mammals, and thus its metabolism presents an attractive target for the control of plant pathogenic insects and fungi.

Chitin synthesis takes place when carbon and nitrogen metabolism pathways are brought together by the organism. The key enzyme involved in the final step of chitin synthesis is chitin synthase. Actually a number of enzymes contribute significantly to the synthesis of chitin, but only glucosamine phosphate synthase and chitin synthase are considered as prime targets for biocontrol agents. Chitin synthesizing systems characteristically include chitin-degrading enzymes. Chitin is hydrolyzed to its monomer by the synergistic and consecutive action of three types of enzymes: endo- and exo-chitinases and chitobiase. Thus, the dual pathogenecity of fungi towards plant pathogenic insects and fungi has been attributed to the various hydrolytic enzymes, such as chitinases, proteases, lipases, etc. which facilitate the entry of pathogen in their respective hosts. Chitin metabolism can be targeted at various stages:

a. Either by using the complex of cuticle degrading enzymes that is involved in the process of degradation of pre-formed chitin.
b. Or by inhibiting the chitin synthase enzyme and other enzymes of chitin metabolism.

The extensive literature review on targeting chitin metabolism as an attractive target with the use of biological preparations for the control of plant-pathogenic fungi and insects has been illustrated in the **Introduction** (CHAPTER 1). An extensive literature survey on important pests and pathogens of agriculture, importance of their control in terms of percent damage, importance of chemical and biological control strategies, disadvantages and limitations of chemical agents, the market status of the different chemical and biological control agents, current and alternative strategies to control plant

pathogenic fungi and insects, eco-friendly technologies, and importance of alternative biocontrol agents like fungi and cuticle/cell wall degrading enzymes along with the chitin metabolism inhibitors with special reference to the host-pathogen interaction, trans-sectorial issues, possibility of use of this eco-friendly package in organic farming and integrated pest management programs has been done.

The **Materials and Methods** (Chapter II) utilized in the course of this investigations include: conditions for growth and maintenance of entomopathogenic fungi: *Metarhizium anisopliae*, *Beauveria bassiana*, *Nomuraea rileyi*, chitinase producer: *Myrothecium verrucaria*, plant pathogens such as *Sclerotium rolfsii*, *Fusarium* sp. and chitosanase and inhibitor producing bacteria such as *Bacillus* sp., *Streptomyces* sp. and fungi such as *Chaetomium* sp, *Volutella* sp. Test organisms such as *Benjaminiella poitrasii*, *Aspergillus niger* etc. were used for the identification of cell wall synthesis inhibitors using the hyphal tip bursting test. The mass production of conidia was carried out by two-stage fermentation, conidia were harvested using mycoharvestor, production of various enzymes and enzyme inhibitors. Biochemical techniques include ultrafiltration, protein electrophoresis, enzyme assays. Analytical methods include Nuclear magnetic resonance (NMR), Infra red spectroscopy (IR), cell wall analysis, and saccharification studies. Field studies and laboratory bioassays include rearing of *H. armigera* under laboratory conditions, pot experiments and field experiments for the control of *S. rolfsii* and *H. armigera* respectively.

Use of mitosporic fungi such as *Metarhizium anisopliae* , *Beauveria bassiana* and *Nomuraea rileyi* for the control of *H. armigera* on pigeon pea and chickpea crops (Chapter III) was explored . More than 40 strains of entomopathogenic fungi,

belonging to genera *Metarhizium*, *Beauveria* and *Nomuraea* were isolated from soil samples using different isolation methods. Numbering of the isolates was done with respect to the field numbers, plot numbers, and sample numbers. Based on the *in vitro* production of cuticle degrading enzyme activities and insect bioassay against *H. armigera*, *M. anisopliae* M 34412 (>95% mortality, chitinase- 3.96 x 10^{-3}U/ml, protease- 3.38 U/ml and lipase- 0.996 U/ml), *B. bassiana* B 3301 (>95% mortality, chitinase- 6.21x 10^{-3} U/ml, protease- 0.995 U/ml and lipase- 0.332 U/ml) and *N. rileyi* N812 (70 % mortality, chitinase- not detected protease- 0.71 U/ml and lipase- 0.498 U/ml) were selected for the large scale production of conidia, formulation and field studies both on pigeon pea and chickpea . The results were compared with endosulfan and *Ha*NPV treatments.

M. anisopliae M34412 conidia in the oil formulation (7:3, diesel: Sunflower oil) were found to be most effective in controlling *H. armigera* on pigeon pea. The percent efficacies were, *M. anisopliae* 66.74%, *N. rileyi* 60.88% and *B. bassiana* 51.25% while endosulfan and *Ha*NPV showed 62.58% and 55.58% efficacy respectively.

The oil formulation of *M. anisopliae* M 34412 conidia was found to be most effective in controlling *H. armigera* on chickpea crop. The percent efficacy was 80.38%, the percent pod damage was 8.54% and the yield was 16.64 q/ ha for the *M. anisopliae* M 34412 treated plot as compared to endosulfan (71.44% efficacy, 10.8% pod damage and yield of 15 q/ha) and *Ha*NPV (70.15% efficacy, 12.37% pod damage and yield of 14.28 q/ha). Untreated plot had 26.73 % pod damage and yield of 9.05 q/ha.

Biochemical studies of chitin metabolizing enzymes of *Metarhizium anisopliae* (Chapter IV) with special reference to enzymes involved in pathogenesis was carried out

in (CHAPTER IV A). Biochemical characterization of cuticle degrading enzymes (CDE) such as chitinase, protease, and lipase, the key enzymes involved in the process of pathogenesis, has been carried out to understand the host-pathogen interaction. Apart from these three enzymes, **importance of extracellularly produced constitutive chitin deacetylase (CDA) and chitosanase by *M. anisopliae* in biocontrol of insect pest, *H. armigera* has been demonstrated for the first time** (CHAPTER IV B). The studies demonstrated that the chitin deacetylase activity was important in initiating the pathogenesis wherein the insect cuticle was softened by the action of CDA to aid mycelial penetration. In short, CDA either prepares a substrate for chitosanase and/or facilitates entry of fungal chitinase produced, if any, for further action.

Use of chitin metabolizing enzymes and chitin synthesis inhibitors, either singly or in combination to arrest the chitin metabolism in insects and fungi has been demonstrated and discussed in CHAPTER V. The extracellular culture filtrate of *M. verrucaria* that had high chitinase (1.8 ± 0.2 U/ml) and appreciable amounts of chitosanase, β-1,3-glucanase, mannanase (0.012± 0.003, 0.214 ± 0.03 and 0.019 ± 0.002 U/ml and alkaline protease (0.007 ± 0.002 U/ml) was used for testing the degradation of cuticular chitin isolated from *H. armigera* and also for the control of *S. rolfsii* on groundnut, either singly or in combination with the *Bacillus* sp. 102 culture filtrate. The reduction in weight of cuticle/larva and chitin content/larval cuticle was observed in cuticle degrading enzyme (CDE) preparation treated *H. armigera* larvae. With the increase in number of sprayings of CDE preparation, the loss in weight of cuticle/larva and chitin content/ larval cuticle became more pronounced suggesting the degradation of chitin in the cuticle due to CDE preparation. Fluorescence microscopy using FITC-WGA

allowed the localization of chitin in the cuticle of treated and control larvae. The sections of control larvae showed continuous band of cuticle with green fluorescence whereas the sections of treated larvae showed non-fluorescent patches in the cuticle. This indicated the alteration in chitin content of test larvae due to CDE preparation. These results confirmed the degradative action of CDE preparation on the insect cuticle that led to death of insects. A combined effect of mycolytic enzyme mixture and the cell wall chitin metabolism inhibitors to control the growth of a root-infecting fungus, *Sclerotium rolfsii* on peanut was also studied. The mixture of two *viz*. *M. verrucaria* and *Bacillus* sp. 102 culture filtrates, controlled 80 percent *S. rolfsii* infection of peanut seeds.

In **Conclusion** (Chapter VI), entomopathogenic fungi have been isolated and tested for the effective control of *H. armigera* on pigeon pea and chickpea crops under field conditions. Further, the role of chitin metabolizing enzymes like chitin deacetylase and chitosanase of *M. anisopliae* in initiating the process of fungus-insect interaction has been evaluated for the first time. The extracellular constitutive production of chitin deacetylase not only helps in softening the insect cuticle but also elicits defense mechanism on part of the fungus by converting its cell wall chitin into chitosan so as to evade the attack from insect chitinases. Pre-formed chitin in insects and fungi can directly be degraded by the mycolytic enzyme complex of *M. verrucaria*. This mycolytic enzyme complex when coupled with the cell wall synthesis inhibitor from *Bacillus* sp. 102 exhibited effective control of *S. rolfsii* as compared to using the enzyme complex or inhibitor alone. Thus, chitin metabolism was shown to be an attractive target for the control of agricultural pests like *S. rolfsii* and *H. armigera*.

Based on the efficacy of entomofungal isolates in the field, novel biocontrol strategies can be employed that comprise of either the cuticle degrading enzyme complex of *M. verrucaria* and inhibitors or cuticle degrading enzyme complex of *M. verrucaria* followed by the application of conidia of entomopathogenic fungi or the use of cuticle degrading enzyme complex of *M. verrucaria*, cell wall synthesis inhibitors and entomopathogenic fungi together for the effective control of pests in agriculture using chitin metabolism as the target.

CHAPTER I

INTRODUCTION

1.1 Origin

With the advent of agriculture, plant diseases became a problem. Thousands of insects, fungi, viruses, bacteria, nematodes and other living forms are potential hazards to agricultural crops. Fungal diseases like rust and smut in wheat were reported in the Bible as curses for disobedience of the commandments. The culprit behind the great Irish Famine of 1840s, was a fungus, which attacked potatoes and caused them to rot. The disease spread rapidly throughout the country. As a result, one million people died and two million people migrated to other countries. While in 1960s, the insect pest on cotton crop in Mexico when became tolerant to organophosphate insecticides, triggered the process of rethinking about use of chemicals for insect control.

Various parts of India, especially Andhra Pradesh faced the problem not only for cotton but also for pigeon pea and chickpea. Pest problems in cotton and pulses are linked to each other, since they usually are cultivated in that order. Frightened by a complete harvest loss in the cotton fields, farmers excessively used insecticides, thus aggravating the insecticide resistance of *H. armigera* in pulses even more. It was a black start for the year 1998 to the cotton growers in Andhra Pradesh, with ladde purugu (*Spodoptera litura*)- the pest that rocked the fertile cotton districts by destroying 370,000 hectares of crop, which led to 44 suicides. (News- Pestilence drives Andhra farmers to suicide, January 19, 1998).

While the year 2002 bought bad timings for the sugarcane farmers of Maharashtra, India. The woolly aphid (*Ceratovacuna lanigera*) infestation on sugarcane not only severely affected the yield but also posed health hazards to the farmers and cattle.

1.2 History

Most of the fights for survival started over millions of years ago in natural ecosystems. They were therefore, rather specific and less likely to produce undesirable side effects. In 1800, Erasmus Darwin, grandfather of Charles Darwin, in his book, *Philosophy of Agriculture and Gardening* hypothesized about the use of natural predators to minimize pests. However, credit for the first experimental proof for such a strategy goes to Italian scientist, Agostino Bassi. He demonstrated the parasitism of the fungus *Beauveria bassiana* on the silk worm *Bombyx mori*. Later other scientists proposed that microbes could be sprayed over infested fields. Louis Pasteur, was the first to use a fungus on grapevines in the vineyards to control the tiny root-inhabiting insects *Phylloxera*. In the mid 1800s, interest in biological pest control received a tremendous boost for the successful control of the cottony-cushion scale insect on citrus trees in California. Charles Valentine Riley of the US Agriculture Department imported the Australian lady bird beetle (*Rodolia cardinalis*) for the control of cottony-cushion scale insect. This novel approach to save the multimillion-dollar Californian citrus industry was the beginning of modern biological pest control (Deshpande, 1998).

1.3 Pests in agriculture

The agricultural pests and pathogens known so far include 2,000 species of insects and 800 fungi. The percent loss in yield worldwide is 5 - 40% for the crops like wheat, rice, millet, cotton, pulses etc. (Table 1.1).

Table 1.1 Loss in yield due to insect pests and fungal pathogens

Crop	Loss in yield	
	In India (%)	Worldwide (%)
Wheat	30.0	5.0
Rice	50.0	26.7
Millet/Sorghum	30.0	9.6
Cotton	>40%	11.0
Pulses	>35.0	30.0
Sugarcane	>40.0*	30.0
Vegetables	30.0	30.0

Major insect pests include:
Helicoverpa on pulses, cotton, vegetables, sunflower
Spodoptera on vegetables, sugarbeet
Pyrilla on wheat, rice, millet, sugarcane
*Woolly aphid (*Ceratovacuna lanigera*) on sugarcane

Major fungal pathogens include:
Verticillium dahliae Kleb, damages around 600 plants with major damage to vegetable crops
Phytopathogenic fungi such as *Sclerotium rolfsii*, *Fusarium oxysporum*, *Rhizoctonia solani* cause wilting in pulses, beans and vegetable crops
Colletotrichum falcatum causes red rot in sugarcane leaves
Pythium sp. causes damping off disease in cucumber, sugarbeet, tomato etc.
Magnaporthe grisiae causes rice blast

Among the insect pests *Helicoverpa armigera* , a lepidopteran insect causes more than 50% loss in yield of important crops such as cotton, pulses, vegetables and sunflower, in India alone (Table 1.1). In recent years, agricultural production of pulses repeatedly suffered average yield losses of 67%, due to high levels of insecticide resistance observed in *H. armigera* (Ranga Rao and Shanover, 1999). Additionally, legumes such as groundnut, cowpea, etc. are prone to fungal attacks by soil - borne plant

pathogenic fungi such as *Sclerotium rolfsii*, *Fusarium oxysporum*, *Rhizoctonia solani* and others.

Sugarcane, *Saccharum afficinarum* is one of the largely cultivated cash crops grown all over India. The major insect pests on sugarcane include *Tryporyza nivella* (Feb.), *Chilo infuscatellus* (Snellen), and *Pyrilla perpusilla* and the fungal pathogens include *Colletotrichum falcatum*, *Ustilago scitaminea*, *Acremonium teriicola* and others. In 1974, from north - east states like Assam, Uttar Pradesh, and Bengal, woolly aphid, *C. lanigera* (Homoptera: Aphididae) was reported as a secondary pest. While in 1995, it became primary pest on sugarcane. In July 2002 for the first time it was seen in Maharashtra as a major pest. Within 6-8 months time it covered almost 700-800 km distance. During 2003, in June itself, it started in almost all sugarcane growing districts of Maharashtra, covering more than 1.5 lakhs ha land. This pest is also serious in northern Karnataka, Andhra Pradesh, south of Gujrat and some part of UttarPradesh and Madhya Pradesh. Aphids cause damage to near about 25% plants on the earth. About 1350 species were recorded in North America and 500 species in England. Most of the species are monophagous and few of them are polyphagous. This pest was considered as a minor pest so no research work was carried out on this pest. However, now both the nymphal and adult stages cause damage to crop as a primary pest and additionally it secretes honeydew which is responsible for the growth of black colored saprophytic fungus *Capnodium* sp. which ultimately affects photosynthesis. Under favorable condition reproduction of the pest is fast and numerous aphids are produced in a short time. About 8000 nymphs are recorded on a single leaf.

1.4 Plant defense

Apart from the mechanical (synthesis of melanin, phenolics etc. that restricts the entry of pests and pathogens) and physiological barriers, plants synthesize a broad range of secondary metabolites, including alkaloids and terpenoids, which are toxic to herbivores and pathogens, and are so believed to act as defense compounds. Defensive chemicals have long been thought to be costly for the plants because of the resources consumed in their biosynthesis, their toxicity to the plant itself or the ecological consequences of their accumulation (Gershenzon, 1994; Purrington, 2000). One way for a plant to reduce these costs is to synthesize defense compounds only after initial damage by the pathogen. This strategy is obviously risky because the initial attack may be too rapid or too severe for such damage -induced defenses to be deployed effectively. Consequently, plants that are likely to suffer frequent or serious damage may be better off investing mainly in constitutive defense, where as plants that are attacked rarely may rely predominantly on induced defenses (Mckey, 1979).

All plant compounds that have negative effects on the growth, development and survival of another organism can be regarded as toxins. The mechanisms of action of some plant toxins are well known. For example, saponins disrupt cellular membranes (Osbourn , 1996), hydrogen cyanide released from cyanogenic glycosides inhibits cellular respiration (Jones *et al*, 2000) .

Recently, some compounds that are well known for their other functions in primary and secondary metabolism have also been found to be involved in plant defense (Green *et al*, 2001; Gronquist *et al*, 2001). Plant growth promoting rhizobacteria (PGPR) belonging to *Pseudomonas* sp. are being exploited commercially for plant protection to

induce systemic resistance against various pests and diseases. Mixtures of different PGPR strains have resulted in increased efficacy by inducing systemic resistance against several pathogens attacking the same crop. Seed treatment with PGPR causes cell wall structural modifications and biochemical/physiological changes leading to the synthesis of proteins and chemicals involved in plant defense mechanisms. Lipopolysaccharides, siderophores and salicyclic acid are the major determinants of PGPR - mediated induced systemic resistance. The performance of PGPR has been successful against certain pathogens, insects and nematode pests under field conditions (Ramamoorthy *et al*, 2001). Plants are not only able to synthesize individual defense metabolites with diverse chemical structures but also produce complex mixtures of defense compounds such as the terpenes of essential oils. Many of the individual components of essential oils are acutely toxic to insects (Isman , 2000) and pathogens (Cox *et al*, 2000). However, the toxicity of these compounds can be potentiated in mixtures, so that the activity of the mixture is higher than would be expected by adding up the activities of the its individual components. This phenomenon of synergism of two different mixtures has recently been demonstrated in larvae of lepidopteran insect, *Spodoptera litura* (Hummelbrunner and Isman ,2001). These mixtures were up to nine times more toxic than would have been expected from the effect of the individual constituents.

Various fungal /plant secondary metabolites have been used as inhibitors of infection-related morphogenesis in phytopathogenic fungi. Fungi that produce spores as an infective propagule are particularly vulnerable during the initial stages of disease cycle, i.e. between spore germination and host penetration. During these stages, the pathogens are reliant almost entirely on endogenous nutrient and energy reserves until

they infect the first host cell. Non-target effects can be reduced by screening for fungicides which do not inhibit vegetative hyphal growth but specifically interfere with developmental events involved in pathogenesis, such as spore germination, formation of penetration structures, or sporulation as indicated in Table 1.2 (Thines *et al*, 2004). Once the pathogen is inside the host cell, it is well shielded from externally applied chemicals and only a few systemic fungicides can eradicate post-penetration stages of infection cycle.

Table 1.2 Effect of various fungal/ plant secondary metabolites on infection-related morphogenesis in phytopathogenic fungi

Stage during penetration	Target pathogen	Secondary metabolite produced by the fungus /plant	Mode of action of secondary metabolite
Adhesion of fungal spore to host surface	*M. grisea*, *C. lindemuthianum*	Tunicamycin and cerulenin	Spores bind to the host surface with the glue that contains mainly glyco-proteins and lipids. This adhesion strength is reduced in presence of the protein glycosylation inhibitor, tunicamycin and lipid biosynthesis inhibitor, cerulenin.
Spore Germination	*Phyllosticta ampelicida, C. graminincola, F. solani*	Gloeosporone produced by *C. gloeosporoides*, Mycosporine-alanine produced by *C. graminicola* Cyclic AMP -dependent	These compounds are the potent self-inhibitors produced by the fungi that inhibit spore germination until the spores become

		protein kinase A inhibitors released by plants	dispersed, Flavonoids stimulate the germination of *F. solani* conidia on host plant , and this can be suppressed by use of protein kinase A inhibitors released by plants.
Appressorium formation	*M. grisea*	Alpha-factor mating pheromone of *Saccharomyces cerevisiae,*	Inhibition was mating type specific for *M. grisea*,
		Glisoprenins produced by *Clonostachys rosea*,	Inhibitors of appressorium formation
		4-Germacradiene-2,6, 12-triol, found in extracts of a basidiomycete *Resupinatus leightonii*	Inhibitor of cAMP-induced appressorium formation
Maturation of appressorium	*M. grisea* *Colletotrichum* sp.	Coumarin, a common secondary metabolite produced by plants ,	All are inhibitors of melanin biosynthesis which is an important step in the maturation of the appressorium
		Scytalol D produced by the fungus *Scytalidium,*	
		Cerulenin obtained from *Cephalosporium caerulens*	
Penetration	*M. grisea*	Melanin biosynthesis inhibitors	Failure in building up the required turgor pressure for the process of penetration

Apart from this, plants are known to produce enzymes such as chitinases that are responsible for the defense in plants against fungal pathogens. It has been observed that purified barley chitinases inhibit the growth of fungal hyphae (Leah *et al*,

1991). Heterologous chitinase gene expression is used in various plants to enhance their defense mechanisms against fungal pathogens (Schickler and Chet, 1997).

Ubiquitination is emerging as a common regulatory mechanism that controls a range of cellular processes in plants. Several putative ubiquitin ligases have been identified as defense regulators in plants that elicit protein-modification system in regulating plant defense against pathogens (Devoto *et al*, 2003). Plant protection delivered via the seed through germplasm modifications provides one of the simplest solutions to disease control (Hammond-Kosack and Parker, 2003). It also reduces the use of chemical fungicides thereby conserving energy, and provides growers with novel options for sustainable disease control. But, it is important to elucidate the molecular processes underlying pathogen adaptation to new plant germplasms or chemical applications.

The involvement of the enzyme in plant-pathogen interactions has been suggested for the chitin deacetylase (CDA) from *C. lindemuthianum*, considering the fungus is a plant pathogen, and the enzyme is extracellular and active on chitin oligomers. Taking into account that chitin oligomers elicit plant-defense mechanisms such as callose formation, lignification, and synthesis of coumarin derivatives (Barber *et al*, 1989; Kauss *et al*, 1989) whereas their deacetylated forms do not (Vander *et al*, 1998; Walker *et al*, 1984), it has been proposed that CDA might deacetylate chitin oligomers that arise from the fungal cell wall subsequent to the activity of plant chitinases , and thereby diminish their elicitor activity (Tsigos and Bouriotis, 1995). CDA is also known to assist in anti-plant defense reaction as demonstrated in the rust fungus-*Uromyces viciae -fabae* (Deising and Siegrist, 1995). (See Chapter IV B for more details)

Pathogens have developed a variety of mechanisms to circumvent plant toxins. Detoxification of plant toxins by pathogens such as plant-pathogenic fungi that are able to metabolize saponin of their hosts has been reported (Morrissey and Osbourn, 1999; Osbourn, 1996). Morrissey *et al*, (2000) have elucidated the role of detoxification enzymes of pathogenic isolates of the fungus *Stagonospora avenae* growing on *Avena sativa*. The antifungal 26-desglucoavenacosides released from the steroidal saponins avenacoside A and B upon pathogen attack of *Avena sativa* are sequentially hydrolyzed by three fungal enzymes, one - rhamnosidase and two β- glucosidases resulting in a strong reduction in antifungal activity (Morrissey *et al*, 2000).

Extensive research on the detoxification of plant compounds by insects has been carried out on the furanocoumarins of the Apiaceae and Rutaceae, which are metabolized by the cytochrome -P450-dependent monooxygenases in larvae of the lepidopteran genera *Papilio* and *Helicoverpa* (Cohen *et al*, 1992; Ma *et al*, 1994). The role of plant chitinases in disease resistance is well-documented (Graham and Sticklen, 1994). Numerous plant and microbial chitinase cDNAs have been cloned. Some of these have been introduced into plants under the control of constitutive promoters resulting in an enhancement of resistance of the host plant to fungal pathogens (Broglie *et al*, 1993; Lin *et al*, 1995, Vierheilig *et al*, 1993). However the role of endogenous chitinases from various sources in mediating plant resistance to insects is less well understood (Kramer and Muthukrishnan, 1997).

Chitin, an insoluble structural polysaccharide that occurs in the exoskeletal and gut linings of insects, is a metabolic target of selective pest control agents. One potential biocontrol agent is the insect molting enzyme, chitinase, which degrades chitin to low

molecular weight, soluble and insoluble oligosaccharides. Insect chitinase and its gene are now available for biopesticidal applications in the integrated pest management programs (Kramer and Muthukrishnan, 1997). Considerable interest in the chitinolytic enzymes has been stimulated by their possible involvement as defensive agents against chitin-containing proliferous and pathogenic organisms such as insects, nematodes, and fungi (Carr and Klessig, 1989; Linthorst, 1991; Sahai and Manocha, 1993). Resistance to undesired organisms can be imparted by degradation of their vital structures, such as the periotrophic membrane/ cuticle of insects, the cell wall of fungal pathogens or by liberation of substances that subsequently elicit other type of defensive responses by the host (Boller, 1987).

The exoskeleton of insects primarily composed of chitin and protein acts as a physicochemical barrier to environmental hazards and predators. However, some entomopathogenic fungi such as *Metarhizium anisopliae*, *Beauveria bassiana*, *Nomuraea rileyi* etc. have overcome these kinds of barriers by producing multiple extracellular degradative enzymes, including chitinolytic and proteolytic enzymes that help the pathogens to penetrate the barriers and expedite infection (EI-Sayed *et al*, 1989; St Leger *et al*, 1986 b; 1991 a, b).

1.5 Pest Control Strategies

1.5.1 Conventional pest control strategies

The control of plant diseases constitutes a broad, highly technical and rapidly developing field of study. The modern tendency for large farming units that are devoted to growing only a single species of crop increases the need for effective disease control as the spread of airborne diseases is encouraged by such uniformity. To counteract this, the

cultural practice is crop rotation. This involves selection of a crop that is not susceptible to the prevailing pathogen. Other measures are burning of infected debris and removal of weeds, which sometimes act as a second host.

1.5.2 Chemical based pest control strategies

Comparatively recent pest-control technology uses pesticides. Agricultural pesticides are organic or inorganic chemicals. The crop-wise consumption of the pesticides in India is illustrated in Table 1.3. Cotton accounts for the maximum pesticide share.

Table 1.3 The crop-wise consumption of pesticides in India

Crop	Pesticide share (%)	Cropped area (%)
Cotton	52-59	5
Rice/Paddy	17-18	24
Vegetables and fruits	13-14	3
Plantation crops	7-8	3
Cereals/Oilseeds/ Pulses	6-7	58
Sugarcane	2-3	2

Source: IARI data, 1999

As early as in 1878, P.M.A. Millardet formulated a spray mixture called Bordeaux that contained copper sulfate and powdered limestone. Initially this blue colored mixture was applied along the roadside edges of vineyards to discourage thievery of grapes.

Fortuitously, it also proved to be useful in controlling downy mildew of grapes (caused by a fungus, *Plasmopara viticola).*

The chemical fungicides are being used in the control of fungal diseases. Fungicides have been used against various diseases of cereals, principally seed-borne diseases. In the 1970s, fungicides were used on routine basis in cereal production. The first systemic fungicide widely used on cereals against foliar disease was ethirimol (Milstem), one of the hydroxypyrimidine group of fungicides and was used to control powdery mildew. This was used as a seed dressing agent and a spray. However, within a few years, mildew pathogen developed resistance to ethirimol.

The benzimidazole fungicides were widely used in the 1970s to control a number of cereal diseases. They controlled eyespot, and also had a good effect on fungi like *Septoria* and *Rhynchosporium* causing foliar diseases, which helped to increase green leaf longevity and hence yield. Because of their broad spectrum of activity against many foliar diseases and their low cost, they continued to be used in mixtures with other fungicide products. Eventually, resistance to benzimidazole fungicides appeared in *Septoria*, *Rhynchosporium*, *Fusarium* and other foliar pathogens and their use had declined.

The mid-seventies saw the introduction of the fungicide group, demethylation inhibitors, which contain the triazole fungicides, and have been the mainstay of cereal disease control. The first of these to appear was triadimefon (Bayleton) and there had been new triazoles appearing on regular basis upto the time of arrival of epoxiconazole (Opus). Triazoles were active against a wide variety of cereal foliar diseases and were used alone or in mixtures with non-systemic fungicides. Over the 25 years since

triadimefon was introduced, some of the triazoles had disappeared due to resistance developed against them.

A new group of fungicides, the STAR or strobilurin type fungicides were introduced in 1997 with azoxystrobin (Amistar) being the first available in Ireland. This was followed by kresoxim-methyl / epoxiconazole (Allegro) and trifloxystrobin (Twist) and famoxadone / flusilazole (Charisma), which is a non-strobilurin but has a strobilurin type of action (Dunne, 2002). Strobilurin types of fungicides were based on natural anti-fungal compounds, which some forest wood decaying mushrooms, secrete to inhibit competitor fungi. They have a novel mode of action and are very safe from an environmental of point of view. The strobilurin group of fungicides are broad-spectrum fungicides, active against a wide range of diseases in many crops. They are excellent inhibitors of spore germination. They all have the same mode of action, i.e. on entering the fungal cell they affect electron transport in mitochondria - which are the energy source in cells- depriving the organism of energy and thereby causing cell death. They largely remain in the plant parts and exert their fungicidal activities there.

The various strobilurins do differ in their systemic properties with some of the group being partially systemic and others redistributing themselves around the plant in the wax layer and epidermal cells by vapor action. They also have other desirable non-fungicidal physiological properties in that they can inhibit ethylene biosynthesis and consequently delay senescence and thus enhance yield. They can also improve the assimilation of nitrogen into plants. As the entire strobilurin group of fungicides has the same mode of action, then if resistance develops in a pathogen to one strobilurin product there will be cross-resistance to all other members of the group. This has already been

reported in case of wheat powdery mildew. Consequently, the use of strobilurins in disease control programs has to be managed to reduce the risk of resistance by other pathogens arising.

In 1873, O.Zeidler, synthesised 2,2 -bis (p-chlorophenyl)-1,1,1-tricholoroethane which enjoys global recognition as DDT (dichloro diphenyl trichloroethane). Of all the synthetic organic insecticides, DDT is the best known and the most widely discussed pesticide (Dhaliwal and Arora, 1994). In agriculture, DDT was used on more than three hundred different agricultural commodities as a general insecticide. Unfortunately, it affected the nervous system of animals. As a result, DDT, the favorite weapon of exterminators, had to be replaced by other synthetic organic insecticides.

During the 1930s, organophosphorous compounds like parathion and schradan were first developed as pesticides by Gerhard Schrader in Germany. Interest in them was generated because of their swift paralyzing action on insects. An added advantage was that the residues which remained in soil were non-toxic. This was a boon from an ecological point of view. Broad-spectrum organophosphorous insecticides, marketed under the trade name parathion are used for quick action. Schradan and its family members are used for selected purposes. Schradans kill sucking insects like aphids, whitefly, and leafhoppers, but caterpillars and beetles that are chewing insects can survive schradans. These chemicals are very toxic to mammals.

Synthetic pesticides became the major tool to control pests and weeds in both developed and developing countries (Flint and Van den Bosch, 1981; Van den Bosch, 1978; Whitten and Oakeshott, 1991). In the early 1950s, organochlorine and organophosphorous insecticides became dominant, starting with the use of DDT in

Switzerland as well as other important organochlorines such as lindane and dieldrin in the Unites States and the organophosphates malathion and parathion in Germany. Because of the high persistence of the organochlorine insecticides in the environment (Römbke and Moltmann, 1966) and their subsequent accumulation in the food chain, the use of DDT was banned in the USA and in Europe in 1973, followed by the ban of aldrin and dieldrin in 1975. However, dieldrin was still frequently used against locust swarms in Africa (Crick, 1990). Table 1.4 indicates the market status of the pesticides from 1990 to the expected market status until year 2005.

Table 1.4 Market status for pesticides

Production cost ($US)	Market status		
	1990	2000	2005*
Chemicals			
Insecticides 35-40	20 B	28 B	30 B
Fungicides (8-12 YR)			
Biopesticides 4-6M	61 M	380 M	3-5 B
(3-4 YR)			
BT	60 M	342 M	2-3 B
Nematodes		19 M	
NPV			
Mycopesticides	1M	19M	1-2 B
Predators			
Plant products			
Others			

***** Predicted figures considering 10-15% increase / year
Compiled from the literature

Organochlorine insecticides used in locust and grasshopper control were after the ban mainly replaced by less persistent but equally or even more toxic organophosphates such as malathion and fenitrothion. Fenitrothion was the most frequently used acridicide

(Greathead, 1992; Prior *et al*, 1992; Steedman, 1990). About 3 million hectares were treated against locusts (*Schistocerca gregaria*) in Africa and the Middle East (Van der Valk, 1999). While DDT and dieldrin allowed applications of small quantities because of their long persistence in the field, treatments with products such as organophosphates and pyrethroids had to be repeated several times and at higher dose rates, which made their environment soundness doubtful.

The indiscriminate use of chemicals created serious problems by causing health hazards. Blindness, asthma, cancer, skin disorders, enlargement of liver, neural malfunction and to some extent even psychological problems. Birds of prey and other vertebrates of higher trophic levels were found to be particularly vulnerable by showing a reduced reproductive success due to the accumulation of DDT, which, among others caused the thinning of eggshells. In some cases, secondary pests of crops became primary pests because their natural enemies were more severely affected than the target pests. Widespread knowledge of groundwater contamination, together with the fact that pesticides are among the most exclusively used synthetic chemicals in agriculture world-wide (Peterson and Highley, 1993), raised public awareness of and opposition against the uncontrolled use of pesticides. The pesticides posed environmental risks due to their toxic nature and the toxic degraded products that impart phytotoxicity, mammalian toxicity etc. Thus attention was diverted towards environment friendly pest control technologies.

1.6 Pest control strategies based on biological control agents

Given the rather depressing scenario of pesticide tolerance, the common man is justified in asking, are there means to displace chemical pesticides from the current agricultural practices? The answer, thankfully, is a reassuring 'Yes'. In 1962, Rachel Carson introduced the concept of biological control methods by providing a very

effective analogy. In *Silent Spring*, she says, " We stand now where two roads diverge. The road we have long been travelling is deceptively easy, a smooth superhighway on which we progress with great speed but its end lies disaster. The other fork of the road- the one "less traveled by" - offers our last, our only chance to reach a destination that assures the preservation of the earth". It is gratifying that from the nineties atleast, scientists are exploring multi-pronged approaches to pest control.

The biochemicals used as a pesticide are environmentally safe, selective, specific in their action, and easily biodegradable. The cost and time of production of biopesticides is low as compared to chemical based control measures, and they can be used in combination with other control measures in integrated pest management programs. They seldom have any effect on non-target organisms, mammals and plants.

The biocontrol agents are based either on plant parts / plant products, micro-organisms such as bacteria, viruses and fungi, fungal and bacterial enzymes, enzyme inhibitors, antibiotics that play an important role in control of plant-pathogenic fungi and insects.

1.6.1 Plants or plant parts for the control of plant diseases and insects

Higher plants are considered as the reservoir of useful products that can be used as biopesticides. Extracts of onion, garlic, eucalyptus, and tobacco are known to control many plant-pathogenic fungi and insects. Pyrethrum is one of the oldest insecticides known. Finely ground, dried flowers of chrysanthemum were marketed as insecticides, which have an active ingredient pyrethrin. Pyrethrins have a rapid paralytic action on insects. Certain essential plant oils, widely used as fragrances and flavors in the perfume and food industries, have been known to repel insects. Isman, (2000) reported the

importance of essential plant oils in the management of tobacco cutworm, *Spodoptera litura* and the green peach aphid, *Myzus persicae*.

Although, a number of plants produce metabolites which are active against insects, the ultimate natural arsenal seems to have been bestowed on the neem (*Azadirachta indica*) tree. For centuries, neem leaves and seeds have helped people to control insect pests. Amongst a host of constituents, a number of triterpenoids, flavonoids, amino acids and sulphur containing compounds have been isolated from various parts of tree. Azadirachtin, which has a disruptive effect on the feeding and growth of insects, is a key constituent of the neem seeds and leaves. A non-toxic insect repellent, thioremone, is produced in the seed kernels. It is suggested that seed extracts are highly effective against more than 100 species of crop pests, including the gypsy moth, Mexican bean bettle, confused flour beetle, citrus mealy bug, aphids, tobacco budworm etc. Neem formulations are mostly non-toxic and biodegradable. The added incentive for using neem seed cake for pest control is its nutritive value for crops. Amadioha , (2000) has demonstrated the potential of neem extracts *in vitro* and *in vivo* in the control of rice-blast fungus *Pyricularia oryzae*.

1.6.2 Importance of micro-organisms in control of plant-pathogenic fungi and insects

1.6.2.1 Bacteria

Introduction of beneficial microorganisms into soil or the rhizosphere has been proposed for biological control of soil-borne fungal crop diseases (Cook, 1993; Dunne *et al*, 1996; Keel and Defago, 1997). Bacteria like *Bacillus*, *Pseudomonas*, *Serratia* and *Arthrobacter* have been proved in controlling fungal diseases (Geoff, 2000; Handelsman and Stabb, 1996; Ordentlich *et al*, 1987,Weller *et al*, 1985). Bacterium such as

Pseudomonas fluorescens, which produces the antimicrobial polyketides was demonstrated to protect cucumber from several fungal pathogens, including *Phythium* sp. (Girlanda *et al*, 2001). Polyketides are the complex natural products that have the ability to treat many diseases. The *Bacillus* sp. is capable of controlling soil-borne fungal phytopathogens (Dileep Kumar, 1996; Kioka *et al*, 1998; Ryder *et al*, 1999) and stimulating plant growth (Podile and Dube, 1988; Utkhede and Smith, 1992). Deka Boruah and Dileep Kumar (2003) have demonstrated the control of wilt disease caused by *Rhizoctonia solani* in brinjal by seed coating using *Bacillus* sp. This *Bacillus* sp. also exhibited *in vitro* antagonism against other fungal plant pathogens such as *Fusarium solani*, *F. semitectum* and others. Saleem and Kandasamy, (2002) reported the antagonism of *Bacillus* sp. towards *Curvularia lunata*, causative agent of grain mold and leaf spot diseases in dry land crops like sorghum. Also, mixtures such as a combination of *Pseudomonas* strains are effective in siderophore-mediated competition for iron and induction of systemic plant resistance to improve control of *Fusarium* wilt of radish (De Boer *et al*, 2003).

The most popular bacterial pesticide is BT, a mixture of *Bacillus thuringiensis* spores and its toxin. As a pesticide, *B*. *thuringiensis* accounts for over 90 percent of total share of today's bioinsecticide market and has been used as biopesticide for several decades. The sporulating cells of *B*. *thuringiensis* contain insecticidal crystal protein, that is toxic to a variety of insect species (Baum *et al*, 1999) and which accounts for the commercial value of BT as a biopesticide. In most countries of the world, products are available for control of caterpillars (var. *kurstaki*, *entomocidus*, *galleriae* and *aizawai*), mosquito and blackfly larvae (var. *israeliensis*) and beetle larvae (var. *tenebrionis*)

(Milner, 1994). Actively growing cells lack the crystalline inclusions and thus are not toxic to insects. The BT preparations remain stable over years of storage and even in the presence of UV rays of the sun. The major drawback of BT, however, is that the insecticidal crystal protein acts only after ingestion by the insect. As the insect feeds on the foliage, the crystals too are eaten up. These are hydrolysed in the insect's midgut to produce an active endotoxin. The active toxin binds to receptor sites on gut epithelial cells and creates imbalance in the ionic make-up of the cell. This is seen by swelling and bursting of the cells due to osmotic shock. Subsequent symptoms are paralysis of the insect's mouthparts and gut. So obviously the feeding process is inhibited. However repeated exposure of an insect population to *B. thuringiensis* induces the emergence of resistant pests. The number of toxin genes, the qualitative and quantitative differences among them and the properties of the resulting toxin, affect the quality of the strains developed. The range of commercial products includes Thuricide, Dipel, Bug Time, Bathurin etc.

1.6.2.2 Viruses

There are more than 1600 different viruses which infect 1100 species of insects and mites. A special group of viruses, called baculovirus, to which about 100 insect species are susceptible, accounts for more than 10 percent of all insect pathogenic viruses (Deshpande, 1998). Baculoviruses are rod-shaped particles which contain DNA. Most viruses are enclosed in a protein coat to make up a virus inclusion body. Alkaline condition of insect's midgut dissolves the protein covering and the viral particles are released from the inclusion body. These particles fuse with the midgut epithelial cells, multiply rapidly and eventually kill the host. But, viral pesticides are more expensive

than chemical agents. Furthermore, many baculoviruses are host specific. Therefore they cannot be used to control several different pests. The action of baculoviruses on insect larvae is too slow to satisfy farmers. These viral preparations are not stable under the ultraviolet rays of the sun. Efforts are being made to encapsulate baculoviruses with UV protectants to ensure a longer field-life.

Insect-specific viruses can be highly effective natural controls of several caterpillar pests. Different strains of naturally occurring nuclear polyhedrosis virus (NPV) and granulosis virus are present at low levels in many insect populations. Epizootics can occasionally devastate populations of some pests, especially when insect numbers are high. Insect viruses need to be eaten by an insect to cause infection but may also spread from insect to insect during mating or egg laying. No threat to humans or wildlife is posed by insect viruses. The successful commercialization of insect-pathogenic viruses has been limited. Thus far, NPV strains have only been mass-produced in living insects, which is a costly procedure. Viral insecticide development is further hindered by the fact that the viruses are specific to one species or genus, ensuring a relatively small market. Naturally occurring viruses may affect many caterpillar pests. NPV affects alfalfa looper, corn earworm, imported cabbageworm, cabbage looper, cotton bollworm, cotton leafworm, tobacco budworm, armyworms, European corn borer, almond moth, spruce budworm, Douglas fir tussock moth, pine sawfly and gypsy moth. Preparations of granulosis virus have been isolated from several caterpillar species, including imported cabbageworm, cabbage looper, armyworm, fall webworm, and mosquitoes, among many others. Viruses invade an insect's body via the gut. They replicate in many tissues and can disrupt components of an insect's physiology,

interfering with feeding, egg laying, and movement. Different viruses cause different symptoms. NPV-infected larvae may initially turn white and granular or very dark. Some may climb to the top of the crop canopy, stop feeding, become limp, and hang from the upper leaves or stems, hence the common name "caterpillar wilt" or "tree top" disease. Victims of a granulosis virus may turn milky white and stop feeding. In both cases, the body contents of the dead larvae are liquefied and the cuticle ruptures easily to release infectious viral particles. Death from a virus infection usually occurs within 3-8 days. A naturally occurring viral epizootic can seriously deplete a pest population. Transmission of the virus through the population may take days or weeks but, if conditions are suitable, the entire population may eventually collapse (Hoffmann and Frodsham, 1993).

1.6.2.3 Fungi

Apart from viruses and bacteria, fungi also affect insects. Two Russian scientists E. Metchnikoff and J. Krassilstchik used *Metarhizium* against two species of beetles, the wheat chafer and sugarbeet carculio respectively. Shortly afterwards, in USA, F. Snow made an attempt to control chinch bug using fungi (Deshpande, 1998). Over 400 different species of fungi have been shown to parasitize insects. For instance, *M. anisopliae* can parasitize as many as 200 different insects.

The viral and the bacterial control agents infect insects via their digestive tract while fungi make entry into the host through the cuticle, which is the outermost covering in insects and fungal cell wall. The pathogenecity of fungi towards insects has been mainly attributed to the various hydrolytic enzymes, such as chitinases, proteases, lipases, etc.

1.6.2.3 A Mycoparasitism

A wide spectrum of fungi is known to show antagonism among them (Table 1.5).

Table 1.5 Examples of mycoparasitic fungi reported in India

Fungus	Host	Disease/ Crop and remarks
Fusarium *F. oxysporum*, *F. semitectum*, *F. udum*	*R. solani*, *Fusarium* sp.	Wilt of radish and banana, Seedling blights of wheat and maize
Myrothecium *M. verrucaria*	*Cochliobolus sativus*	Seed rot, seedling blights, and root rot in cereals
Gliocladium *G. virens*	*Pythium ultimum*, *R. solani*	Damping-off disease of cotton, groundnut wilt
Piptocephalis *P. virginiana*	*Choanephora cucurbitarum*	Fruit rot, leaf blight of okra, squash, pumpkin, beans, cucumber, etc.
Pythium *P. nunn*, *P. oligandrum*, *P. acanthophoron*	*F. solani*, *Pythium myriotylum*, *P. ultimum*, *Sclerotinia sclerotiorum*	Browning root rot of cereals, rhizome rot of ginger, damping -off of cucumber, sugar beet and cress
Rhizopus *R. nigricans*	*F. oxysporum var. lycopersici*	Tomato wilt
Trichoderma *T. harzianum*, *T. viride* are most well studied	*R. solani*, *Sclerotium rolfsii*, *F. solani*, *F. culmorum*, *P. ultimum*,	Potato wilt, Damping-off of cucumber
Tremella *T. mesenterica* *T. encephala*	*Peniophora laeta* *P. incarnata* *Phlebia radiata*	Occurs on hardwoods with basidiomycetes and competes with them for nutrients
Pythium paroecandrum	*Botrytis cinerea*	Botrytis blight in cucumber, grapes, etc.

Source: Modified from Whipps and Lumsden, (2000)

In 1932, Weindling first time noted the potential of *Trichoderma* and *Gliocladium* in controlling plant-pathogenic fungi. It was due to the toxic metabolites secreted by these two fungi. Initially parasitic fungus locates the host mycelium or spores, using chemical signals originating from the host fungus. *Trichoderma,* for instance, attaches to the host hyphae by coiling, hooks or appressorium-like bodies and penetrates the host by secreting cell wall lytic enzymes. On the other hand, fungi like *Sporidesmium sclerotivorum* produces haustoria in the host *Sclerotinia minor* and triggers autolysis. The involvement of extracellular metabolites produced by the mycoparasite in the process of parasitism is well documented (Jeffries, 1997). The cell wall lysis is a common feature of such interactions that may be mediated through the action of enzymes like chitinase, ß-1, 3 glucanase, and protease, popularly known as mycolytic enzyme complex.

The infective propagules produced by mycoparasitic fungi mainly include conidia, chlamydospores, oospores. Different mycoparasitic *T. harzianum* strains develop chlamydospores in submerged fermentation while conidia on solid substrates. Chlamydospores of *G. virens* were produced in stirred tank fermentor and the different fermentation stages were used to make the effective biocontrol formulation. While sexually produced spores of *Pythium oligandrum*, oospores were used for the seed treatment of sugarbeet, cress and carrot to control damping-off diseases (McQuilken *et al*, 1990).

Myrothecium verrucaria, can parasitize hyphae of *Cochliobolus sativus* (Campbell, 1956), and this high chitinase- producing fungus, was also successfully used for the control of *Drechslera teres* infection of barley leaves by the seed treatment (Mostafa,

1995). Biocontrol of cocoa pod diseases with mycoparasite mixtures has been suggested by Krauss and Soberanis, (2001). Cocoa pod diseases include: moniliasis caused by *Moniliophthora roreri*; witche's broom caused by *Crinipellis perniciosa* and black pod caused by *Phytophthora palmivora*. Five mycoparasitic strains of *Clonostachys rosea* and three of *Trichoderma* sp. were compared singly and in combination for their potential to control cocoa pod diseases. Control of moniliasis and yield were positively co-related to the number of mycoparasites in the inoculum.

1.6.2.3 B Entomopathogenesis

Recognition that fungi have potential for biological control dates back to Louis Pasteur. During 1880s *Metarhizium* species was used to control wheat chafer, *Anisoplia austriaca* and the sugarbeet curculio, *Cleonis puncitiventris*. The genera such as *Metarhizium*, *Beauveria*, *Nomuraea*, *Verticillium*, *Entomophthora*, and *Neozygites* are commonly encountered in nature. In most of the cases, spores are the means for the spreading of infection in an insect population. Pre-infection requirements are adhesion and germination of spores to form germ tubes or appressoria.

Once the fungus breaks through the cuticle and underlying epidermis, it may grow profusely in the blood, in which case death is probably the result of starvation or physiological/biochemical disruption brought about by the fungus. Alternatively, insecticidal secondary metabolites may contribute to the demise of the insect. The life cycle is completed when the fungus sporulates on the cadaver of the host. Under the right conditions, particularly, high relative humidity, the fungus will break out through the body wall of the insect, producing aerial spores. This may allow horizontal or vertical transmission of the disease within the insect population. The common insect pathogenic fungi reported in India are listed in Table 1.6.

Table 1.6 Examples of entomopathogenic fungi reported in India

Fungus	Host	Crop	Remarks
Beauveria	Spider, beetles, brown plant hoppers	Sugarcane, rice, cardamom, cabbage	*B. bassiana,* Commercial use
Cephalo-sporium	Coffee green bug, brinjal , leaf beetle	Coffee, brinjal vegetables	
Conidiobolus	Aphids and diverse insects		*C. coronatus,* weak pathogen
Entomophthora	Aphids ,chest - nut beetle, coffee green bug	Alfa alfa, chest-nut, cruciferous vegetables, wheat, coffee	*E. muscae, E. grylli* potential for commercialization
Hirsutella	*Pyrilla* , mango leaf hopper, mites	Sugarcane, coffee, mango, coconut	Effectively used in Kerela for control of coconut mites
Isaria	*Pyrilla*	Sugarcane	Distinct erect sporophore
Metarhizium	*Pyrilla*, coconut beetle, Brinjal mealy bug, *H. armigera*	Sugarcane, coconut, vegetables Pulses	*M. anisopliae* and *M. flavoviride* commercial use
Neozygites	Mite, thrips, mealy bugs *Helicoverpa,*	Vegetables Pulses	Important for commercial exploitation
Nomuraea *Paecilomyces*	*Spodoptera* White fly	Sugar beet Fruit plants	
Verticillium	Leaf hopper, banana aphid, rust fungi	Mango, Banana, vegetables	*V. lecanii* commercially important

Source: Modified from Butt *et al*, (2000)

Recently, entomopathogenic fungi such as *Metarhizium, Beauveria, Nomuraea, Verticillium,* and *Paecilomyces* have been studied on a large scale in the field of pest control. Epizootics caused by *N. rileyi* and *B. bassiana* have been observed on boll worms and *Spodoptera litura* in South Indian fields since last 15 years (Uma devi *et al*, 2003) as a result the insect population is highly susceptible to these fungi. Quesada-moraga and Vey, (2003) have showed the variation of *B. bassiana* isolates in the control of *Locusta migratoria*, and this variation was mainly due to the variation in their virulence and their ability to produce in vitro toxic metabolites.

M. anisopliae is being used in Australia as a bio-pesticide for control of sugarcane whitegrubs in soil (Milner *et al*, 2003). *B. bassiana* and *M. anisopliae* have also been tested against the spotted stalk borer , Chilo partellus (Lepidoptera: Pyralidae) (Tefera and Pringle, 2003). Tounou *et al*, (2003) demonstrated the efficay of *M. anisopliae* strain Ma43 and *Paecilomyces fumosoroseus* strain Pfr 12 against adults of green leaf hopper *Empoasca decipiens* (Homoptera : Cicadellidae).The pathogenecity of *M. anisopliae* and *B. bassiana* was also demonstrated against African Fruit fly puparia and adults (Ekesi *et al*, 2002). Infection of the coffee berry borer, *Hypothenemus hampei* (Coleoptera :Scolytidae) by Brazilian isolates of the entomopathogenic fungi *B. bassiana* and *M. anisopliae* was demonstrated by Samuels *et al*, (2002). The pathogenecity of five species of entomopathogenic fungi, *B. bassiana*, *M. anisopliae*, *M. flavoviride*, *P. fumosoroseus* and *V. lecanii* to the various developmental stages of *Boophilus annulatus* ticks under laboratory conditions showed that *M. anisopliae* and *B. bassiana* were most virulent to engorged females and caused 85-100% mortality within 7-10 days post-inoculation. All the tested fungi also prevented or reduced the egg laying capability of the ticks several

days before their death (Gindin *et al*, 2001). Recently, Shi and Feng, (2004) have demonstrated the lethal effects of *B. bassiana*, *M. anisopliae* and *P. fumosoroseus* on the eggs of the carmine spider mite, *Tetranychus cinnabarinus*. During a 12-day observation period after spray, the infected eggs shrunk in shape, then turned orange brown for *B. bassiana* and *P. fumosoroseus* or dark-gray for *M. anisopliae*, and eventually had outgrowths of the sprayed fungus when maintained under moist conditions.

The fungi can be produced easily on a large-scale, they are easy to apply, do not leave toxic residues, are environmentally safe, and on top of all, there is no known resistance in insect community against the fungal preparations. However, they are effective within limited temperature range and their efficacy is influenced by biotic and abiotic factors, and have limited shelf-life (Table 1.7). The fungal kingdom is a diverse assemblage of organisms with a great variety of structural types (Deshpande, 2000). The vegetative growth forms are mycelia and unicellular yeast, while asexual spores such as conidia, and sporangiospores are produced for the dispersal. The lower fungi sexually produce resistant zygospores that are commonly used in the biocontrol formulations. Other resistant structures like blastospores and chlamydospores are also used as infective propagules.

P. fumosoroseus , a biocontrol agent against sweet potato whitefly can be grown in form of blastospores, which are resistant to simple drying (Jackson et al, 1997). *P. fumosoroseus* like many other entomopathogenic fungi is dimorphic in nature , that means it can exhibit mycelium and yeast form in liquid culture. The yeast-form cells eventually lead to the production of blastospores or hyphal bodies. Since the

blastospores are produced from yeast-form cells, media, which contained high concentration of nitrogen and carbon, supported maximum blastospore production.

Table 1.7 Characteristic properties of mycopesticide in production and use

Advantages	Special features
No accumulation in food chain	Limited shelf life
No evaporation	Efficacy influenced by biotic and abiotic factors
No toxic residues	Effective within limited temperature range
No risk of leaching into groundwater or drinking water	Efficacy influenced by fungicides and other plant protection agents
Safe for humans, warm-blooded species and plants	Contact effect, death delayed in accordance with disease course
Simple detection by re-isolation on selective media	
Easy to apply	Forms toxic metabolite products on proliferation
Mass production is possible	Allergy risk
Relatively narrow host spectrum	

Source: Deshpande M.V., (1998)

Entomophthora virulenta is pathogenic to aphids that can produce zygospores in surface and submerged fermentation (Latge and Soper, 1977). Under submerged condition, using dextrose and yeast extract, a high number of zygospores were obtained in 9 days having 70% germination rate. Therefore, these zygospores can be used in biopesticide formulations.

Another pathogen commonly occurring on aphids is *Erynia neoaphidis* . Similar to other entomophthorales, the primary conidia of *E. neoaphidis* eject actively and start another cycle if they land on the host. Although the mycelial phase can be produced *in vitro*, it cannot be used directly in biocontrol formulation (Shah *et al*, 1998). Therefore, algination, that is entrapment of mycelia in the alginate beads, for conidia production was used to apply *E. neoaphidis* for the biocontrol of aphids (Shah *et al*, 1998).

N. parvispora, a biocontrol agent against *Thirps tabaci*, possesses most of the typical features of entomophthoralean fungi, such as multiplication as hyphal bodies in host tissue, and formation of ballistospores and secondary capilliconidia that are infective propagules. This fungus produces zygospores, which after germination reestablish itself in the host population (Grundschober *et al*, 1998).

Limited success has been achieved in production of conidia under submerged conditions, but conidiation of *Hirsutella thompsonii*, a pathogen of several aphids and mites was achieved in submerged conidiation (Van Winkelhoff *et al*, 1984). Thomas *et al*, (1987) reported production of *B. bassiana* conidia in submerged fermentation by carefully manipulating medium ingredients. Although the surface of conidia that were produced was different than the aerial conidia, they exhibited similar level of virulence (Thomas *et al*, 1987).

1.6.3 Use of mycloytic and cuticle degrading enzymes and their inhibitors in pest control

1.6.3.1 Structure of fungal cell wall and insect cuticle

The protective covers, that are, cuticle in insects and cell wall in fungi share common structural components (Figure 1.1). The cell wall is not an inert protective shell but rather, a dynamic organelle required for the viability of the cell. Its complex structure

serves many functions, such as osmotic protection to the protoplast, transport of molecules, growth, conjugation and spore formation. This means, any major disruption in its organization or metabolism will be deleterious to the cell.

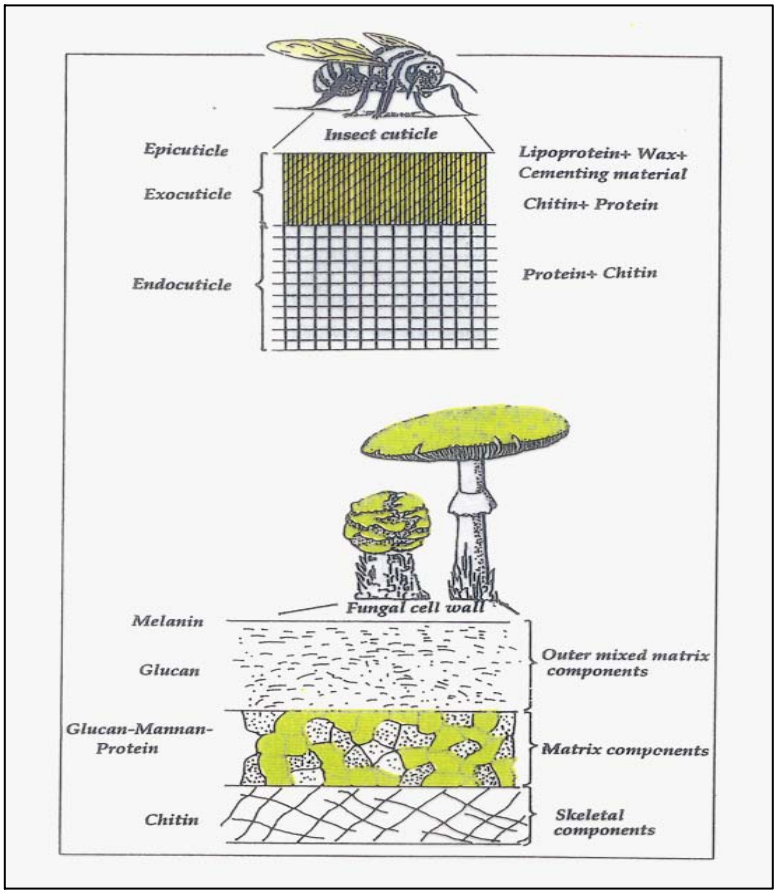

Figure 1.1 Insects and fungi share a common bond

Like bacteria and plants, fungal cell walls are primarily composed of polysaccharides. These are divided into two groups on the basis of their physical form, such as skeletal and matrix components. The skeletal components provide strength to the cell wall. These are the highly crystalline polymers, chitin and glucans. Chitin is a very interesting and widely distributed polymer. Chemically it is a nitrogen containing polysaccharide with long fibrous molecules. It forms material of considerable mechanical

strength and resistance to chemicals. The insect cuticle is composed of wax, lipids, protein and chitin. The main component being chitin, its share is 25-50% of dry weight of insect cuticle. Insect cuticle is a composite material consisting of a thin lipid-protein rich epicuticle covering the bulky procuticle. The pro-cuticle is composed of the exo- and endo-cuticle are mainly composed of chitin and protein, wherein the exo-cuticle is generally melanized (Andersen, 2002). Fungal cell wall components are divided into two groups, such as skeletal and matrix components. Chitin and R-glucans are the main structural components while S-glucans and mannoproteins are the matrix components. Thus, insects and fungi share a key structural polymer, chitin, a β- 1,4-linked *N*-acetylglucosamine polymer that is related to cellulose, with the C-2 hydroxyl groups replaced by acetamido residues. The partially deacetylated form of chitin is chitosan (the corresponding glucosamine polymer) (Shaikh and Deshpande, 1993). It is advantageous to develop a common agent against both insects and fungi and furthermore chitin is absent in plants and mammals, and thus its metabolism presents an attractive target for the control of plant pathogenic insects and fungi (Deshpande, 1998). The chitin content of fungal cell walls varies widely from 3-5 % to about 60 %. In *S. rolfsii*, a root-infecting peanut pathogen, 60 % of the cell wall is made up of chitin (Patil *et al*, 2000). The matrix components are polysaccharides called mannans linked with proteins which form a sort of cementing material in the cell and which also contribute to the flexibility of cell walls. Other constituents, such as lipids and pigments like melanins, though quantitatively minor, contribute a lot to the protective mechanisms. Of course, the first step towards cell wall formation would be the synthesis of chitin. Chitin synthesis takes place when carbon and nitrogen metabolism pathways are brought together by the organism (Figure 1.2).

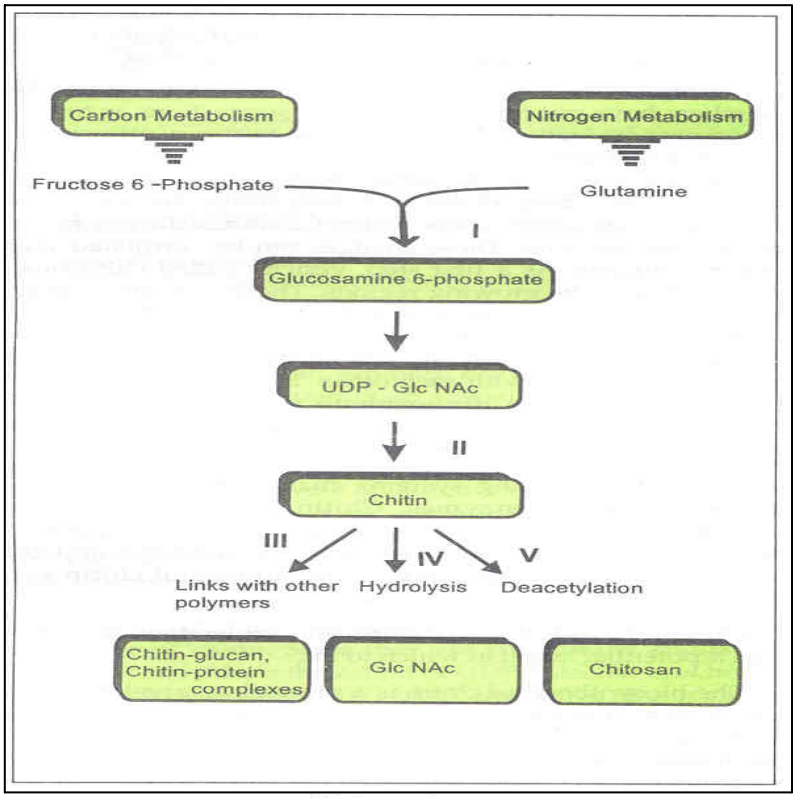

Figure 1.2 Chitin Synthesis

The key enzyme involved in the final step of chitin synthesis is chitin synthase. Actually a number of enzymes contribute significantly to the synthesis of chitin, but only glucosamine phosphate synthase and chitin synthase are considered as prime targets for biocontrol agents. Chitin synthesis is immediately followed by certain modifications to form the cell wall. Chitin either forms bonds with matrix components or deacetylates to form chitosan. Insect cuticle may be visualized in a simplified manner as containing alternate layers of protein and chitin. It has also been regarded as a plasiticized, protein sheet, variously subdivided to give a layered structure. The outer surface has waxes and the inner parts chitin. The hardening of cuticle is either due to its impregnation with calcium salts and further polymerization. The thick cuticle consists of three layers. From

the outside going in, the layers are called epicuticle, exocuticle and endocuticle, respectively. The epicuticle is a thin, dark pigmented multilayered membrane made up of cuticulin (lipoprotein), wax and cementing material of uncertain composition. The exocuticle is amber or black colored. Its main constituents are chitin and protein. The endocuticle is double the thickness of the exocuticle, but colorless and is made up of chitin and protein. Chitin usually accounts for only 25-50% of the dry weight of the cuticle. Below the cuticle are present the cells involved in synthesis of cuticle. These are bound to it by a membrane. The rigidity of the cuticle is due to exocuticle. Since the cuticle is incapable of growth, it must be shed from time to time as the insect grows, and a new and larger cuticle is laid down in its place. This is called as molting or ecdysis. The molting process uses glycogen reserves for chitin synthesis. When a new cuticle is being formed, the epicuticle is laid down first, then the exo- and endo-cuticles are laid down. Finally the structure is layered with wax and cement coating. Glycogen reserves are re-accumulated after the molting is complete. Synthesis and breakdown of chitin are active processes in insects and intimately linked to survival.

Chitin synthesizing systems characteristically include chitin-degrading enzymes. The complete enzymatic hydrolysis of chitin to free *N*-acetyl-D-glucosamine is performed by a chitinolytic system, the action of which is known to synergistic and consecutive. The endochitinase (poly-ß-1-4- (2-acetamido-2-deoxy)-D-glucoside glyconohydrolase; E.C. 3.2.1.14) hydrolyses the polymers of *N*-acetyl-D-glucosamine, including tetramers and to a lesser extent, trimers. Chitobiase (ß-D-*N*-acetyl-gluocosaminidase, chitobiose acetamidodeoxy glucohydrolase: E.C. 3.2.1.30) hydrolyses chitobiose and chitotriose.

Thus, the dual pathogenecity of fungi towards plant pathogenic insects and fungi has been attributed to the various hydrolytic enzymes, such as chitinases, proteases, lipases, etc. which facilitate the entry of pathogen in their respective hosts, e.g. *V. lecanii* is useful in the control of rust fungi and insects such as white flies and aphids. Chitin metabolism can be targeted at various stages:

a. Either by using the mycolytic enzyme complex that is involved in the process of degradation of pre-formed chitin, e.g. to control root infection caused by fungi like *S. rolfsii* and *R. solani* of peanut, beans and cotton, daily use of dilute *S. marcescens* chitinase was tried. On similar lines, the antifungal enzymes of *M. verrucaria* have been applied to control *S. rolfsii* infection of peanut .
b. Or by inhibiting the chitin synthase enzyme and other enzymes of chitin metabolism e.g. various compounds including chlorinated hydrocarbons, triazines, nitrophenols, organophosphates are now known to inhibit chitin synthase activity. The organophosphates , kitazin-P , parathion, sulfenimide , and captan selectively prevent chitin formation in insects. But because of the harmful effects of the chemical agents, there is a need to screen for bioactive metabolites of microbes, which have distinct advantages of selectivity, specificity and ease of production.

Other than insects and fungi, chitin is present in shells of marine invertebrates such as crabs, oysters, lobsters and shrimps. Billions of tons of chitin are produced annually by marine animals. Since 1970, dumping of chitin wastes into waters has been considered illegal. In an attempt to find alternative methods to dispose of chitin waste, it was serendipitously discovered that flooding agricultural land with this waste helped combat root-infecting fungi. A marked reduction of root-rot in beans as well as wilt in

radish, both caused by *Fusarium* species was achieved by this means. Scientists later found that land filling by chitin has an indirect effect on plant pathogenic fungi. The addition of chitin suppresses total fungal population and stimulates lytic enzymes and antibiotic producing microbes. Chitinous material is also useful in controlling plant parasitic nematodes. Tomato root-knot nematodes can be effectively reduced by addition of chitin. The different level of susceptibility of fungi and nematodes to chitin suggested modifications in land filling process.

1.6.3.2 Enzymes involved in degradation of fungal cell wall

The extracellular fungal cell wall hydrolytic enzymes of *T. harzianum* act synergistically *in vitro*. The combined inhibitory effect of purified endochitinase and *N*-acetylglucosaminidase was found to be greater on the sporulation and growth of different plant pathogenic fungi. Nevertheless, the enzymes from different sources also act synergistically to control the growth of plant-pathogenic fungi (Goldman *et al*, 1994). However, in view of the observations of Lorito *et al,* (1993) regarding the wider range of inhibition for the enzymes from *T. harzianum* , the combination of the enzymes from the mycoparasitic organisms will be more effective than the similar mixtures from saprophytic ones. A *F. chlamydopsorum* strain, a mycoparasite of groundnut rust, (caused due to *Puccinia arachidis*) produces an endo-chitinase that inhibits the germination of the rust fungus (Mathivanan *et al*, 1998).

It has been pointed out that chitinase contributed significantly in the killing process (Mendonsa *et al*, 1996). Therefore, most of the fermentations with the biocontrol organisms include chitin as the inducer to increase the levels of chitinases (Domnas and Warner , 1991; Vyas and Deshpande, 1989). Chitin also supports production of other cell

wall-degrading enzymes such as protease, ß-1, 3 –glucanase and ß -1,6 glucanse in *T. harzianum* (De la Cruz *et al*, 1995) and ß-1,3 –glucanase in *M. verrucaria* (Vyas and Deshpande, 1989). Most of the cell wall-degrading enzymes, including endo- ß -1,6 and ß-1, 3 glucanases in *T. harzianum* , have been found to be produced on the autoclaved mycelia of *B. cinerea* and *Phytophthora syringae* when used as a sole carbon source (De la Cruz *et al*, 1995).

1.6.3.3 Enzymes involved in degradation of insect cuticle

Entomopathogenic fungi in submerged fermentation produce extracellularly CDEs when locust cuticle is used as a sole carbon source (St Leger *et al*, 1986). Extracellular enzymes appear sequentially in *Metarhizium* and *Beauveria* cultures. Proteolytic enzymes such as esterase, endopeptidase, aminopeptidase and carboxypeptidase are produced within first 24 h of growth. *N*-acetylglucosaminidase appears next and endo-chitinase which attacks chitin polymer randomly, is produced in significant quantities after 4 days. Lipases are undetected upto 5 days. This order of extracellular production of the CDEs is parallel to the sequence of cuticle constituents (Charnley, 1989). A significant degree of strain variability was noted in the production profiles of *B. bassiana* and *M. anisopliae* (Gupta *et al*, 1992). Variability was not only in the quantities of enzymes but also with regard to their secretion pattern on different growth media. Five strains of *B. bassiana* grown in a medium containing purified cuticle from either *Galleria mellonella* and/or *Trichoplusia ni* produced different quantities of CDEs. Biodochka and Khachatourians, (1988) reported that *B. bassiana* GK2016 strain for protease production required gelatin as on glucose medium it did not produce any proteolytic activity. However, some strains of *B. bassiana* produced proteases when grown on glucose medium (Gupta *et al*, 1992;

Samuels and Paterson,1995). Glucose medium in combination with nitrate did not support the chitinase production in *B. bassiana*. Similar to glucose, *N*-acetylglucosamine also regulated the chitinase and protease production. These observations can certainly be useful to tailor biological control agents against specific pests. Natural variation in different strains could be used to rationally develop improved mycoinsecticides. However, Heale *et al*, (1989) reported that it was difficult to correlate activities of these enzymes alone with pathogenecity of the biocontrol organism. For instance, they reported that the mutants of *B. bassiana* that produced reduced levels of cuticle degrading enzymes were still pathogenic to their hosts.

Chitin is present in the exoskeleton and gut lining of insects. Therefore, biosynthesis of chitin is a promising target for pesticide action. The insect molting enzyme, chitinase has been described from *Bombyx mori*, *Manduca sexta*, and several other species. Similarly, chitinases have been implicated in different morphogenetic events in fungi (Villagomez-Castro *et al*, 1993). The allosamidin was found to be inhibitory after ingestion to the growth of mite, *Tetranychus urticae* and a larva of a housefly, *M. domestica* (Sakuda, 1996). Nevertheless, chitinase inhibitors can be explored as potential biopesticides.

1.6.3.4 Use of enzymes and enzyme inhibitors in biocontrol of pathogenic fungi and insects

1.6.3.4 A Importance of chitinases

Chitinases (E.C. 3.2.1.14) catalyse the hydrolysis of chitin between the C1 and C4 of two consecutive *N*- acetylglucosamines. The enzyme is regarded important for the extensive carbon and nitrogen recycle in nature. Chitinase occurs widely in soil microorganisms, and also in plants and insects, fulfilling a possible defense role in the

plant. Chitinase having very diverse characteristics are known and may be of value in basic studies related to their biological role. Considerable interest in the chitinolytic enzymes has been stimulated by their possible involvement as defensive agents against chitin-containing pathogenic organism such as insects, nematodes and fungi (Carr and Klessig, 1989; Linthorst, 1991; Sahai and Manocha, 1993; Shaikh and Deshpande, 1993).

Several microorganisms, including bacteria such as *Bacillus lichiniformis* (Takayanga *et al*, 1991), *Bacillus pabuli* (Frandberg and Schnurer, 1994), *Bacillus thuringiensis* (Thamthiankul *et al,* 2001), *Serratia marcescens* (Young *et al*, 1985; Young *et al*, 1985), *Nocardia orientails* (Usui *et al*, 1984), *Vibrio alginolyticus* (*Ohishi et al*, 1996) and many species of fungi such as, *Myrothecium verrucaria* (Vyas and Deshpande,1989), *Stachybotrys elengans* (Tweddell *et al*, 1994), *Streptomyces cinereoruber* (Tagawa and Okazaki, 1991), *Streptomyces lydicuis* (Mahadevan and Crawford, 1997), *Trichoderma harzianum* (Felse and Panda, 1999), *Trichoderma viride* (Rogalski *et al*, 1997), *Verticillium lecanii* (Bing-Lan *et al*, 2003; Fenice *et al*, 1998) produce significant levels of extracellular chitinolytic enzymes. Chitinase activity in plant (Krishnaveni *et al*, 1999; Park *et al*, 2002; Pirttila *et al*, 2002) and human serum has also been described (Patil *et al*, 2000).

In the late 1970s, chitinases were shown to degrade insect gut peritrophic membranes *in vitro*. Brandt *et al*, (1978) proposed that chitinases cause perforations in the membranes, thus facilitating entry of the pathogens onto the tissues of susceptible insects. The addition of exogenous chitinase from *Streptomyces griseus* to the blood meal of the mosquito, *Anopheles freeborni* , prevented the peritrophic membrane from forming (Shahabuddin *et al*, 1993). *Escherichia coli* - produced recombinant endo-chitinase

ChiAII , encoded by *Serratia marcescens* was found by scanning electron microscopy to perforate *Spodoptera* larval midgut peritrophic membranes after *in vitro* incubations (Regev *et al*, 1996). Moreover, perforation of peritrophic membranes also occurred *in vivo* after fifth instar larvae were fed on a diet containing recombinant *ChiAII*. Chitinases also facilitate the penetration of the host cuticle by entomopathogenic fungi (Coudron *et al*, 1989; St. Leger *et al*, 1991 a, b). Chitinases and ß-*N*-acetylglucosaminidases were secreted when the entomopathogens *M. anisopliae*, *B. bassiana*, *V. lecanii* and *N. rileyi* are grown on insect cuticles (St. Leger *et al*, 1986; Coudron *et al*, 1989). At the time of cuticle penetration, virulent isolates of *N. rileyi* exhibited substantially higher levels of chitinase activity than avirulent strains (EI-Sayed *et al*, 1989). Chitinase gene expression in entomopathogenic fungi is believed to be controlled by a repressor-inducer system in which chitin or some oligomeric products of degradation serve as inducers (St. Leger *et al*, 1986).

1.6.3.4 B Importance of chitin deacetylase and chitosanase

Chitin deacetylase (CDA, E.C. 3.5.1.41) catalyses the hydrolysis of *N*-acetamido bonds in chitin to produce chitosan. Chitosan is further acted upon by chitosanase (E.C. 3.2.1.132) enzyme to form free glucosamine residues. The presence of CDA activity has been reported in several fungi (Araki and Ito, 1975; Kafetzopoulos *et al*, 1993; Martinou *et al*, 1993; Gao *et al*, 1995; Alfonso *et al*, 1995, Tsigos and Bouriotis, 1995; Tokuyasu *et al*, 1996) and insect species (Rajulu *et al*,1986).The most well studied chitin deacetylases are those from the fungi *Mucor rouxii* (Araki and Ito, 1975; Kafetzopoulos *et al*, 1993; Martinou *et al*, 1993) *Absidia coerulea* (Gao *et al*, 1995), *Aspergillus nidulans* (Alfonso

et al, 1995) and two strains of *Colletotrichum lindemuthianum* (Tsigos and Bouriotis, 1995; Tokuyasu *et al*, 1996).

Two different biological roles have been suggested for fungal CDAs, namely, their involvement in cell wall formation and plant-pathogen interactions. The involvement of CDA in cell wall chitosan biosynthesis was demonstrated for the first time during studies to investigate chitin and chitosan biosynthesis in fungi. In *M. rouxii*, it was revealed that chitin synthase operates in tandem with CDA. Chitin synthase synthesizes chitin by the polymerization of *N*- acetyl-D-glucosamine (UDP-GlcNAc), and CDA hydrolyses the *N*-acetamido bonds in the chitin chains, acting more efficiently on nascent rather than microfibrillar chitin (Davis and Bartnicki- Garcia, 1984). Similar results were also obtained for the fungus *A. coerulea* , where it was found that CDA was localized near the inner face of the cell wall (Gao *et al*, 1995). A similar biological role has been reported for the two CDAs from *S. cerevisiae*. It was shown that these enzymes are required for correct ascospore wall formation (Christodoulidou *et al*, 1996).

An alternative biological role, namely the involvement of the enzyme in plant-pathogen interactions has been suggested for the CDA from *C. lindemuthianum*, considering the fungus is a plant pathogen, and the enzyme is extracellular and active on chitin oligomers. Taking into account that chitin oligomers elicit plant-defense mechanisms as mentioned earlier in plant defense, where as their deacetylated forms do not, it has been proposed that CDA might deacetylate chitin oligomers that arise from the fungal cell wall subsequent to the activity of plant chitinases , and thereby diminish their elicitor activity (Tsigos and Bouriotis, 1995). CDA is also known to assist in anti-plant defense reaction as demonstrated in the rust fungus *Uromyces viciae -fabae* (Deising and

Siegrist, 1995). The possible contribution of the extracellular constitutively produced CDA along with chitosanase by *M. anisopliae* in the process of insect pathogenesis has been evaluated for the first time (Nahar *et al*, 2004).

1.6.3.4 C Use of non-enzymic components and chitin metabolism inhibitors for the control of plant pathogenic fungi and insects

One of the first scientists to envisage use of microbes for pest control was the 19th century zoologist Elie Metchnikoff. Some microbes produce antibiotics for their own defense. Relatively few antibiotics are routinely used to control plant diseases. These antibiotics can help man to combat viral, bacterial and fungal diseases of crops. For example, streptomycin can be used to control bean blight caused by the bacterium, *Erwinia*. Streptomycin is also effective against a few fruit pathogens, such as blights and cankers, particularly powdery mildews and rusts. Soaking of paddy seeds in aureofungin helps reduce incidence of seed-borne fungal infection. This can be used as a control measure. A mixture of cycloheximide and griseofulvin has been tried to ruin fungal pathogens. In Japan, sheath blight of rice was effectively controlled by replacing arsenic fungicides with polyoxins and validamycin (Deshpande, 1998). In field, these antibiotics are used either as wettable powders or as dust.

The insecticidal antibiotic, tetranactin, has also been used to control carmine mites of fruits and tea. Most of these antibiotics impair membrane function thus impeding cellular functions by disrupting movement of molecules into and out of cells. Simultaneously, they may also inhibit synthesis of important cellular components such as sterols, protein or nucleic acid.

The biosynthesis of chitin is a promising target for pesticide action. Many currently used pesticides inhibit chitin synthesis directly or indirectly. Inhibition of chitin biosynthesis with different inhibitors has been illustrated in Table 1.8.

Table 1.8 Inhibition of chitin biosynthesis

Type of inhibitors	Examples with mode of action
Non-specific inhibitors	Kitazin-P, Parathion etc. that block chitin synthase action
Indirect inhibitors	Benzoyl phenylurea, Dimilin that inhibit proteinase which activates chitin synthase, Neurotoxins and respiration inhibitors, that distort the membrane site of the enzyme
Competitive inhibitors	Polyoxins and Nikkomycins that compete with the substrate UDP-GlcNAc required for chitin synthase activity

Source: Deshpande M.V., (1998)

Various chemically synthesized organophosphorous compounds are being used in agriculture for the control of insect and fungal pest (Binks *et al*, 1993). For instance, edifenphos is being used to control rice blast caused by *Magnaporthe grisea* (earlier called *P. oryzae*). The organophosphorous fungicides like Kitazin-P inhibited incorporation of ^{14}C-glucosamine into the fungal cell walls which can be attributed to inhibition of chitin synthesis either directly by inhibiting chitin synthase or indirectly by altering membrane permeability (Binks *et al*, 1993).

Two classes of antifungal antibiotics, namely polyoxins and nikkomycins have been isolated from streptomycetous cultures. Both of them are structurally very similar to

UDP-GlcNAc, an active monmer of chitin. Since they are structurally similar, the antibiotic can mimic the action of UDP-GlcNAc and bind to chitin synthase. This would block the active site of the enzyme and thus make the enzyme unavailable to the monomer for polymerization. Thus the synthesis of chitin would be blocked. Polyoxin D is most effective against *Pellicularia sasakii*, a rice sheath blight pathogen, while polyoxin B and L inhibit pear black spot and apple cork-spot fungi, respectively. Nikkomycins are structurally closely related to polyoxins and are very specific inhibitors of the enzyme chitin synthase. In case of fungi, the observed effect on species exposed to polyoxins and nikkomycins is swelling and bursting of growing hyphae while in case of insects, egg or cyst formation is inhibited (Deshpande, 1998). Compounds such as pseurotin A and 8-O-demethylpseurotin A have been isolated from a fungus *Aspergillus fumigataus*, and known to show inhibition of chitin synthase activity, however, they do not show any direct antifungal activity. These antibiotics cannot enter into the fungal cell without a facilitating compound easing their entry. Therefore, a combination of Amphotericin B is used to overcome this problem and to render chitin synthase accessible to pseurotins (Wenke *et al*, 1993).

Other than streptomyces strains, antifungal activity is found in bacteria such as *Bacillus* sp. too. Although, these antibiotics inhibit chitin synthesis, their target of action is not the enzyme chitin synthase. For example, Tetaine, a compound isolated from *Bacillus pumilus*, inhibits an enzyme which brings together carbon and nitrogen pathways for chitin synthesis. This enzyme is glucosamine 6-phosphate synthase. Tetaine, a dipeptide, as such, is not an enzyme inhibitor, however, when it enters into the fungal cell, it is broken down by cellular enzymes, to alanine and anticapsin. Anticapsin is the

compound responsible for enzyme inhibition. However, the fact remains that these enzyme inhibitors merit more research if we are to win the fight against pests (Milewski *et al*, 1993).

Chitinases are widely distributed in nature and have different roles to play in different systems. Chitinase is widely distributed in plants, microorganisms and insects and is a specific and key enzyme for the ecdysis of insects through hydrolysis of integumental chitin. Therefore, chitinase is good target for insecticides, but an effective inhibitor of it was not found until Daizo *et al*, (1987) demonstrated specific inhibition of *B. mori* chitinase by allosamidin. Surprisingly, allosamidins are specific inhibitors of insect chitinases. Allosamidin, an inhibitor of chitinase, blocks the development of malarial parasites trapped in the peritrophic membrane that forms around the blood meal (Shahabuddin *et al*, 1993). Allosamidin, produced by *Streptomyces* species, is structurally very closely related to acetylglucosamine, a basic unit of chitin. In other words, allosamidin is a molecular mimic and therefore, is a powerful competitive inhibitor of chitinase. Thus, there is a need to screen for more compounds that can target the chitin metabolism either by inhibiting the process of chitin synthesis or the chitinases in both insects and fungal pathogens.

1.7 Trans-sectorial issues related to application of biocontrol agents

1.7.1 Researchers

They are required to understand the safety measures to be taken while dealing with the production of biopesticide based preparations. The knowledge of quality control e.g. viable count of organism in case of microbes, virulence and/or insecticidal /

fungicidal activity of the preparation, purity of the product etc. is essential. The intellectual property right (IPR) issues are also of major concern.

1.7.2 Policy related studies

It is essential to understand the structure of the society, the inter-relation of the end-users and policy makers' etc. in different states so as to promote the product to at all possible locations.

1.7.3 End users

1.7.3.1 Biosafety

This is concerned with the intrinsic safety properties and safe handling of living organisms. When discussing biosafety in relation to modern biotechnology it concerns predominantly the safety of genetically modified organisms. It also involves the testing of the product towards phytotoxicity to plants, mammalian toxicity, and effect on non-target organisms so as to preserve the nature's balance.

1.7.3.2 Bioethics

Bioethics in biotechnology in particular is concerned with the ethical aspects of activities involving genetic modification, but also deals with for instance animal welfare issues. This stresses especially on the use of natural strain, which is not genetically modified and the strain selected should be such that it will not disturb the nature's balance with respect to plant ecosystem, animal ecosystem etc.

1.7.3.3 Socio-economics

Agriculture is the backbone of Indian economy. About 80% of the population of India are associated with farming. Of these, major role is played by the women farmers' who work in the field for the whole day to earn their living. It is very essential on the

part of the government to initiate certain policies or training workshops to literate such population and direct them towards upliftment. One good example in this direction is the Mushroom cultivation training program where in the people in a particular region get an opportunity of going to their respective agricultural university and learn this "do it yourself technology". Here they are trained in all possible aspects such as mushroom cultivation knowledge, actual mushroom cultivation, preservation, packaging, marketing etc. so that they can start on their own and proceed further for the improved financial status.

Similar efforts, if directed towards the training to the farmers' and their families at the respective agricultural universities for the production and application of promising eco-friendly biopesticides, will open the doors towards the green revolution. It is essential on the part of the enduser / farmer to know what type of product has to be used at what time of the crop, in what proportion it has to be applied, how many times it has to be applied etc. and to understand this, it is essential on their part to learn what's going on in nature, what types of host-parasite interactions exist naturally, and how do they help in maintaining nature's balance. Once they have thorough knowledge about this system, it is essential to make them available the raw materials and infrastructure at nominal charges along with availability of other cheaper and effective disease control measures, that could be employed together in integrated pest management programs and knowledge about their easy application, and production and effectiveness will help in poverty alleviation thereby raising the standard of living of women farmers' especially and open the doors towards the path of "DO IT YOURSELF TECHNOLOGY" and gain "SUSTAINABLE AGRICULTURE" (SAMPARK).

1.8 Present investigations

Objectives of the present investigation are as follows:

1. Use of *Metarhizium anisopliae , Beauveria bassiana, and Nomuraea rileyi* for the control of insect pest, *H. armigera* on pulses under field conditions.
2. Biochemical studies of chitin metabolizing enzymes of *M. anisopliae*.
3. Use of chitin metabolizing enzymes and chitin synthesis inhibitors, either singly or in combination to arrest the chitin metabolism in insects and fungi.

CHAPTER II

MATERIALS AND METHODS

2. 1 Materials

The materials and chemicals used in the studies were purchased from suppliers as shown in Table 2.1.

Table 2.1 Source of chemicals and materials

Chemicals, Materials	Source
Bovine serum albumin , Calcoflour White M2R , chitin, chitosan, ethylene glycol chitosan, glucosamine, gum arabic, fluorescein isothiocyanate-wheat germ agglutinin (FITC-WGA), laminarin , melanin , 3-methyl -2-benzothiazoline hydrazone (MBTH), 4-methylumbelliferyl -ß-D-glucosaminide, 4-MU-ß-D-N,N',N''- triacetylchitotrioside, *N*-acetyl glucosamine (GlcNAc), UDP-C^{14} - GlcNAc, tyrosine	Sigma Chemical Co. St. Louis, MO, USA
Ultra filtration membrane	Millipore, Molsheim, France
Endosulfan	Excel Industries, Mumbai
HaNPV	MPKV, Rahuri
Pre-stained molecular weight markers for SDS-PAGE	Bio-Rad, Hercules, CA
Unicorn bags	Unicorn Imp and Mfg. Corp., USA

All other chemicals used were of analytical grade

2.2 Organisms

The organisms used during the course of this work are listed in Table 2.2.

Table 2.2 List of organisms used

Purpose	Organisms used
Chitin metabolism inhibitors	*Bacillus* sp. 101, *Bacillus* sp. 102, *Streptomyces* sp. NCL1, *Chaetomium* sp. MY3, *Volutella* sp.
Model for hyphal tip bursting test	*Aspergillus niger, Benjaminiella poitrasii, Fusarium* sp.,
Root infecting plant pathogen	*S. rolfsii* NCIM 1084
Entomopathogenic fungi used for the control of *H. armigera*	
M. anisopliae	M 34412 , M 1333, M 1311, M 1322, M 34311, M 3419 (Isolate 4), M 34210, M 34513, M 2305 (Isolate 1), M 42014 (Isolate 5), M 79120, M 79221, M 45115, M 51219, M 45317, M 79322, M 45216, M 2416 (Isolate 2), M 2508, M 2104, M 51118 , M 2427 (Isolate 3)
B. bassiana	B 3301, B 3426, B 3437, B 22504, B 23405,B 20202, B 20603
N. rileyi	N 81, N 82, N83, N84, N85, N86, N87,N88, N89, N810, N811, N812, N813, N814, N815
High level extracellular chitinase producer	*M. verrucaria* NCIM 903

2.2.1 Media

The media used during the course of this work are listed in Table 2.3.

Table 2.3 Media

Name	Constituents/100 ml
Potato dextrose agar (PDA)	3.9 g (Himedia)
Sabouraud malt yeast extract peptone medium (SMYP)	0.3g Sabouraud malt extract, 0.3 g Yeast extract, 0.5 g peptone, 1g glucose, 2 g agar
Yeast extract peptone glucose medium (YPG)	0.3 g Yeast extract, 0.5 g peptone, 1g glucose
Malt glucose yeast extract peptone medium	0.3 g malt extract, 0.3 g Yeast extract, 0.5 g peptone, 1g glucose, 2 g agar
Chitin medium	0.3 g KH_2PO_4; 0.1 g K_2HPO_4; 0.07g $MgSO_4$; 0.14g $(NH_4)_2 SO_4$; 0.05 g NaCl ; 0.05g $CaCl_2$; 0.05 g yeast extract; 0.05g bacto-peptone, and 0.5g chitin. The medium also contained trace metal solution (0.1 ml/100 ml). The pH of the medium after autoclaving was 5.5.
Trace metal solution	(mg/litre), 5.0 $FeSO_4.7H_2O$; 1.56 $MnSO_4.7H_2O$;3.34 $ZnSO_4.7H_2O$; 2.0 $CoCl_2$. 2 H_2O
Inhibitor production medium	1 g Soyabean meal; 0.5 g starch; 1.0 g yeast extract; and 1.5 g mannitol

2.2.2 Maintenance

All the *M. anisopliae* and *B. bassiana* isolates were maintained on PDA, while for the maintenance of *N. rileyi* isolates, SMYP agar was used. Numbering of the isolates was done with respect to the field numbers, plot numbers and sample numbers. The stock cultures were maintained at 4°C until used. Sub culturing was done on the host insect (*H. armigera*) after every three months and the passaged culture was re-isolated and maintained on potato dextrose agar and preserved by lyophilisation. The passaged stock of entomopathogenic fungi was used for all the experiments.

The mycolytic enzyme producing fungus, *M. verrucaria* NCIM 903, and a root infecting plant pathogen, *S. rolfsii*, NCIM 1084 were maintained on PDA slants. The bacterial and fungal cultures screened for anti-fungal activity such as *Bacillus* sp. 101, *Bacillus* sp. 102, *Chaetomium* sp., *Volutella* sp., *Streptomyces* sp., *A. niger* and *Fusarium* sp. were isolated from soil using soil dilution method (Goettel and Inglis, 1996) and maintained on MGYP agar.

B. poitrasii, used for hyphal tip bursting test (HTB) was maintained on YPG containing glucose, (1%), Difco yeast extract, (0.3%) and Difco peptone,(0.5%).

2.2.3 Isolation of entomopathogenic fungi

The soil samples were collected from the different regions around Pune, Maharashtra. For the isolation of entomopathogenic fungi two different methods namely, soil dilution method (Goettel and Inglis, 1996) and *Galleria* bait method (Zimmermann, 1986) were used. In *Galleria* bait method, in a vial containing 60 g of soil sample, four middle aged G*alleria* larvae were added and the vials were kept at 25 ± 2^0 C for a period of 14 days. The vials were moved upside down everyday. After 14 days, the soil samples

were screened for the presence of mycosed *Galleria* larvae and the respective fungus was isolated .On the basis of conidial morphology (Lomer and Lomer , 2001), isolates were identified as *M. anisopliae* , *B. bassiana* and *N. rileyi* .The *N. rileyi* strains were isolated from mycosed *Spodoptera litura* (Fabricius) larvae found in sugar beet fields near Pune.

2.2.4 Mass production of conidia of entomopathogenic fungi by solid state fermentation

Three strains namely *M. anisopliae* M34412, *B. bassiana* B3301 and *N. rileyi* N812 were selected for the large-scale production of conidia. The mass production of conidia was carried out in Unicorn-bags (32x64cm, Type 14 with a membrane for gaseous exchange) filled with 2 kg of rice soaked overnight in 1000 ml distilled water, as a substrate (Hassani *et al*, 2004). After autoclaving at 15 lb 40 minutes, bags were inoculated with 200 ml of biomass grown on YPG medium for *M. anisopliae* and *B. bassiana* and SMYP for *N. rileyi* in shake flasks for 48-72 h. The inoculated bags were incubated in humidity chamber with 25±2° C and relative humidity (70±10) for 14 days. After 14 days, the sporulated substrate was dried at 30° C for 2-3 days to reduce the moisture content (<20%). The conidia were then harvested with a myco-harvester (a unit specially designed to remove conidia from the biomass under vacuum, CABI Bioscience, UK) and stored at 4°C until used.

2.2.5 Formulation studies of conidia of entomopathogenic fungi

The viability and virulence (% mortality of *H. armigera,* see chapter III) of conidia for *M. anisopliae* M34412, *B. bassiana* B3301 and *N. rileyi* N812 were tested in different formulations such as diesel, sunflower oil, diesel: sunflower oil in the ratio 7:3, 0.1% Tween 80 etc. For this study, the conidial suspensions (1 x 10^7 conidia/ml) were

prepared in different formulations, kept at room temperature for 1 h and their germination was monitored on YPG agar for *M. anisopliae* M 34412 and *B. bassiana* B 3301 at 25 ± 2 ^{0} C for 24 h and on SMYP agar for *N. rileyi* N 812 at 25 ± 2 ^{0} C for 72 h .The germ tube formation was observed under the microscope (40X) and the percent germination was then calculated.

2.2.6 Germination studies of conidia of entomopathogenic fungi in presence of chickpea leaf extract

The conidial germination was carried out on YPG agar for *M. anisopliae* M 34412 and *B. bassiana* B 3301 and on SMYP agar for *N. rileyi* N812 with and without chickpea leaf extract. The chickpea leaf extract was prepared by crushing 5g chickpea leaves in 100 ml water. The germination studies were carried out at 25 ± 2 ^{0} C and incubated for 24 h for *M. anisopliae* M 34412 and *B. bassiana* B 3301 and for 72 h for *N. rileyi* N812.

2.3 Enzyme production

2.3.1 Production of cuticle degrading enzyme complex of entomopathogenic fungi

The cuticle degrading enzyme complex was produced by growing entomopathogenic fungi in chitin medium (Vyas and Deshpande, 1989) using chitin as the sole carbon source. The flasks were inoculated with 1 x 10 ^{7} spores/ml and incubated at 28 ° C for 96 h in case of *M. anisopliae* and *B. bassiana* isolates and for 120 h in case of *N. rileyi* isolates. The culture filtrate was used for the estimation of chitinase, protease and lipase activities.

2.3.2 Extracellular constitutive and induced production of chitin metabolizing enzymes of *M. anisopliae*

The extracellular constitutive production of chitin metabolizing enzymes such as chitinase, chitosanase and chitin deacetylase was studied in YPG (medium under shaking condition at 28 ° C for 72 h. The chitin medium was used for induced production of chitinolytic enzymes as described above at 28 ° C for 96 h.

2.3.3 Production of mycolytic enzyme complex of *M. verrucaria*

M. verrucaria was grown on a chitin (0.5% w/v) containing medium under shaking conditions (200 rpm) at 28° C for 7 days as described above. The cell-free broth, obtained after centrifugation at 5000 g, was used for the estimation of mycolytic enzyme activities *viz*. chitinase, chitosanase, β-1,3 glucanase, protease and mannanase .

2.3.4 Inhibitor production

All the microbial cultures were grown in inhibitor production medium under shaking conditions (200 rpm) at 28° C for 96 h. The supernatant was separated by centrifugation at 10,000 rpm for 10 min, and was used for further studies.

2.4 Enzyme assays

2.4.1 Chitinase assay

The chitinase activity in the culture supernatant was estimated as described earlier using acid swollen chitin as the substrate (Vyas and Deshpande, 1989). To prepare acid swollen chitin , the chitin (10g, purified powder from crabshells,) was suspended in chilled *O*-phosphoric acid (88%, w/v) and left at 0°C for 1h with stirring. The acid swollen chitin was repeatedly washed with chilled distilled water, followed with a 1% (w/v) $NaHCO_3$ wash and further dialyzed against cold distilled water. After homogenization in Waring blender (1 min), 50 mM acetate buffer, pH 5.0, was added to

the suspension so that 1ml of suspension contained 7 mg of chitin. The reaction mixture for chitinase assay contained 1ml 0.7% acid swollen chitin, 1ml 50 mM acetate buffer, pH 5.0 and 1ml enzyme solution that was incubated at 50°C for 1h. The GlcNAc produced was estimated colorimetrically with *p*-dimethyl amino benzaldehyde (DMAB) (Reissig *et al.*, 1955). One international unit was defined as the activity that produced 1μmol of GlcNAc per min.

The endo-chitinase and *N*-acetylglucosaminidase assays were performed using fluorogenic glycosides, 4-methyl-umbelliferyl-β-D-*N*-acetylglucosaminide (4-MU-GlcNAc) and 4-MU-β-D-*N, N′, N″*-triacetyl chitotrioside (4-MU-$(GlcNAc)_3$, prepared in

50% (v/v) ethanol, respectively (Jackson *et al*, 1996) .The 20 μl of 700 μM substrate was incubated with 50 μl of enzyme in 130 μl of 50mM sodium acetate buffer, pH 5.0, at 37 ^{0} C for 30 min. The reaction was then stopped by adding 2.3 ml 200 mM Na_2CO_3. The fluorescence was measured in a Perkin-Elmer fluorescence spectrophotometer by excitation at 360 nm and reading the emission at 445 nm.

2.4.2 Chitosanase assay

The chitosanase activity was estimated using acid swollen chitosan as the substrate. The acid swollen chitosan was prepared following the protocol used to make acid swollen chitin. The assay mixture contained 1ml 0.7 % acid swollen chitosan, 1ml 50mM acetate buffer, pH 5.0 and 1ml enzyme that was incubated at 50°C for 1h. The reducing sugars produced were estimated using Somogyi method (Somogyi, 1952) at 520nm. One international unit of enzyme was defined as the activity that produced 1 μmol glucosamine equivalents per min.

2.4.3 Chitin deacetylase assay

The chitin deacetylase (CDA) activity was estimated using ethylene glycol chitin (EGC) as the substrate prepared according to the method of Araki and Ito, (1975). Ethylene glycol chitosan (40mg) was treated at 4°C in a mixture containing 400 mg of $NaHCO_3$ and 4.5ml of acetic anhydride and kept at 4°C for 24 h. After addition of 200µl of acetic anhydride the mixture was allowed to stand for further 24 h at 4°C. After dialysis acetylated ethylene glycol chitosan (1mg/ml) was used as a substrate for the assay of CDA. The assay was carried out according to Kauss and Bausch (1988) with 100µl 50 mM sodium tetraborate buffer, pH 8.5, 100µl 1mg/ml EGC and 50µl enzyme incubated at 37°C for 30 min and the reaction was terminated by the addition of 250µl 5%(w/v) potassium hydrogen sulfate ($KHSO_4$). For color development 250µl of 5%(w/v) $NaNO_2$ was added and allowed to stand for 15 min, and 250µl 12.5%(w/v) ammonium sulfamate ($N_2H_6SO_3$) was further added. After 5 min freshly prepared 250µl 0.5% (w/v)3-methyl-2-benzothiazoline hydrazone (MBTH) was added and the mixture was heated in a boiling water bath for 3 min. The tubes were cooled in tap water and 250µl 0.5% (w/v) $FeCl_3$ was added and estimated spectrophotometrically at 650 nm. One international unit was defined as the enzyme activity that produced 1µmol of acetate per min.

2.4.4 *N*-acetylglucosaminidase activity

The *N*-acetylglucosaminidase activity was determined by measuring the amount of *p*-nitrophenol released in a reaction mixture containing 0.9ml of 5mg/ml of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 50 mM acetate buffer, pH 5.0 and 0.1ml of

enzyme, incubated at 50°C for 30min. The reaction was terminated by adding 2 ml of 0.2 M Na_2CO_3 . One international unit was defined as enzyme activity that produced 1μmol of *p*-nitrophenol per min.

2.4.5 Protease assay

Protease activity was measured using Hammerstein casein as a substrate (Vyas and Deshpande, 1989). The reaction mixture, 2ml, contained an aliquot of a suitably diluted enzyme solution, 10mg Hammerstein casein and 0.2mM of sodium carbonate buffer, pH 9.7. Enzyme reaction was carried out at 35°C for 20 min and terminated by the addition of 3ml trichloroacetic acid (TCA) (2.6 ml 5%TCA + 0.4 ml 3.3N HCl). The absorbance of the TCA soluble fraction was measured at 280nm. One international unit was defined as enzyme activity that produced 1 μmole of tyrosine per min.

2.4.6 Lipase assay

Lipase activity was determined as described by Pignede *et al*, (2000). The substrate emulsion was prepared with olive oil , 50 ml and gum arabic , 50 ml (10% w/v). The reaction mixture contained 1ml enzyme, 5ml substrate emulsion and 2ml of 50 mM phosphate buffer, pH 6.8 and was incubated for 1h at 37°C with shaking. The reaction was stopped with 4ml of acetone-ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity was determined by titration of the fatty acid released with 50 mM sodium hydroxide. One international unit was defined as enzyme activity that produced 1μmole of fatty acid per min.

2.4.7 Laminarinase assay

The extracellular, β-1,3-glucanase, was estimated using laminarin, as substrate (Vyas and Deshpande, 1989). An aliquot of 0.5 ml enzyme solution was mixed with 0.5 ml of 1% laminarin prepared in 50mM acetate buffer, pH 5.0 and incubated at 50 ^{0} C for 30 min. Reducing sugars were estimated as glucose equivalents as described by Somogyi, (1952). One international unit was defined as the enzyme activity that produced 1 μmole of glucose per min.

2.4.8 Mannanase assay

The mannanase activity was estimated using yeast mannan as a substrate (Mendonsa *et al*, 1986). The 0.5 ml of crude enzyme was mixed with 0.5 ml of yeast mannan (1%) prepared in potassium phosphate buffer (200 mM, pH 5.8) and was incubated at 50° C for 1h. Reducing sugars were estimated as mannose equivalents as described by Somogyi, (1952). One international unit was defined as the enzyme activity that liberated 1μmole of mannose per min .

2.4.9 Chitin synthase assay

The intracellular chitin synthase activity was estimated as described earlier. To estimate chitin synthase activity, UDP-C ^{14} -NAG was used as a substrate. Each assay mixture was 50 μl containing 5 μl of the enzyme preparation and final concentration of Tris-HCl ((50 mM, pH 8.0), $MgCl_2$ (10 mM), GlcNAc (25 mM), UDP- C ^{14} -*N*-acetylglucosamine (1 mM) containing 25nCi UDP- C ^{14} -GlcNAc. The assay mixture was incubated at 37 ^{0} C for 1 h and the reaction was terminated by the addition of 66% (v/v) ethanol. The assay mixture was filtered through glass microfibre filters (presoaked with 10% TCA; Whatman GF/C, 2.5 cm) on a Millipore Manifold system. The reaction tubes

were washed twice with 1 ml 1% Triton X-100 and the filters were washed with 4 ml 66% ethanol. The filters were dried at 80 ^{0} C for 8 h and transferred to scintillation vials containing 3 ml scintillation fluid (4 g PPO (2,5-diphenyloxazole) and 0.1 g POPOP (91,4-bis{ 5-phenyl -2-oxazoyl}-benzene) in 1000 ml toluene) for radioactive counting. The total and specific activities were calculated as described earlier (Gooday and de Rousset-Hall, 1975). Chitin synthase activity was expressed as pkat and is defined as the amount of enzyme activity that produced 1 pmole of the product per second.

2.5 Hyphal tip bursting test

For the hyphal tip bursting test the four fungi namely, *B. poitrasii*, *S. rolfsii, A. niger,* and *Fusarium* sp. were selected .To obtain actively growing hyphal tips the fungal cultures were inoculated on MGYP agar plates and these plates were incubated at 28° C for 16-18 h. The hyphal tip elongation of *B. poitrasii* was approximately 1.5 divisions/ 2 min, while time period required to advance 1 division was 3-5 min for *S. rolfsii, A. niger* and *Fusarium* sp. The 10 µl culture filtrate of the potential antifungal organism in presence of sorbitol (0.6 M) was added to the plates. The bursting of the hyphal tips was monitored microscopically up to 3 h. The hyphal tips (15-20) per field were counted and the number of tips bursted in 10 fields were counted to find hyphal tip bursting test (% HTB) in a stipulated time as indicated.

2.6 Biochemical techniques

2.6.1 Isolation of cell wall

The cell wall analysis was carried out to analyze the chitin and chitosan contents of *M. anisopliae* as described by Khale and Deshpande (1992). The YPG grown (72 h at 28 ^{0}C) mycelium was used for the cell wall analysis. The mycelium was crushed with

mortar and pestle in the presence of liquid nitrogen. The crushed mycelial mass was transferred to a centrifuge tube and sedimented in cold distilled water at 1500g for 10 min and pellet was washed 5-6 times in cold distilled water. The pellet was further subjected to and washed with decreasing concentration of cold NaCl solution (5%, 2% and 1%, w/v) followed by (10-12 times) cold distilled water. The cell wall preparation was clean and free from cytoplasmic material as determined by light microscopy. The purified cell walls were lyophilized and stored at -10^{0}C.

2.6.2 Hydrolysis of chitin

The enzymatic hydrolysis of the practical grade chitin was carried out using extracellular broth of *M. anisopliae* grown on YPG (72 h) and chitin medium (96 h) under shaking condition .The enzyme activities in the broth of *M. anisopliae* grown in YPG were (U/ml): CDA, 0.26; chitosanase, 0.31; *N*-acetylglucosaminidase, 0.0034; protease, 0.1; lipase, 0.312 and in chitin medium were (U/ml): chitinase, 0.01; *N*-acetylglucosaminidase, 0.0016; CDA, 0.07; chitosanase, 0.15; protease 0.1; lipase, 0.15. For the hydrolysis experiment, the reaction mixture contained chitin, 5 g, culture filtrate ,100ml (either singly or 50 ml of each from YPG and chitin medium grown). Bacterial contamination was avoided by the addition of sodium azide (0.01 g). The reaction mixture was incubated at 28° C under shaking conditions for 24 h.

2.6.3 Estimation of reducing sugars

The reducing sugar was estimated by Somogyi , (1952), GlcNAc and glucosamine were measured by modified Elson-Morgon method (Reissig *et al* , 1955). All the above estimations were done in triplicate. Percentage of hydrolysis was determined using the following formula:

$$\% \text{ Hydrolysis} = \frac{\text{Amount of reducing sugars}}{\text{Amount of substrate}} \times \frac{203}{221} \times 100$$

2.6.4 Protein estimation

Protein was estimated according to Lowry *et al*, (1951) using crystalline bovine serum albumin as standard.

2.6.5 Solubilization of melanin

Melanin was solubilized with 0.5M NaOH at 60°C for 24h. The solution was centrifuged and the clarified solution was adjusted to pH of 7.2 and stored at 4°C.

2.6.6 Ultrafiltration

The crude culture filtrate of *M. anisopliae* was concentrated using Amicon ultrafiltration unit with YM10 (10,000 mol. wt cut-off) membrane.

2.6.7 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was performed in 7.5% (w/v) polyacrylamide slab gel at pH 8.9 (Trudel and Asselin, 1990).

2.6.7.1 Detection of chitin deacetylase activity after PAGE under native conditions

After electrophoresis, gels were incubated in 50 mM Hepes-KOH at pH 7.0 (200 ml per gel) with gentle shaking for 5 min at room temperature. They were then put on a clean glass plate (80 x 170 mm) and covered with a 7.5% (w/v) polyacrylamide gel containing 0.1% (w/v) ethylene glycol chitin in 50 mM Hepes-KOH buffer at pH 7.0. The close contact between the two gels was made by gently sliding a 12x75-mm test tube on top of the overlay gel, which was still wet with buffer. Gels were incubated at 37^0C for 4-5 h in plastic container under moist condition. After incubation, gels were transferred in a fresh solution of 0.01% (w/v) Calcoflour white M2R in 0.5 M Tris-HCl

(pH 9.0 buffer). The gels were removed after 5 min and washed with several changes of distilled water for at least 2 h. Proteins exhibiting activity were visualized under UV light and photographed under gel documentation system. The CDA activity appeared as a blue-white band, which was more intense than the background fluorescence observed due to intact chitin still remaining in the gel. Depolymerisation of chitosan generated after enzymatic deacetylation was made by exposure to nitrous acid at acidic pH. After incubation of native gels, gels were exposed to nitrous acid generated by mixing just before use 72 ml of 5.5 M $NaNO_2$ with 28 ml of 1 N H_2SO_4. After 10 min in nitrous acid, gels were washed in distilled water for 5 min and processed for staining with Calcoflour white M2R as previously described. Chitin deacetylase activity was detected after SDS-PAGE by renaturation with 1% Triton X-100 in 50mM Hepes-KOH buffer (pH 7.0) according to Trudel and Asselin, (1990).

2.6.7.2 SDS-PAGE

The PAGE under denaturing conditions was performed in 7.5% (w/v) polyacrylamide gels at pH 8.9. Samples were not heated prior to electrophoresis. Pre-stained molecular weight markers (Bio-Rad) were lysozyme (20,700), soybean trypsin inhibitor (29,100), carbonic anhydrase (34,000), ovalbumin (52,000), bovine serum albumin (70,000), β-galactosidase (121,000) and Myosin (205,000).

2.6.7.3 Protein staining after electrophoresis

Protein staining was done using silver nitrate (Blum *et al,* 1987).

2.6.8 Determination of chitin and chitosan contents in cell wall

The total hexosamines from the cell walls (10mg) were extracted by hydrolysis in sealed tubes under nitrogen with 1ml of 6M HCl at 105^0Cfor 8h. HCl was evaporated *in vacuo*, the residue was dissolved in distilled water and insoluble material was removed by centrifugation. Chitin and chitosan contents were estimated by suspending 10mg of cell walls in 1ml of distilled water and treating with 1.5ml of 2M $NaNO_2$, and 0.5ml of 2M HCl (4h at room temperature) The residue obtained after centrifugation was hydrolyzed in 6M HCl at 100^0C for 14h. After removing HCl *in vacuo* the glucosamine contents were determined for two fractions: the fraction insoluble in HNO_2 corresponding to chitin and the rest to chitosan. The respective controls were prepared according to Khale and Deshpande, (1992). The methods of Good and Bessman, (1964) and Reissig *et al,* (1955) were followed for total glucosamine and GlcNAc estimation.

2.6.9 Thin layer chromatography of cell wall synthesis inhibitor(s)

The 10 times concentrated *Bacillus* 102 culture filtrate by freeze-drying was used for liquid-liquid extraction for 14 h using different organic solvents (8 times) such as ethyl acetate, chloroform, hexane, benzene, etc at room temperature.

The two layers were separated, solvent was evaporated and the residue was dissolved in the distilled water containing 0.6 M sorbitol for HTB test. On a large scale, the 1000 ml cell-free culture filtrate was concentrated to 100 ml and extracted for 15 h with 500 ml chloroform at room temperature. The chloroform layer was separated, dried over anhydrous sodium sulfate and evaporated to dryness under *vacuo*. The crude residue (1.2 g) revealed 6 different spots by Thin Layer Chromatography (TLC). Further separation was achieved on Silica Gel (60-120 mesh) column eluted with an

increasing gradient elution of ethyl acetate: petroleum ether (1:9 → 10:0) and then with methanol: ethyl acetate (1:9 → 5:5). Total 30 fractions of 50 ml each were collected, using TLC analysis. One main fraction was further purified by preparative TLC [Silica Gel PF 254, solvent system ethy1 acetate: petroleum ether (20:80)]. This fraction furnished two components. The one major spot was characterized using Infrared (IR), Nuclear Magnetic Resonance (NMR) and mass spectrometry.

All experiments were carried out three times in duplicate, unless otherwise mentioned.

2.6.10 Microscopy

2.6.10.1 Detection of CDA activity on insect (*H. armigera*) cuticle using light microscopy

M. anisopliae spores (10^6) were spread on the isolated *H. armigera* third instar larval cuticle stretched on a slide with a thin layer of YPG with 2 % agar. The spores were allowed to germinate and grow for 48 h and the cuticle with and without spores were subjected to MBTH staining specific for chitosan. The staining was visualized and photographed using Leitz Laborlux microscope with a WILD MPS32 camera.

2.6.10.2 FITC-WGA staining of the insect (*H. armigera*) cuticle treated with cuticle degrading enzyme complex of *Myrothecium verrucaria*

The bioassay was performed with CDE preparation containing chitinase activities 0.8, 1.6, 3.2 and 6.4 U/ml. Using automizer, the CDE preparation was sprayed on 3rd instar larvae of *H. armigera.* The inactivated CDE preparation and distilled water were used as controls. Each treatment consisted of 10 larvae and repeated thrice. Immediately after CDE treatment, the chitinase on larval cuticle was determined by dipping these

larvae in chitinase assay reaction mixture. The mortality was recorded after 72 h, 120h and 144h.

To determine the effect of CDE preparation on insect cuticle, 3rd instar *H. armigera* larvae were treated with CDE preparation containing 6 U/ml chitinase. One set of larvae received single spraying while the other received two sprayings at an interval of 48h. Larvae in control set were treated with distilled water. Each treatment consisted of 30 larvae and replicated 4 times. Larvae were observed for mortality up to 4 days and dead larvae were kept at 4°C. After 4 days all surviving larvae were killed by chloroform treatment. The larval cuticle was removed in Ringer's solution (NaCl, 0.9%, KCl, 0.0429%, $CaCl_2$, 0.025%). The amount of chitin in larval cuticle was estimated according to Ishaaya and Casida, (1974).

Some of the larvae from test and control sets were fixed in Carnoy's solution (Glacial acetic acid 1 part, chloroform 3 part and absolute ethanol 6 part). Dehydration of the tissue was done by deeping the tissue in v/v (30%, 50%, 70%, 85%, 90%, 100% ethanol, each step is for 30 min and then in 3:1, 1:1, 1:3 ethanol: Xylol, each step is for 1h and then embedded in paraffin wax. 5-7μ thick transverse sections of larvae were taken using microtome as described by Sharma and Sharma, (1980). Deparaffinised sections were then stained using a fluorescent lectin, FITC-WGA which specifically binds to chitin. (Ludmilla *et al*, 2002) The fluorescence was observed by epifluorescence microscope (Leitz Labor Lux) using the I3 filter with an excitation range of 450-490nm. The photographs were recorded by WILD MPS 32 camera unit.

2.7 Application of biocontrol agent

2.7.1 Laboratory studies

2.7.1.1 Insect culture

The initial culture of *H. armigera* was established by collecting larval and pupa stages from the fields. The rearing of larvae was done individually in plastic vials on vegetable diet (Okra) disinfected for 10 min with 0.5 % sodium hypochlorite as described by Ignoffo *et al*. (1975). The temperature and relative humidity in the insect rearing room were maintained at 25±2 ° C and 65±5 %, respectively.

2.7.1.2 Insect bioassay

The fungal isolates were grown on PDA and SMYP agar, incubated in dark for 14 days at 25^{o}C. Viability of conidia powder was tested in different formulations as described by Ibrahim *et al,* (1999). For the bioassay, conidial suspensions (1 x 10^{7} conidia /ml) of the different isolates were made by scraping conidia from 14 days old culture in 0.1 % Tween 80. Susceptibility was evaluated by directly dipping third instar larvae of *H. armigera* in 30 ml of conidial suspension for 5 sec. Three replicates of 20 larvae were used in each experiment. As a control, 3 batches of 20 larvae were treated with 0.1% Tween 80 in sterile distilled water. After treatment, each larva was kept in a separate plastic vial (42 x 65 mm) containing moist Whatman No.1 paper and allowed to feed on disinfected okra pieces and was incubated at 25±1^{0}C, 70±10 percent RH and 16:8(L:D). The mortality was recorded upto 14 days. The dead larvae were placed in a sterile petri plate containing a moist cotton swab to allow mycelial growth over the cadaver for 3 days. Mortality was corrected by Abbott's (1925) formula.

2.7.2 Pot experiment

The plant pathogen, *S. rolfsii* was grown on YPG medium for 48 h at 28°C. The garden soil was sterilized by autoclaving and then infested with mycelial mass of *S.*

rolfsii. In each pot four peanut seeds were sown. The plants were irrigated with, 1) culture filtrate of *M. verrucaria* diluted to give chitinase activity, 0.04 U/ 50 ml;
2) culture filtrate of organism containing potential cell wall synthesis inhibitor (1 mg/ 50 ml) and 3) combination of 1 and 2. Two control pots (C1and C2) *viz.* seeds sown in sterile soil and infested soil were irrigated with tap water. The observations were noted after 21 days.

2.7.3 Field trials

The field evaluation of three strains of entomopathogenic fungi to control *H. armigera* infestation on pigeon pea and chickpea were carried out in randomized block design with four replications .The normal agronomy practices except plant protection measures. The oil formulation of conidia (5 x 10^{12} conidia/3L Diesel: Sunflower oil, 7:3) was sprayed with the Ultra Low Volume (ULV) sprayer (70ml/min; 3L/ha). The endosulfan (2ml/L, 500 L/ha) and *Ha*NPV (250 LE/ha) (MPKV, Rahuri) were sprayed with Hand Compression Knapsack sprayer. The persistence of inoculum in the field was determined by collecting *H. armigera* larvae after 0,3,5,7 and 14 days after spraying. These larvae were then kept under observation for a period of 14 days and after death were kept in a plastic vial containing moist filter paper and incubated at 25 $\pm$ 2^0C, relative humidity (70$\pm$10) to observe mycosis. The persistence of the inoculum on the larval population was adjudged based upon the percent mortality data of the larvae collected from the field after spraying. Field studies were evaluated on the basis of percent efficacy (Henderson and Tilton, 1955), percent pod damage and percent yield which were determined according to Hassani , (2000).

CHAPTER III

USE OF *Metarhizium anisopliae, Beauveria bassiana* AND *Nomuraea rileyi* FOR THE CONTROL OF *H. armigera* ON PIGEON PEA AND CHICKPEA CROPS.

3.1 Introduction

The gram pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is a cosmopolitan, polyphagous pest attacking more than 182 host plants belonging to 47 botanical families in the Indian subcontinent (Pawar , 1998). A pulse crop, Cajanus *cajan* (pigeon pea) is an important crop in semi-arid tropical and sub-tropical farming systems, providing high quality vegetable protein, animal feed and firewood. Insects feeding on flowers, pods and seeds are the most important biotic constraints affecting pigeon pea yield. Insects feed on all parts of the pigeon pea plant. Pigeon pea is heavily infested by two important insect species, mainly *H. armigera* and *Maruca vitrata* (=testulalis) (Pyralidae). *H. armigera* is a major biotic constraint to increasing pigeon pea production. The loss in yield of pigeon pea, all over world has been estimated to be 45 % (Bhatnagar *et al*, 1982), while in India, > 60% loss in yield (Anonymous, 1994) has been reported. Chickpea, (*Cicer arietinum*) is the premier pulse crop contributing 45 % to the National pulse basket followed by pigeon pea with 22 %. Especially southern and central part of India face a problem of *H. armigera* infestation on pulses. More than 50 species of insects infest chickpea under field and storage conditions. Most of the insect damage, however, occurs in the reproductive phase, mainly on pods and seeds and can significantly reduce the crop yields of chickpea as there is not much time left for plant to compensate this loss at later stage of plant maturity. The major insect pests on chickpea include , the gram pod borer, *H. armigera;* the black cutworm*, Agrotis ipsilon;* the black aphid , *Aphis craccivora* ; the termite , *Odontotermes obsesus* and the semilooper , *Autographa nigrisigna* (Asthana *et al*, 1997) .

The key pest status of *H. armigera* is due to larval preference for feeding on plant parts rich in nitrogen such as reproductive structures and growing tips. The life cycle of *H. armigera* is as depicted in Figure 3.1.

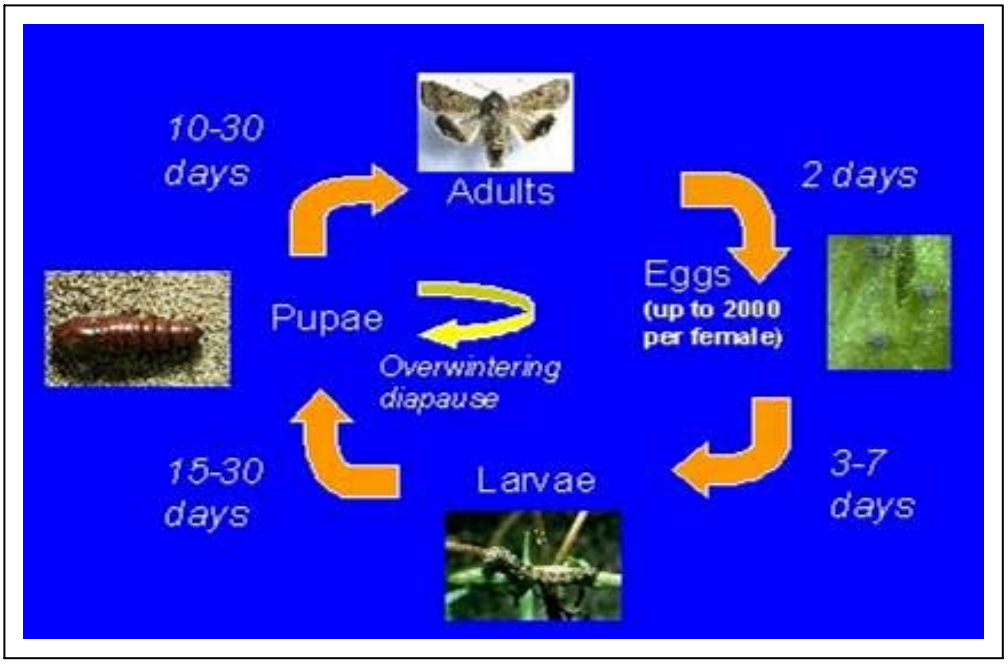

Figure 3.1 Life cycle of *Helicoverpa armigera*

Females oviposit at night and fecundity is high, with upto 2000- 3000 eggs reported from a single female. The eggs are white and nearly spherical when freshly laid and darken with age. Eggs hatch in 3-7 days, and the number of instars from 5 to 7 varies with temperature and host plant. The generation time of *H. armigera* is variable. It ranges from 28-42 days. Pupation occurs in a pupal cell 2-18 cm below ground. The prepupal stage lasts for 1-4 days. The pupal stage requires 10-14 days for nondiapausing individuals but may last several months during diapause. Usually, after the pigeon pea crop *H. armigera* shifts on to chickpea crop which is another susceptible host.

In India, for the *H. armigera* management, calendar sprays are recommended and followed, with the first application at 50% flowering and second and third application at 15 days intervals (Sachan, 1992). In Asia, *H. armigera* has developed high levels of resistance to organophosphates and synthetic pyrethroids (Armes *et al*, 1996). This has resulted in failure in control measures and a lack of confidence in insecticide (Reynolds and Armes, 1994). The rapid increase in pesticide use on pulses is alarming and emphasizes farmers' concern with insect pests. Thus, there is a need for the safe and effective pest management strategies.

The difficulty in managing insecticide-resistant populations of *H. armigera* has given impetus to the development and use of alternative insecticides such as plant derived-products e.g. Neem (*Azadiracta indica*) and insect pathogens particularly the *Helicoverpa* nuclear polyhedrosis virus (NPV). The use of NPV to control *H. armigera* has received much attention, particularly in India, though reliable control on pigeon pea has not been obtained (Shanower *et al*, 1997). Both neem and NPV products suffer from poor and highly variable quality, development of resistance, and a more limited distribution network than conventional insecticides. Alternative pest control strategies have to be employed to overcome such problems. In these regards, use of fungi-based preparations can play an important role because they are safe, act by contact, and kill the insect in 3-4 days time with no resistance reported so far.

The mycoinsecticides based on deuteromycetous fungi such as *Metarhizium anisopliae* (Agarwal, 1990), *Beauveria bassiana* (Sandhu *et al*, 2001), *Nomuraea rileyi* (Tang *et al*, 1999) have been reported to be useful to control insect pest. However, the practical utility of these entomopathogenic fungal strains against *H. armigera* on pulses

was not demonstrated (Patel, 1975). One of the reasons could be the possible inhibition of fungal growth in the presence of acidic exudates of the chickpea leaves.

In the present chapter, indigenous isolates of entomopathogenic fungi have been isolated, and selected on the basis of their pathogenecity towards *H. armigera* under laboratory conditions, and then used to determine their effectiveness in the control of *H. armigera* in pigeon pea field and these entomopathogenic fungi have been tested for their growth in the presence of chickpea leaf extract and subsequently have been used to control *H. armigera* infestation on chickpea crop under field conditions. Furthermore, the biochemical characterization of cuticle degrading enzymes such as chitinase, protease and lipase has been carried out to understand the host-pathogen interaction.

3.2 Results and discussion

3.2.1 Isolation and selection of best strains of entomopathogenic fungi

A total of 44 cultures were isolated using different methods of isolation as described under Materials and Methods. *M. anisopliae* (22), *N. rileyi* (15) and *B. bassiana* (7) isolates were identified (Figure 3.2) on the basis of morphology and then analyzed on the basis of percent mortality and biochemical activities. The *M. anisopliae* and *B. bassiana* isolates from custard apple fields showed > 70% mortality of *H. arimgera* in the bioassay. Furthermore, all the *M. anisopliae* isolates were isolated by the soil plating method, while *B. bassiana* strains by *Galleria* bait method. It has been reported that the success of *Galleria* bait method was the isolation of maximum number of *B. bassiana* isolates as compared to the other two entomopathogenic fungi (Maranhao and Santiago-Alvarez, 2003). The *N. rileyi* strains were isolated directly from the dead *Spodoptera* larvae from the fields. The *N. rileyi*

strains could not be isolated by soil dilution method that could in turn be attributed to its slow growth rate.

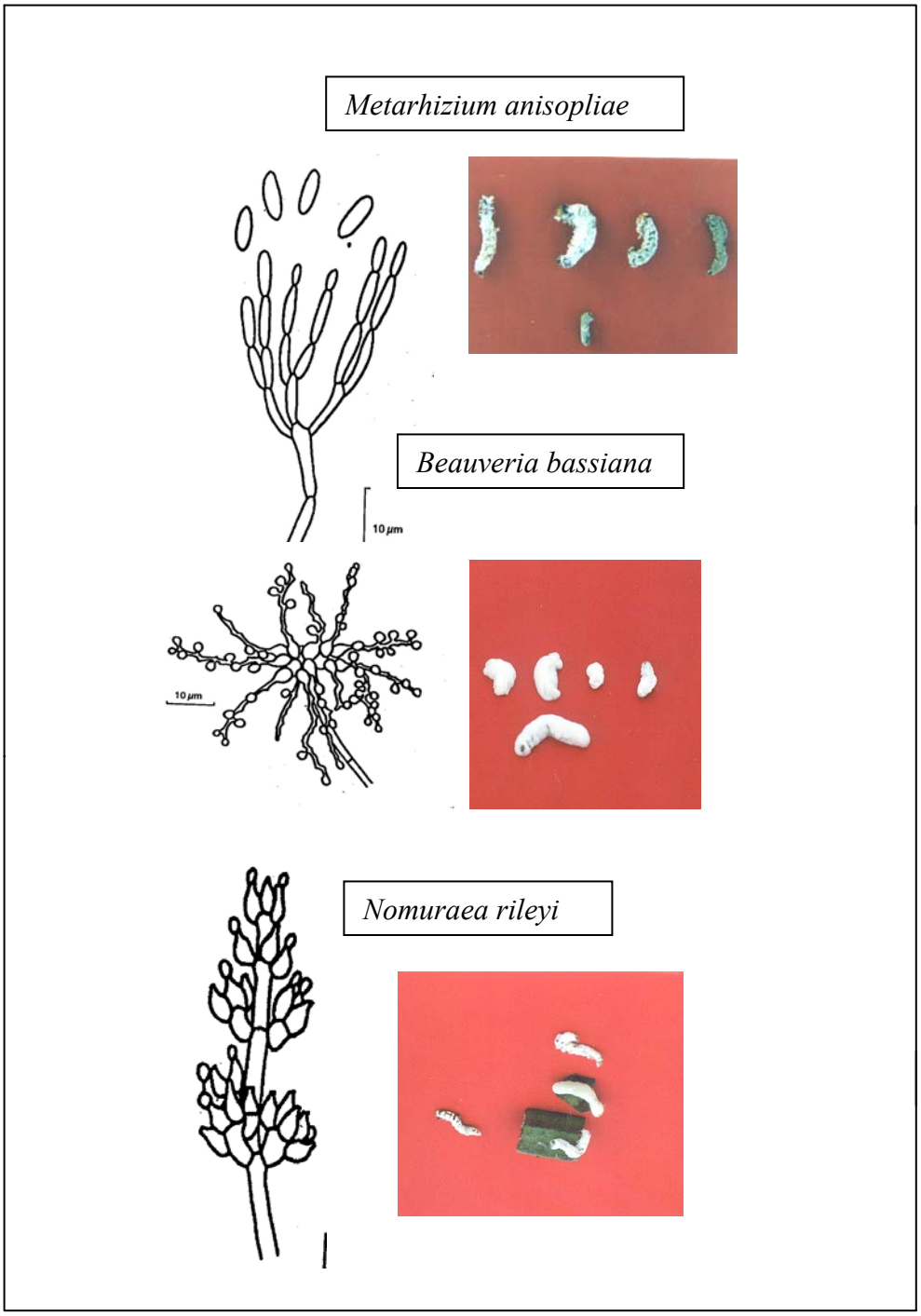

Figure 3.2 Morphology of *M. anisopliae, B. bassiana, N. rileyi* and their pathogenecity towards *H. armigera*

3.2.2 Insect bioassay and cuticle degrading enzyme activities of entomopathogenic fungi

All the isolates were tested for their comparative infectivity against *H. armigera* and were also analyzed for the production of cuticle degrading enzymes. For penetration through the insect cuticle, deuteromycetous fungi such as *Metarhizium* and others produce chitinase, protease and lipase, popularly known as cuticle degrading enzymes, in a co-ordinated manner (Krieger de Moraes *et al*, 2003; St Leger *et al*, 1986 a). It can be seen from Table 3.1 that *M. anisopliae*, *B. bassiana* and *N. rileyi* isolates exhibited differential levels of cuticle degrading enzyme activities for the different % percent mortality values. *M. anisopliae* isolates exhibiting > 70% mortality had high levels of chitinase (2.91 ± 1.05 U/ml x 10^{-3}), protease (2.49 ± 0.89 U/ml) and lipase (0.70 ± 0.29 U/ml) while there was a decrease in enzyme levels in the isolates exhibiting less % mortality (50-70% and 50%) (Table 3.1). *B. bassiana* strains with > 70 % mortality against *H. armigera* exhibited higher levels of chitinase (6.54 ± 0.33 U/ml x 10^{-3}), and lower levels of protease (0.91± 0.08 U/ml) and lipase (0.35 ± 0.02 U/ml) activities in comparison with *M. anisopliae* isolates exhibiting > 70% mortality . Also, when the % mortality declined, there was a decrease in the enzyme activities of *B. bassiana*. *N. rileyi* isolates did not exhibit detectable chitinase levels even upto 120 h. St. Leger *et al* (1986) reported the early appearance of protease and lipase during *in vitro* production of cuticle degrading enzymes in *M. anisopliae.* This has been attributed to the layering of cuticular components. The absence of detectable level of chitinase activity in *N. rileyi* strain can be co-related to its slower growth. *N. rileyi* N 812, the only *N. rileyi* isolate exhibiting > 70% mortality against *H. armigera*, did not show any chitinase activity, but had low levels of protease (0.71± 0.08 U/ml) and lipase (0.50 ± 0.06 U/ml)

activities in comparison with *M. anisopliae* and *B. bassiana* isolates and with the reduction in % mortality , the enzyme activities were also affected (Table 3.1). This suggests that the role played by different cuticle degrading enzymes in various organisms is likely to be one of the important factors responsible during the insect-pathogen interaction.

This indicated that cuticle-degrading enzymes are important in the process of insect pathogenesis. Sequential production of these enzymes in higher amounts at an early infection stage facilitates faster kill of the insect. These enzymes play a multipurpose role in the biology of insect pathogens. Under *in vitro* conditions, fungi may be induced to produce extra cellular chitinases that digest available chitin substrates. Alternatively, constitutive chitinases, localized in the fungal fungal cell wall or in the periplasmic spaces, play a role in fungal cell wall softening allowing for mycelial branch initiation or release of daughter cells (Cabib *et al*, 2001). *In vivo*, chitinases could potentially disrupt the cuticle barrier, providing the penetrant germ tube access to nutrients. Under *in vitro* conditions, *Metarhizium anisopliae* ME1 produced multiple chitinase isozymes including both endochitinases and an exochitinases. It has been proposed that a cocktail of serine- proteases, metalloproteases, subtilisin-like proteases and aminopeptidases act on peptides cross-linking the chitin fibrils providing substrate for the fungal chitinases. However, Jackson *et al*, (1985) reported that chitinase deficient mutants of the insect mycopathogen *Verticillium lecanii* were able to penetrate and infect aphids. Furthermore, recombinant *M. anisopliae* strains engineered to overproduce chitinase did not possess altered virulence to *Maduca sexta* larvae (Screen *et al*, 2001).

Table 3.1 Comparative analysis of insect bioassay and biochemical characteristics of *M. anisopliae, B. bassiana,* and *N. rileyi* isolates

Organism	% mortality	Activities ± SE			Strains
		Chitinase U/ml x 10^{-3}	Protease U/ml	Lipase U/ml	
M. anisopliae	>70	2.91±1.05	2.49 ± 0.89	0.70 ± 0.29	M 34412, M 1333, M 2427, M 1322, M 34513, M 34210, M 34311, M 2104, M 2416, M 45317
	50-70	1.24 ± 0.49	0.92 ± 0.48	0.30 ± 0.16	M 1311, M 3419, M 2305, M 2508, M 45216, M 79322, M 45115, M 79120, M 42014, M 51118
	< 50	0.52 ± 0.06	0.36 ± 0.06	0.08 ± 0.01	M 51219, M 79221
B. bassiana	>70	6.54 ± 0.33	0.91 ± 0.08	0.35 ± 0.02	B 3301, B 3437
	50-70	4.79 ± 1.27	0.57 ± 0.18	0.23 ± 0.07	B 3426, B 22504, B 23405, B 20603
	< 50	2.7 ± 0.02	0.33 ± 0.01	0.13 ± 0.01	B 20202
N. rileyi	>70	ND	0.71 ± 0.08	0.5 ± 0.06	N 812
	50-70	ND	0.58 ± 0.08	0.37 ± 0.08	N 81, N 82, N 83, N 84, N 85, N 86, N 810, N 814, N 815
	< 50	ND	0.29 ± 0.14	0.20 ± 0.06	N 87, N 88, N 89, N 811, N 813

ND- Not detected, SE- Standard error
The values presented are the average of three replications.

In view of the biochemical studies and insect bioassay results, one isolate from each group, M. *anisopliae* M 34412, *B. bassiana* B 3301 and *N. rileyi* N812 were

selected for the further studies such as large scale production of spore, formulation studies, and field experiments.

3.2.3 Solid-state fermentation

The production of conidia was significantly different among the fungal isolates on different agricultural products (Vimala Devi *et al*, 1994; Zhang and Watson, 1997). The solid-state fermentation studies were carried out on different substrates like sorghum, wheat, beaten rice, and rice. Maximum conidia production was found after 14 days on rice in *M. anisopliae* M34412 (40 g/kg), *B. bassiana* B3301 (10g/kg) and *N. rileyi* N812 (6.47g/kg) with spore count of 5.61x 10^{10}, 1 x 10^{11}, 6.47x 10^{10} spores/g of dry powder, respectively. The protocol used for conidia production is depicted in Chart 3.1.

Chart 3.1 Production of conidia of entomopathogenic fungi

Stock culture (7 d)

↓

Liquid fermentation (48-72 h)

↓

Solid state fermentation on rice (2 weeks) using
Unicorn bags (64 x 36 cm) with, Membrane type M.

↓

Mycoharvestor (after drying)

↓

Yield

↓

Dry spore powder

↓

Storage at 4 ^{0} C until used

3.2.4 Formulation studies

The combination of formulation, application and the selection of the strain is one of the key steps for field trials. Use of different oil based formulations for mycoinsecticides has been extensively studied (Lomer and Lomer, 2001). Table 3.2 shows that the conidial germination of *M. anisopliae* M34412 in sunflower oil, diesel: sunflower oil mixture (7:3) and Tween 80 (0.1%) was >90% in 12 h.

Table 3.2 Effect of different oil formulations on the germination of conidia of the selected fungal isolates

	Conidial germination (%) ± SE		
Formulation [a]	*M. anisopliae* M34412[b]	*B. bassiana* B3301[b]	*N. rileyi* N812 [c]
Diesel	86.7 ± 1.3	47.3 ± 2.2	72.6 ± 2.3
Sunflower	92.7 ± 1.9	55.8 ± 1.8	81.8 ± 1.4
Diesel : Sunflower oil (7:3)	95.9 ± 1.6	66.7 ± 1.7	84.7 ± 1.7
Safflower oil	86.1 ± 2.1	41.8 ± 1.3	79.8 ± 1.7
Diesel : Safflower oil (7:3)	78.1 ± 3.2	52.2 ± 2.1	78.0 ± 2.8
Groundnut oil	88.9 ± 1.8	43.8 ± 2.1	50.7 ± 3.7
Tween 80 0.1%)	99.4 ± 0.4	84.9 ± 1.0	85.1 ± 1.6

a, conidia were suspended in the formulation (1 x 10^7/ml) at room temperature for 1h; b, percent germination after 12h; c, percent germination after 36h. SE - Standard error

There was significant difference in the percentage of germination and percent mortality in different oil formulations. In case of *B. bassiana* B3301, as compared to Tween 80 (84.9%), the diesel: sunflower oil mixture showed lower % germination (66.7%) in 12 h. In case of *N. rileyi* N 812 the conidial germination was relatively slow. It was >80% in the presence of sunflower oil, diesel: sunflower oil mixture (7:3), diesel: groundnut oil mixture (7:3) and Tween 80 (0.1%) after 36h. The less percent germination was seen in safflower oil, groundnut oil and their combination with diesel which can be attributed to the high viscosity of the oil which aggregated spores and reduced the germination. Similar observations were also reported by Ibrahim *et al,* (1999) for *M. anisopliae*. It has been suggested that oil formulation can prevent conidial desiccation, and improve adhesion of conidia to the hydrophobic surface of insect cuticle (Inyang *et al*, 2000; Vimala Devi and Prasad, 1996).

Patel, (1975) suggested that the acidic exudates of the chickpea plant affected the activity of the parasitoids, which was the reason for their failure. However, as shown in Table 3.3, presence of the chickpea leaf extract did not affect the conidial

Table 3.3 Effect of chickpea leaf extract on the conidial germination of entomopathogenic fungi

Isolate	% Germination	
	YPG/SMYP agar	Chickpea leaf Extract
M. anisopliae M 34412	97 ± 1.5	97 ± 2.5
B. bassiana B 3301	84.22 ± 0.78	78.11 ± 2.5
N. rileyi N 812	75.11 ± 2.2	69.66 ± 1.6

The values indicated are the average of three replicates with < 15% variation.

germination of the selected fungal isolates when compared with the conidial germination on YPG/SMYP medium.

Inyang *et al*, (2000) reported that sunflower oil/ Shellsol T formulations enhanced the infectivity of *M. anisopliae* for mustard beetle, *Phaedon cochleariae*. Figure 3.3 depicts the percent mortality of *H. armigera* with the three isolates in different oil-based formulations. In the dip method, though all the tested formulations were found to be effective (>50% mortality with all the isolates), in a diesel: sunflower oil mixture and Tween 80 (0.1%) ≥90% mortality with all the three isolates was observed. For the field studies, the conidia were mixed with the formulation just before application in the field.

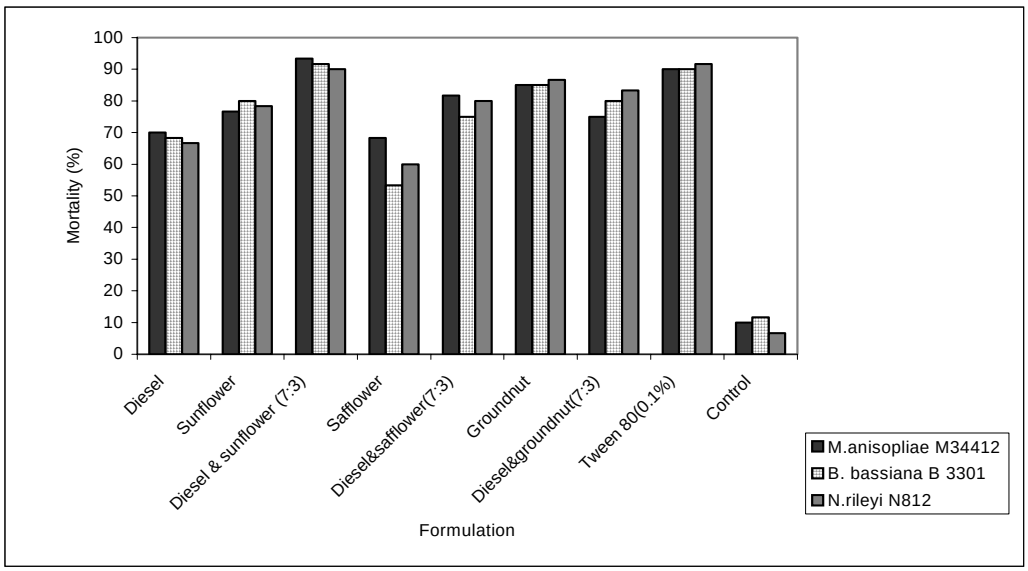

Figure 3.3 Percent mortality of *Helicoverpa armigera* with different oil formulations of entomopathogenic fungi ,Control (0.1%, Tween 80 without conidia)

3.2.5 Field evaluation

The results of the field trial were assessed using three different parameters *viz*; percent efficacy, percent pod damage and yield (q/ha).

3.2.5.1 Pigeon pea

The percent efficacy, percent pod damage and yield (q/ha) obtained by *M. anisopliae* M34412*, B. bassiana* B3301 and *N. rileyi* N812 is presented in Table3.4.

Table3.4 Efficacy, pod damage and yield of different treatments against *H. armigera* infestation on pigeon pea under field conditions

Treatment	Cumulative efficacy (%) *± SE	Pod damage (%)± SE	Yield (q/ha) ± SE
M. anisopliae M34412 ($5x\ 10^{12}$ conidia/ha)	70.93 ± 4.19	8.76 ± 1.10	14.04 ± 1.25
B. bassiana B3301 ($5x\ 10^{12}$ conidia/ha)	55.51 ± 4.27	17.27 ± 1.17	10.18 ± 0.75
N. rileyi N812 ($5x\ 10^{12}$ conidia/ha)	62.95 ± 2.08	12.08 ± 1.72	11.61± 1.64
Endosulfan (2 ml/L, 500L/ha)	63.43 ± 0.85	10.24 ± 0.78	12.78 ± 0.95
*Ha*NPV (250 LE/ha)	57.66 ± 1.98	15.20 ± 0.91	10.64 ± 0.55
Control		23.63± 1.76	7.31±0.76

*After Henderson and Tilton, (1955), SE- Standard error

In the pooled mean, percent efficacy from the various treatments after two sprays was found to be in the range from 55.51% to 70.93% against *H. armigera*. The treatment with *M. anisopliae* M34412 was found to be the most effective showing maximum efficacy of 70.93%. Earlier research workers reported 76.7% control of *Rhammatocerus schistocecoides* with *M. anisopliae*, (Magalhaes *et al*, 2000).

The % pod damage in the *M. anisopliae* M34412 treated plot was found to be least (8.76%). As compared to the control plot (23.63% pod damage) all other treatments showed % pod damage in the range 10.24%- 17.27%. The average yield (q/ha) in the control was 7.31 q/ha while it was increased up to 14.04 q/ha in case of *M. anisopliae* M34412 treatment. The treatment with endosulfan showed the yield of 12.78 q/ha while that for *N. rileyi* N 812, *Ha*NPV and *B. bassiana* B 3301 were 11.61, 10.64, and 10.18 q/ha, respectively.

It can be seen from Figure 3.4 that the % mortality after first spraying was highest with *M. anisopliae* M34412 (87.50 %) while with *N. rileyi* N812 and *B. bassiana* was 85% and 80 %, respectively which decreased subsequently. The inoculum formulated in oils increased the efficacy of pathogen and prolonged viability of conidia (Daoust and Roberts, 1983). The persistence of the inoculum declined (<50 percent) after 7th day collection which suggested that all the three isolates exhibit 50 percent of viable inoculum upto 8 days which was proved in terms of mortality (%) (Deshpande *et al*, 2004). Vimala Devi, (1994) recorded that at the end of 6 days, the persistence of *N. rileyi* was less than 50 percent against *S. litura* on castor under field condition.

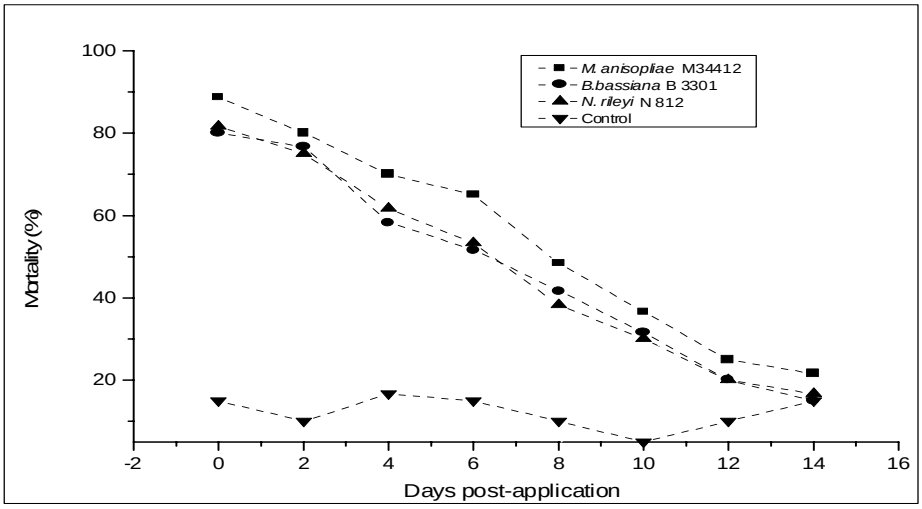

Figure 3.4 Persistence of insect pathogenic fungi after first spray in terms of % mortality of *Helicoverpa armigera* in the field
(Control, unsprayed plot)

3.2.5.2 Chickpea

The results of the field trial were assessed using three different parameters *viz.* percent efficacy, percent pod damage and yield (q/ha). It can be seen from Table 3.5 that all the treatments gave 50-80% reduction in the *H. armigera* population as compared to the control on chickpea plants under field conditions. The percent efficacy for *M. anisopliae* M 34412 was 80.38% while for Endosulfan 71.44% and for *Ha*NPV it was 70.15%. The percent efficacy for *B. bassiana* B3301 was 52.23 % and *N. rileyi* N 812 was 61.83%. In the control, percent pod damage was 26.73 % while it was 8.54%, 18.28% and 15.48% for *M. anisopliae* M34412, *B. bassiana* B3301 and *N. rileyi* N812 isolates, respectively. The percent pod damage in endosulfan and *Ha*NPV treated plots was 10.8% and 12.37%, respectively.

Regarding yield (q/ha), the yield for Endosulfan and *Ha*NPV treatment was 15 q/ha and 14.28 q/ha, respectively as compared to the control (9.05 q/ha). In case of

fungal spore treated plots, the yield for *B. bassiana* was 11.9 q/ha and for *N. rileyi* N 812 was 12.62 q/ha. *M. anisopliae* M34412 was found to be the best treatment with highest yield of 16.64 q/ha (Nahar *et al*, 2003).

Table 3.5 Efficacy, pod damage and yield of different treatments against *H. armigera* infestation on chickpea under field conditions

Treatment	Cumulative efficacy (%) *± SE	Pod damage (%)± SE	Yield (q/ha) ± SE
M. anisopliae M34412 ($5x\ 10^{12}$ conidia/ha)	80.38 ± 8.27	8.54 ± 1.41	16.64± 1.97
B. bassiana B3301 ($5x\ 10^{12}$ conidia/ha)	52.23 ± 3.07	18.28 ± 2.14	11.90 ± 1.22
N. rileyi N812 ($5x\ 10^{12}$ conidia/ha)	61.83 ± 2.54	15.48 ± 2.29	12.62 ± 1.42
Endosulfan (2 ml/L, 500L/ha)	71.44 ± 2.4	10.8 ± 1.28	15.00 ± 0.92
*Ha*NPV (250 LE/ha)	70.15 ± 4.19	12.37± 1.53	14.28 ± 1.36
Control	--	26.73 ± 4.94	9.05 ± 1.64

*After Henderson and Tilton, (1955), SE- Standard error

Thus, in the present chapter, use of indigenous mitosporic fungi for the control of *H. armigera* infestation on pigeon pea and chickpea has been demonstrated with the

successful use of oil formulation (Diesel: Sunflower oil ,7:3) , thereby raising the hopes of use of entomoptathogenic fungi in pest control. The cuticle degrading enzyme activities of all the isolates were found to be in accordance with their ability to kill the target insect, suggesting that it would be interesting to find the effect of supplementing the conidial formulation of other isolates with higher levels of cuticle degrading enzymes to increase their efficacy in pest control. Further studies on the effect of conidia on non-target organisms, stability under field conditions, etc. are essential to understand the behavior of the fungus after application. Though the oil-based formulation of *M. anisopliae* M34412 showed better performance than the chemical insecticide, endosulfan, the stability of the spore preparation under field conditions is the major concern to make the technology cost effective and viable.

Apart from the development of the effective biocontrol package, it is essential to under take studies on trans-sectorial issues for transfer of the knowledge about the production of the biopesticides, its application etc. to the farmers', socio-economic considerations etc. This requires the participatory field trials on large scale involving farmers' from different locations, teach them the application of the formulation and make them aware of the ideal spraying schedules, and help them to understand the host-pathogen interactions, so as to reduce the pest population and eventually to have better yield.

CHAPTER IV

BIOCHEMICAL STUDIES OF CHITIN METABOLIZING ENZYMES OF *Metarhizium anisopliae*

A. CUTICLE DEGRADING ENZYME COMPLEX OF *M. anisopliae* ISOLATES

B. IMPORTANCE OF EXTRACELLULARLY PRODUCED CONSTITUTIVE CHITIN DEACETYALSE AND CHITOSANASE IN BIOCONTROL OF INSECT PEST, *H. armigera*

4.1 Introduction

The major concern of developing biopesticides commercially is the speed of kill as compared to the chemical insecticides. *Bacillus thuringiensis* takes 3-4 days and NPV (nucleopolyhedroxy viruses) 5-7 days for effective control of insect pest. However, both of them face limitations in their mode of action *i.e*. the need to be ingested by the pest. On the other hand, the insect-pathogenic fungi act by contact. The myco-insecticide formulations generally comprise of spores that settle on the insect cuticle to germinate, penetrate and cause subsequent death of the 'host' within 4-7 days, (Arcas *et al*, 1999; Moorhouse *et al*, 1993) which is relatively a longer time too. The main barrier in the penetration process is the chemical composition of the cuticle. The melanization of the cuticle makes it comparatively resistant to the proteolytic and chitinolytic attack (St Leger *et al*, 1986 b). Moreover, the melanin incorporated in the exo- and endo-cuticle hampers the chitinase action (Lemburg, 1998). Bull, (1970) reported that chitinase from a streptomycetous source was most susceptible to melanin isolated from cell walls of the fungi *Aspergillus nidulans*. Therefore, it has prompted the investigation of the role of other chitin metabolizing enzymes such as chitin deacetylase and chitosanase in the penetration process of *Metarhizium* isolate that killed third instar larvae of *Helicoverpa armigera.* Chitin deacetylase converts chitin, a β- 1,4 - linked *N*-acetylglucosamine polymer into its deacetylated form chitosan, a glucosamine polymer, which is amorphous and can be degraded easily by chitosanase. In case of plant pathogen like *Uromyces viciae –fabae,* chitin deacetylase is known to adopt a defensive role against the host resistance mechanisms (Deising and Seigrist, 1995). It was interesting to find out if an analogous system existed in the fungus-insect host system.

In the present chapter, *in vitro* differential expression of cuticle degrading enzymes of *M. anisopliae* in induced and constitutive media has been studied and an attempt has been made for the first time to demonstrate the importance of chitin deacetylase in softening the insect cuticular chitin, thereby facilitating the entry of the fungus into the insect.

4.2 Results and discussion

4.2 A Cuticle degrading enzyme complex of *M. anisopliae* isolates

4.2.1A Time course for constitutive and induced production of cuticle degrading enzymes *of M .anisopliae*

The time course for extracellular production of chitin deacetylase, chitosanase and chitinase enzymes in *M. anisopliae* was studied in YPG and in chitin medium. As shown in Figure 4.1, *M. anisopliae* in YPG, in absence of a potential chitin-based inducer, exhibited constitutive production of enzymes such as CDA and chitosanase. However, chitinase activity was not detected upto 120 h. Maximum CDA (0.26 ± 0.03 U/ml) and chitosanase activity (0.31± 0.04 U/ml) were observed in 72 h.

On chitin medium, the extracellular chitinase activity appeared after 72 h, which increased, to 0.01 ± 0.001U/ml in 96h which remained same upto 120h. The production of CDA (0.07 ± 0.01 U/ml) and chitosanase (0.15 ± 0.02 U/ml) was maximum in 96 h (Figure 4.2) which were however, lower and produced later as compared to those in YPG grown culture filtrate.

The *N*-acetylglucosaminidase, protease and lipase activities in YPG (72h) were 0.0034, 0.01 and 0.312U/ml and in chitin medium were 0.0016, 0.01 and 0.015 U/ml.

In the present investigations the chitinase activity of *M. anisopliae* in the chitin containing medium was 0.01 U/ml in 120h. St Leger *et al*, (1986 a) reported chitinase activity of 0.027 U/ml in *M. anisopliae* after 120h.

St Leger *et al*, (1986 a) observed that the appearance of extracellular enzymes in entomopathogenic fungi was paralleled by the sequential solubilization of cuticle constituents. The production profile for *M.anisopliae* strain used in this study showed that CDA and chitosanase activities appeared in 24h. The CDA and chitosanase activity peaked in 72h (0.26 and 0.31U/ml) and then reduced to 0.13 and 0.15U/ml respectively, in 120h. The proteolytic activity made its appearance in 24h and was constant till 120h 0.01 U/ml, (data not shown).

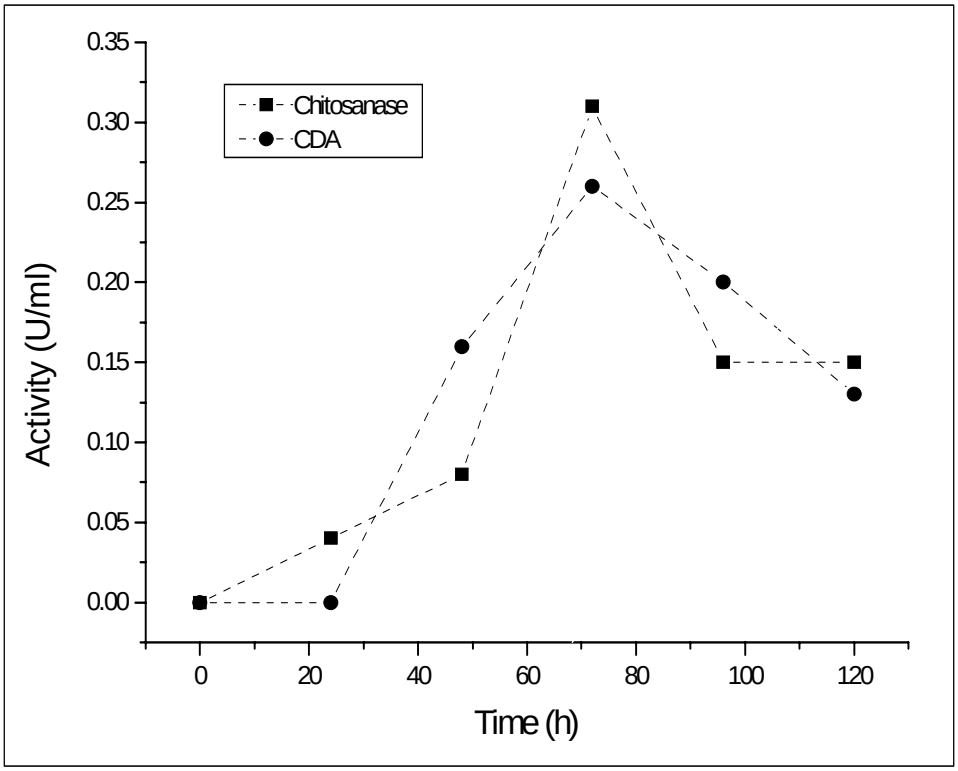

Figure 4.1 Time course for constitutive production of chitin metabolizing enzymes of *M. anisopliae* in YPG medium

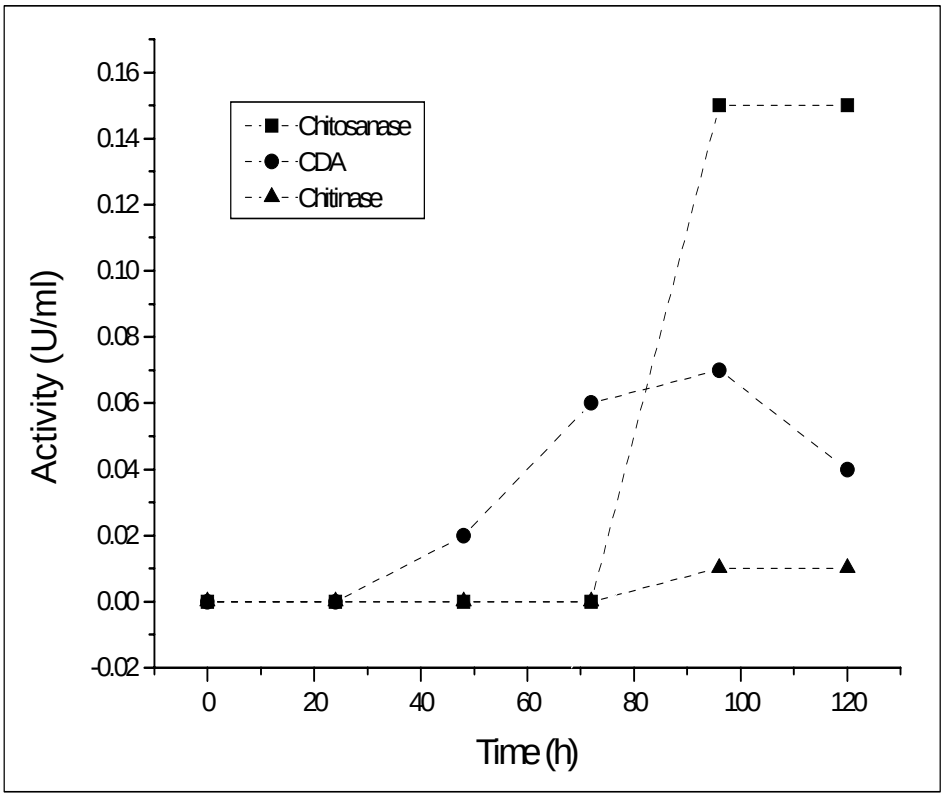

Figure 4.2 Time course for induced production of chitin metabolizing enzymes of *M. anisopliae* in chitin medium

The CDA and chitinase activities from other strains of *M. anisopliae* isolated indigenously were estimated. Interestingly other *M. anisopliae* strains which showed high constitutively produced CDA activity had low induced chitinase activities (Table 4.1).

Entomopathogenic fungi invade the insects by a combination of mechanical pressure and enzymatic degradation of the cuticle. St Leger *et al,* (1986 b) suggested that proteases play a major role in cuticle degradation by *M. anisopliae* as chitinolytic enzymes appear after the enzymes of the proteolytic complex protein that was in accordance with the cuticular structure wherein the protein masked the chitin. Recently Screen *et al*, (2001) suggested that the wild –type levels of chitinase was not limiting for cuticle penetration.

The transformants of *M.anisopliae* showing early induction of overexpressed chitinase did not show increase in pathogenicity against *M.sexta*.

Table 4.1 Constitutive chitin deacetylase and induced chitinase activities in different *M. anisopliae* isolates

Isolate No.	Chitin deacetylase activity in YPG U/ml (72 h)	Chitinase activity in Chitin medium U/ml (96 h)
M. anisopliae	0.26	0.01
M. anisopliae Isolate1	0.046	0.0284
M. anisopliae Isolate2	0.057	0.0398
M. anisopliae Isolate3	0.1	0.0212
M. anisopliae Isolate4	0.038	0.0313
M. anisopliae Isolate5	0.055	0.0325

The melanin incorporated in the exo- and endo-cuticle hampers the chitinase action (Lemburg, 1998). Bull, (1970) reported that chitinase from a streptomycetous source was most susceptible to melanin isolated from cell walls of the fungi *Aspergillus nidulans*. Therefore, it has prompted the investigation of the role of other chitin metabolizing enzymes such as chitin deacetylase and chitosanase in the penetration process of *M. anisopliae* isolate that killed third instar larvae of *Helicoverpa armigera* within 3 days.

Detection of higher levels of constitutive CDA and chitosanase activities in *M. anisopliae* suggested that CDA can be one of the important enzymes involved in cuticle degradation process (Nahar *et al*, 2001).

4.2 B Importance of extracellularly produced constitutive chitin deacetyalse and chitosanase in biocontrol of insect pest, *H. armigera*

4.2.1 B Bio-assay with *H .armigera*

The conidia of *M.anisopliae* were used for the bioassay with third instar larvae by the dip method. The 70% mortality was observed in 3 days.

4.2.2 B Detection of chitin deacetylase activity after PAGE under native conditions

Activity staining (Figure 4.3) of the enzyme by the method of Trudel and Asselin, (1990), identified presence of three CDA bands corresponding to their

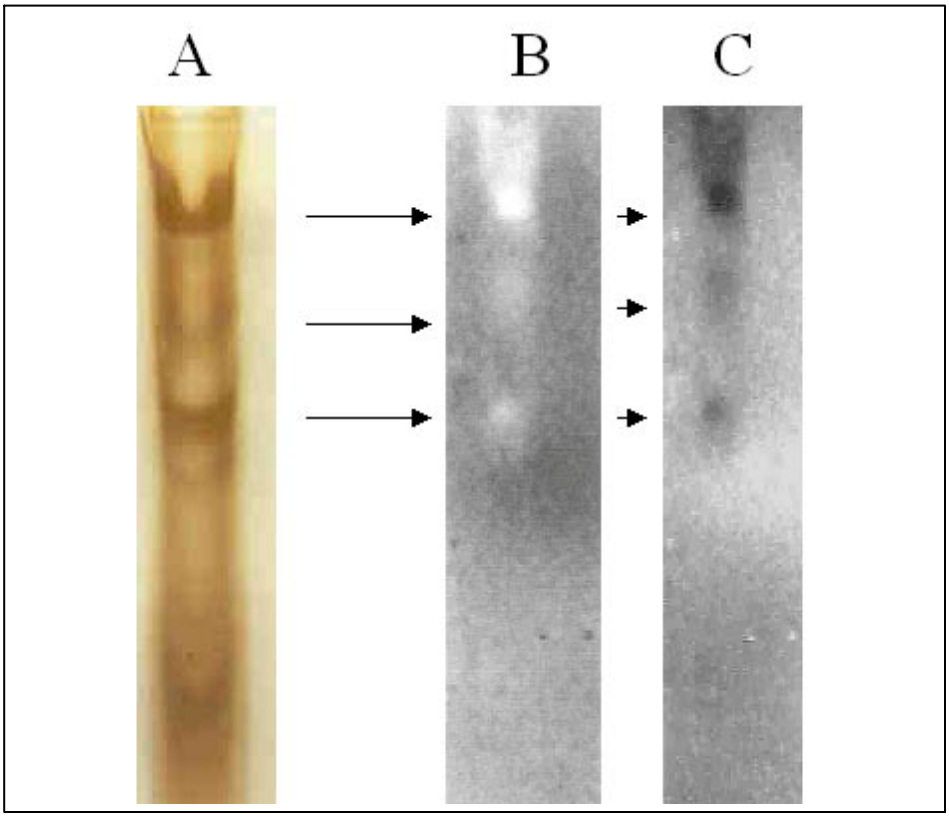

Figure 4.3 Visualization of chitin deacetylase activity on a 7.5% PAGE gel. (A) Silver staining for protein; (B) Activity staining was observed by incubating the PAGE gel with an 7.5% overlay gel containing 0.1% ethylene glycol chitin for 15-18h at 37°C. The fluorescent activity bands were visualized in UV light using calcoflour white staining (CFW, 0.01%); (C) After nitrous acid treatment, CDA activity was detected as dark bands against a fluorescent background.

respective protein bands on native-PAGE (Figure 4.3).

4.2.3 B Biochemical characteristics of chitin deacetylase from *M. anisopliae*

Some of the biochemical properties of the CDA from 72 h old extra cellular broth of *M. anisopliae* in YPG were determined. The optimum temperature for the activity was found to be 30^0 C. The substrate specificity for the enzyme was also determined using different commercially available chitin products (Table 4.2). The CDA activities with all the commercially available substrates were in the range of 0.023 ± 0.003 to 0.12 ± 0.003 U/ml (Table 4.2). This variation can be attributed to processes involved in their isolation and purification, crystallinity of the substrate and the number of deacetylated residues. Activity was undetected in case of γ-chitin. The maximum CDA activity (0.26 ± 0.003 U/ml) was detected on a soluble form of chitin, ethylene glycol chitin (Table 4.2).

Table 4.2 Substrate specificity for extracellular chitin deacetylase of *Metarhizium anisopliae*

Substrate	Activity U/ml
Practical chitin(BDH)	0.12 ± 0.003
Gentle purified crab chitin	0.063 ± 0.005
Katakura chitin	0.023 ± 0.003
Sigma α chitin	0.023 ± 0.004
Chitin CHA-2	0.115 ± 0.004
γ-Chitin	ND
Ethylene glycol chitin	0.26 ± 0.006

ND-Not detected, YPG grown (72h) culture filtrate was used as a source of CDA activity. Results are an average ± S.D. of 3 sets of triplicate experiments. Katakura, Chitin CHA-2 (Katakura, Japan), γ -chitin (Calbiochem, USA)

The *M. anisopliae* CDA activity was not inhibited in the presence of solubilized synthetic melanin and of sodium acetate added during the reaction. Melanin (10-250 μg) did not affect the CDA activity while *M. anisopliae* chitinase was inhibited by 58% (0.006 U/ml; 10μg melanin). Melanin is known to inhibit chitinolytic enzymes such as chitinase (Bull, 1970). Kuo and Alexander, (1967) demonstrated the incubation of DOPA- melanin with chitinase markedly inhibited the activity due to prevention of acetylhexosamine formation. It has been suggested that melanin may either combine with the biodegradable polysaccharides or may inhibit the enzymes participating in the wall lysis by producing transient molecules. Furthermore, Lemburg, (1998) demonstrated that the sclerotized cuticle layer in the fixed insect cuticle of *Halicryptus spinulosa* and *Priapulus caudatus* was resistant to chitinase hydrolysis.

Tokuyasu *et al*, (1996) reported that the extracellular CDA from plant pathogenic fungus, *Collectotrichum lindemuthianum* was not inhibited by sodium acetate while Kafetzopoulos *et al*, (1993) has shown that the intracellular CDA of dimorphic fungus *Mucor rouxii* was inhibited. *M. anisopliae* extracellular CDA was not inhibited in the presence of sodium acetate (1-5 mM).

Chitin deacetylase of the *C. lindemuthianum* had an optimum pH of 8 while CDA from the *M. rouxii* had an optimum pH of 5.5 (Tokuyasu *et al*, 1996; Kafetzopoulos *et al*, 1993). Extracellular chitin deacetylase from *M. anisopliae* also showed activity in the pH range 7.5-9.2. The optimum pH for the enzyme activity was found to be 8.5-8.8. It

will be interesting to find that the localization of enzyme had any influence on the optimum pH.

In case of *Uromyces viciae-fabae,* the broad bean rust fungus, five isoforms of chitin deacetylase were produced in co-ordination with the penetration of the fungus through the leaf stomata (Deising and Seigrist ,1995). The apparent molecular masses for the five CDA bands ranged from 12.7 – 48.1 kDa separated on SDS-PAGE**.** Trudel and Asselin, (1990) reported the three isoforms of CDA in *Mucor racemosus* with molecular weights of 64,000, 30,000 and 26,000 dalton and four isoforms in *Rhizopus nigricans* with molecular weights of 64,000, 35,000, 30,000 and 26,000 dalton. In case of purified CDA from *C. lindemuthianum* it was between 31.5-33 kDa while it was 80kDa for *M. rouxii* (Tokuyasu *et al*, 1996; Kafetzopoulos *et al*, 1993). The SDS–PAGE activity gel showed the presence of three bands and the apparent molecular weight was 70,000, 37,000 and 26,000 kDa (Figure 4.4). The activity observed in the presence of SDS was less as compared to the native gel.

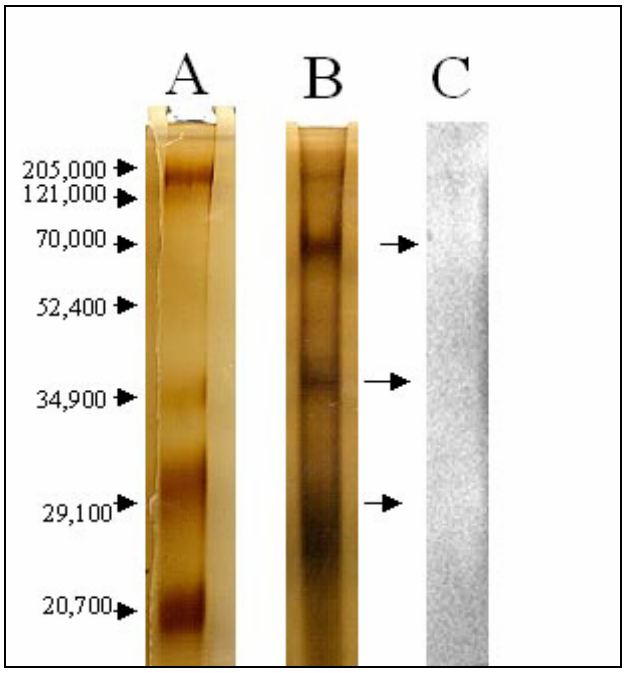

Figure 4.4 SDS-polyacrylamide gel electrophoresis
(A) Molecular weight markers (B) Protein
(C) Activity staining with CFW

4.2.4 B Hydrolysis of chitin

Using extracellular crude culture filtrate of *M. anisopliae* grown on different media, the hydrolysis of chitin was carried out. It can be seen from Table 4.3 that when the crude filtrate from the YPG grown culture was used for hydrolysis, the amount of reducing sugars produced in 24 h was more (12.85 ± 1.02 mg/ml) than the hydrolysis using enzymes from chitin medium (6.18 ± 0.67 mg/ml, Table 4.3).

Table 4.3 The enzymatic hydrolysis of chitin with *Metarhizium anisopliae* culture filtrates

	Reducing sugars (mg/ml)	Glucosamine (mg/ml)	GlcNAc (mg/ml)	%Hydrolysis*
YPG grown CF	12.85± 1.02	10.85± 0.97	ND	23.13 ± 1.156
Chitin medium Grown CF	6.18 ± 0.67	4.25 ± 0.34	0.9± 0.09	11.20 ± 0.56
YPG and chitin grown CF (1:1)	9.50 ± 0.85	8.30 ± 0.75	0.8 ± 0.06	17.10 ± 0.86

ND - Not detected ; CF - Culture filtrate. * Calculated as described under Materials and Methods. The experiment was carried out in triplicate as described under Materials and Methods.

The glucosamine contents estimated using the Good and Bessman, (1964) method, were 10.85 ± 0.97 mg/ml and 4.25 ± 0.34 mg/ml, respectively. The increase in the glucosamine contents in the hydrolysate of the YPG grown culture can be attributed to the involvement of CDA and chitosanase in the chitin modification and degradation. The GlcNAc was not detected during the hydrolysis with YPG grown culture filtrate, which can be due to the absence of detectable chitinase activity in the YPG grown CF. The 1:1 mixture of both produced reducing sugar (9.5 ± 0.85 mg/ml), glucosamine (8.3 ± 0.75 mg/ml) and GlcNAc (0.8 ± 0.06 mg/ml) during hydrolysis, suggesting that all the chitin metabolizing enzymes contribute significantly in the hydrolysis. The % hydrolysis, calculated according to the formula given in Materials and Methods, using 5% chitin as a substrate was maximum (23.13 ± 1.16 %) with the constitutively produced enzyme mixture. The % hydrolysis was increased from 11.2 ± 0.56 % to 17.1 ± 0.86 % when the chitin grown CF was supplemented with YPG grown CF.

The low level of chitinase in the induction medium indicates that the fungus inherently produced low amounts of chitinase. This may be due to the possible reliance on CDA in the cuticular degradation process for insect pathogenesis. The high amounts of CDA may be compensating for the low chitinase levels.

4.2.5B Detection of CDA activity on insect (*H. armigera*) cuticle

M. anisopliae spores were spread on the isolated larval cuticle stretched on YPG agar medium and incubated at 28 ^{0} C for 48 h. As shown in Figure 4.5, the formation of bluish patches with MBTH staining indicated conversion of chitin to chitosan due to the

action of CDA, produced during germination of spores on insect cuticle. This demonstrates that the chitin deacetylase activity is important in initiating the pathogenesis wherein CDA action softens the insect cuticular chitin to aid mycelial penetration.

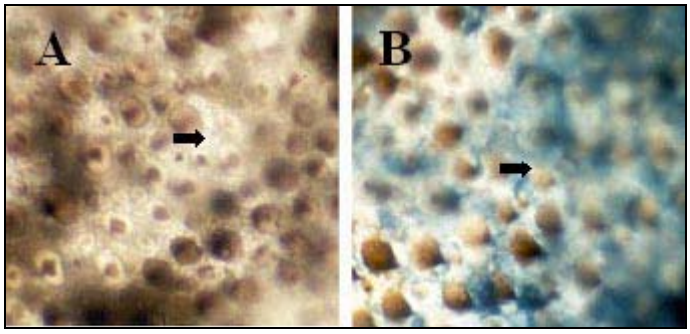

Figure 4.5 The detection of chitosan formed on the cuticle due to the germinating *M.anisopliae* spores using MBTH staining. (A) Control, absence of chitosan (B) Chitosan formation indicated by bluish patches

4.2.6B Cell wall analysis

The chitin deacetylase was reported to play an important role in plant-pathogen interaction (Kauss *et al*, 1982; Tsigos *et al*, 2000). For instance, the chitin deacetylase deacetylates the cell wall chitin of the penetrating fungal hypha for protection against plant endochitinases. The insects produce chitinase to degrade old cuticle during molting which can also act on fungal cell wall chitin. To combat the insect chitinases entomopathogenic fungi can adopt this as one of the defense mechanisms too. Therefore the cell wall analysis was carried out. The cell wall analysis showed that in *M. anisopliae* mycelium, the total hexosamine contents was 21.45% of the cell wall (Table 4.4). Chitin composed 53.62% of the total hexosamines while chitosan was 46.38% (Table 4.4). This suggests that the activity of chitin deacetylase could be playing a dual role, in the cuticle softening as well as in self- defense.

Table 4.4 Chitin and chitosan contents of cell wall of *M. anisopliae* mycelium

Cell wall	10 mg
Total hexosamine	2.145 mg (100%)
GlcNAc	1.15 mg (53.62%)
Glucosamine	0.995 mg (46.38%)

Total hexosamines, *N*-acetylglucosamine and glucosamine were determined spectrophotometrically as described under Materials and Methods .The values in parenthesis are of % chitin and chitosan contents of the total hexosamines

It has been suggested that the constitutive production of chitin deacetylase could be the primary step in the process of cuticle degradation in the fungi that show low or delayed chitinase production.

Thus in the present chapter, the production of constitutive chitin deacetylase from *M. anisopliae*, in the absence of induced chitinase, has been demonstrated. CDA converted cuticular chitin to chitosan and is thus suggested to play an important role in the cuticle softening and pathogenesis (Nahar *et al*, 2004). CDA was important in initiating the process of pathogenesis in *M. anisopliae* by softening the insect cuticle to aid mycelial penetration. Chemical assays of *M.anisopliae* cell wall composition revealed the presence of chitosan. Thus, CDA may have a dual role in modifying the insect cuticular chitin for easy penetration as well as for altering its own cell walls for defense from insect chitinases.

In view of the rising concern for pest management one of the feasible alternatives would be the isolation and identification of insect-pathogens, which can secrete high levels of chitin metabolizing enzymes, particularly chitin deacetylase. It would enable the

use of chitin deacetylase spray in conjunction with the mycopesticide formulation in the IPM to facilitate the faster kill. Of course, the cost-effective production of these enzymes needs further evaluation.

CHAPTER V

USE OF CHITIN METABOLIZING ENZYMES AND CHITIN SYNTHESIS INHIBITORS, EITHER SINGLY OR IN COMBINATION TO ARREST THE CHITIN METABOLISM IN INSECTS AND FUNGI

5.1 Introduction

The biocontrol of root-infecting fungi has been achieved successfully using mycoparasitic fungi like *Trichoderma harzianum* and *Gliocladium virens* (Papavizas, 1985). The relationship between the mycoparasitism and fungal cell wall degrading enzymes, mainly chitinases, β-1,3-glucanase and proteinases has been well established. Lorito *et al*, (1993) demonstrated a wide spectrum of antifungal activity of the chitinolytic enzymes of *T. harzianum*. While using *Serratia marcescens* chitinase, it was shown that disease development in beans by *S. rolfsii* was prolonged (Ordentlich *et al*, 1988). *Myrothecium verrucaria* produced extracellularly a complete complex of mycolytic enzymes, *viz*., chitinase, chitosanase, β-1,3-glucanase, mannanase and proteinase that significantly degraded mycelia of *S. rolfsii* and *Fusarium* sp. (Deshpande, 1999; Vyas and Deshpande, 1989). Alternately, one can attack the process of chitin synthesis using the inhibitors of chitin metabolizing enzymes. In Japan against rice - plant diseases (Fiedler *et al*, 1982), the inhibition of cell wall polymer, particularly chitin, synthesis is one of the novel targets suggested for antifungal antibiotics produced by microorganisms (Cohen, 1993; Deshpande, 1998; Groll *et al*, 1998). In the present chapter, to control the growth of root-infecting fungus, a two-pronged attack *viz*., inhibition of cell wall synthesis and degradation of pre-formed cell walls with special reference to chitin has been used.

Therefore, in the present chapter fungal and bacterial cultures have been screened for the potential cell wall synthesis inhibitors and the use of *M. verrucaria* cuticle degrading enzyme mixture alone in degradation of *H. armigera* cuticle and use of *M. verrucaria* mycolytic enzyme mixture alone and in combination with one of the potent

microbial preparations of chitin metabolism inhibitors have been demonstrated to control root-infecting fungus *S. rolfsii*, which causes wilting and stunted growth of peanut.

5.2 Results and discussion

5.2.1 Mycolytic enzyme activities of *M. verrucaria* NCIM 903

Along with high chitinase (1.8 ± 0.2 U ml^{-1}) activity, the culture filtrate of *M. verrucaria* had appreciable amount of chitosanase, ß-1,3-glucanase, mannanase (0.012 ± 0.003, 0.214 ± 0.03, and 0.019 ± 0.002 U ml^{-1}, respectively) and alkaline protease (0.007 ± 0.002 U ml^{-1}) activities. As chitinase is the key activity in the fungal cell wall degradation in the pot experiments the chitinase activity (0.04 U/ 50ml) was considered as a standard for the watering of the seedlings.

5.2.2 Screening of micro-organisms for cell wall synthesis inhibitors

The supernatants of 4 days old cultures were used for the hyphal tip bursting test using 4 different fungi (Table 5.1). Among the tested bacterial cultures, two *Bacillus* strains, 101 and 102 showed positive hyphal tip bursting test for all the four fungal species. While *Streptomyces* isolate NCL1 exhibited HTB for *B. poitrasii* and *S. rolfsii*. The *Actinomyces* isolate NCL 2 and fungal isolates, *Aspergillus* MY2 and *Chaetomium* MY3 showed positive test against *B. poitrasii* only. *M. verrucaria* culture filtrate did not indicate any positive HTB in *B. poitrasii* even after longer incubation (1h). It can therefore be attributed to the absence of cell wall synthesis inhibitor in the culture filtrate.

The *Bacillus* sp. though produced chitinase activity, it was 50 fold less (0.028 ± 0.002 U ml^{-1}) as compared to *M. verrucaria* when grown under the same experimental conditions. The chitosanase, protease and mannanase activities were not detected. However, it produced marginally higher ß-glucanase (0.323 ± 0.08 IU ml^{-1})

activity as compared to *M. verrucaria Bacillus* sp.102 culture filtrate exhibited maximum HTB when it was heated at 90 ^{0} C for 10 min. It showed 70 ± 10 percent HTB with *B. poitrasii* (Table 5.1).

Table 5.1 Hyphal tip bursting test of extracellular broth of microbial cultures

Culture	Hyphal tip bursting test (%)			
sp.	*B. poitrasii* (3-5 min)	*S. rolfsii* (50-60 min)	*A . niger* (8-10 min)	*Fusarium* (5-7 min)
Bacillus sp.101	50 ± 10	40 ± 10	25 ± 5	25 ± 5
Bacillus sp.102	80 ± 5*	70 ± 5	45 ± 5	55 ±5
Streptomyces sp.NCL	45 ± 5	20 ± 5	-	-
Aspergillus sp. MY 2	25 ± 5	-	-	-
Chaetomium sp. MY3	60 ± 10	-	-	-
Volutella sp.	-	-	-	-

-, Not detected.
*The culture filtrate heated at 90 ^{0} C for 10 min also showed 70 ± 10 % HTB with *B. poitrasii*.

The *Bacillus* sp. 102 preparation exhibiting maximum HTB was tested for its inhibitory effect on intracellular chitin synthase, endo-chitinase and *N*-acetylglucosamindase activities of a test fungus *B. poitrasii* along with other crude preparations. The culture filtrates of other isolates, such as *Bacillus* sp. 101, *Streptomyces* sp. NCL1 and *Chaetomium* sp.MY3 inhibited the chitin synthase activity to 20-50 percent. However, *Bacillus* sp. 102 preparation did not inhibit chitin synthase activity. Instead it inhibited endo-chitinase and *N*-acetylglucosamindase activities to 30-40 percent as compared to the control .The effect of *Bacillus* sp. 102 culture filtrate

on *M. verrucaria* chitinase activity was to be unaffected when the total chitinase activitiy was measured on acid swollen chitin and *N*-acetylglucosamindase on *p*NP- *N* acetylglucosaminide were found to be unaffected. Koga *et al*, (1987) had also reported such type of selectivity in the inhibition of insect chitinase by allosamidin (a specific endo-chitinase inhibitor). It has been suggested that chitin hydrolysis may be one of the regulating processes for chitin synthesis in fungal cell wall (Adams *et al,* 1993; Gooday *et al*, 1992; Ghormade *et al*, 2000). Therefore, hyphal tip bursting can also be correlated to the inhibition of *N*-acetylglucosaminidase and endo-chitinase activities which by supplying *N*-acetylglucosamine contribute in hyphal tip growth.

5.2.3 Isolation of chitin metabolism inhibitor(s)

The *Bacillus* sp. 102 preparation exhibiting maximum HTB (Table 5.1) was used for further purification. The inhibitor was extracted in different organic solvents and it was found that in case of extraction with chloroform, the HTB of *Bacillus* sp. 102 was not affected (Table 5.2) and so this solvent was used for further large scale extraction of the inhibitor.

Table 5.2 Extraction of chitinase inhibitor(s) of *Bacillus* sp. 102

Organic solvent	Hyphal tip bursting test (%)
Culture filtrate	80 ± 5
Ethyl acetate	55 ± 10
Benzene	ND
Hexane	ND
Chloroform	70 ± 5

ND, not detected

Using silica gel column chromatography, 6 fractions were tested for antifungal activity using HTB test with *B. poitrasii*. The fractions (1,2 and 5) showed good HTB activity. The first fraction exhibiting maximum HTB showed two spots in preparative TLC. Further characterization of the major fraction using IR, NMR and mass spectrometry revealed that it had characteristics similar to oleic acid. It was confirmed by comparing the spectral data of the standard oleic acid as well as overlaying HPLC . The HTB activity using *Benjaminiella* of the fraction 1 was maximum without separation (Chart 5.1).

Chart 5.1 Isolation of chitinase inhibitor(s) of *Bacillus* sp. 102

Culture filtrate
HTB +++ (100%)
↓
Extracted with chloroform (1:5)
HTB ++ (80%)
↓
Silica gel (60-120 mesh) column eluted with
ethyl acetate: petroleum ether (1:9 → 10:0) and
then with methanol: ethyl acetate (1:9 → 5:5)
↓

	↓	↓	↓	↓	↓	↓
Fraction	1	2	3	4	5	6
HTB	++ (80%)	+ (50%)	-	-	+ (50%)	-

Though fractions 1,2 and 5 only showed positive HTB test, all the fractions inhibited endo-chitinase and *N*-acetylglucosaminidase activities to a large extent (30-70%).

5.2.4 Effect of CDE preparation on *H. armigera*

The CDE preparation when used against the 3rd instar larvae of *H. armigera*, showed a dose dependent percentage mortality. At higher concentrations (3.2 U/ml chitinase and above), damage to larval cuticle was more pronounced resulting in insect

death within 72h. The CDE preparation containing 6.4 U/ml chitinase enzyme was found to be effective against *H. armigera* (73.33% mortality). Using this preparation ≥60% mortality was achieved within 72 h (Table 5.3).

Table 5.3 Effect *M. verrucaria* CDE preparation on 3rd instar *H. armigera* larvae

Chitinase (U/ml)	Chitinase (U/larva)	Mortality (%)		
		72 h.	120 h.	144 h.
0.8	0.003	ND	ND	ND
1.6	0.007	ND	ND	16.66
3.2	0.015	36.66	46.66	50.00
6.4	0.032	60.00	66.66	73.33
Control	ND	3.33	3.33	3.33

Arithmetic mean of three replications; each replication contains 10 larvae, ND: not detected

The reduction in weight of cuticle/larva and chitin content/ larval cuticle was also observed in CDE treated *H. armigera* larvae. With the increase in number of sprayings of CDE preparation, the loss in weight of cuticle/ larva and chitin content/ larval cuticle became more pronounced suggesting the degradation of chitin in the cuticle due to CDE preparation (Table 5.4).

Table 5.4 Cuticle degradation of *H. armigera* using CDE preparation

CDE treatment	Wt. of cuticle	Chitin content
(Chitinase 6.00 U/ml)	(%)*	of cuticle (%)*
Control	100.00	100.00
Spraying I	83.83	76.9
Spraying II	68.7	57.1

*Arithmetic mean of four replications; each replication contains 30 larvae. Observations recorded after 96h.

Fluorescence microscopy studies using FITC -WGA allowed the localization of chitin in the cuticle of treated and control larvae. The sections of untreated (control) larvae showed continuous band of cuticle with a green fluorescence whereas the sections of treated larvae did not show any fluorescent patches (Figure 5.1). This indicated that the alteration in chitin content of cuticle of test larvae was due to CDE preparation.

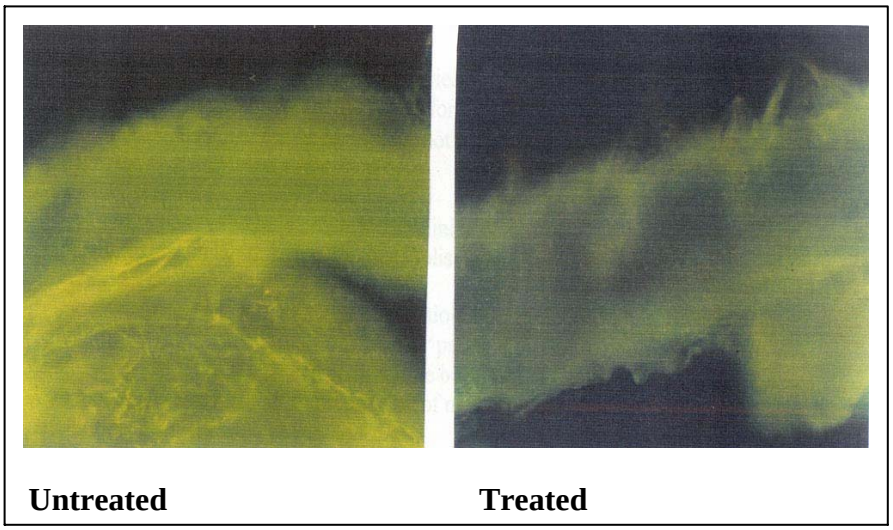

Figure 5.1 Chitin localization in cuticle of *H. armigera* using FITC-WGA

Both these results confirm the degradative action of CDE preparation on the insect cuticle, which ultimately led to insect death. Brandt *et al*, (1978) proposed that chitinases cause perforations in the insect gut peritrophic membranes thus facilitating the entry of the pathogens into the susceptible hosts. Mendonsa *et al*, (1996) have demonstrated the ability of *M. verrucaria* cuticle degrading complex in the control of first and fourth instar larvae of a mosquito. But so far, the effect of cuticle degrading enzyme complex of *M. verrucaria* in the control of *H. armigera* was not demonstrated. Implication of chitinases in degradation of insect cuticle could thus be an important tool in the knockdown of insects, especially *H. armigera* in shorter time. However, stability of the enzyme preparation under field conditions would be a major concern.

5.2.5 Pot experiment

In the sterilized-non infested soil the germination of peanut seeds was 90 percent. The disease development was maximum in the infested soil irrigated with water. As shown in the Figure 5.2, the irrigation with a mixture of two culture filtrates was useful to protect peanut plants from the *Sclerotium* attack (85 percent). The irrigation of infested soil separately with mycolytic enzyme preparation of *M. verrucaria* and crude inhibitor preparation of *Bacillus* sp. 102 resulted in the germination of peanut seeds 40 and 70 percent, respectively.

Thus, the mycolytic enzymes and chitinase inhibitor exhibited a better control of the spread of actively growing mycelium of *S. rolfsii* in combination than in isolation. The identification and structure-function relationship studies of the pure inhibitor from *Bacillus* sp.102 will be useful to identify its role explicitly.

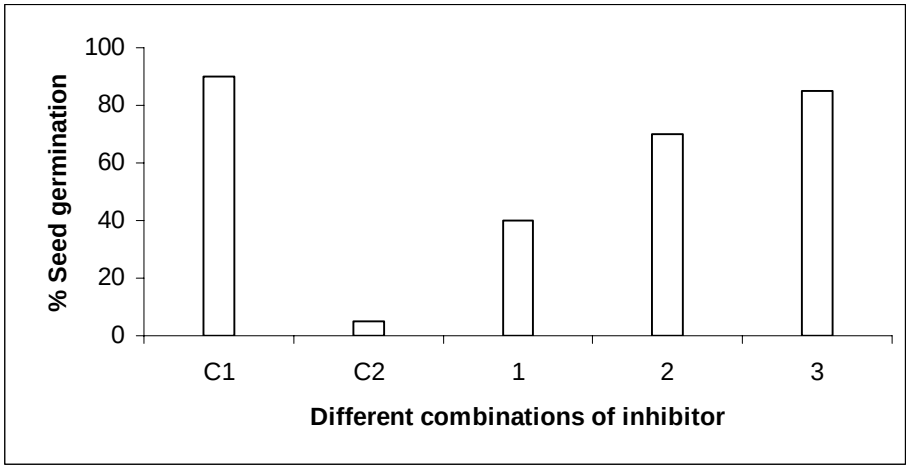

Figure 5.2 Effect of daily irrigation with mycolytic- and cell wall synthesis inhibitor- preparations, singly and in combination, on the peanut seed germination in the infested soil with *S. rolfsii* in pot experiment. C1, seeds sown in sterile soil irrigated with tap water; C2, seeds sown in infected soil irrigated with tap water; 1-3, irrigation of infested soil with *M. verrucaria* and *Bacillus* sp. 102 culture filtrates, and their mixture, respectively as described under Materials and Methods.

Koga *et al,* (1987) have reported the inhibitory effect of allosamidin on insect chitinase. Therefore, the further investigations on the contribution of chitinase inhibitor from *Bacillus* sp.102 for the control of insect pest will be useful to design an integrated approach. Apart from this, it is also essential to demonstrate the application of cuticle degrading enzyme complex of *M. verrucaria* , either singly or in combination with the inhibitor for the effective control of polyphagous insect pest, *H. armigera* under field conditions.

CHAPTER VI

CONCLUSION

Conclusion

Pulses (lentils/ chickpea and others), vegetables etc. are the major source of nutrition for the population in India. In recent years, the agricultural production of these crops suffered average yield losses of > 50%, half of which were caused by the caterpillar *H. armigera* due to high levels of insecticide resistance. Other pests mainly include the soil borne fungal pathogens such as *S. rolfsii* , *F. oxysporum* , *R. solani* etc. that result mainly in wilting of plants , and thereby affect the yield. Due to failure in the performance of chemical insecticides in the control of pests and pathogens, there is a need to use novel biological approaches that could lead to sustainable agriculture.

Chitin is a unique aminosugar (a β-1,4-linked GlcNAc) polymer that is present in fungi and insects and absent in vascular plants and animals. In fact, the presence of chitin as main structural polymer in the protective covers *viz*. cell wall of fungi and cuticle of insects, make these two groups distinct from others in agriculture. Therefore, the investigations in the present thesis are targeting chitin metabolism: use of entomopathogenic fungi for control of insect pest, cuticle degrading enzyme/ mycolytic enzyme preparation to control insect pest or fungal pathogen and chitin metabolism inhibitors for the control of fungal pathogens. Chitin can be targeted with the help of organisms that secrete the enzymes involved in the hydrolysis of pre-formed chitin, or with direct application of the enzyme preparation to facilitate the process of hydrolysis and with the use of the enzyme preparation along with chitin metabolism inhibitors ultimately leading to degradation of fungus and death of insects.

With this approach in mind, attempts were made to screen for entomopathogenic fungi that have the ability to kill the leidopteran insect *H. armigera*. The

entomopathogenic fungi produce enzyme such as chitinases, proteases and lipases that play an important role in the penetration process. It is evident from the work presented in chapter III that the differential level of production of these enzymes in *M. anisopliae*, *B. bassiana*, and *N. rileyi* affected their ability to kill *H. armigera*. Apart from the key enzymes of the cuticle degrading enzyme complex, the present work highlights for the first time, the importance of extracellular, constitutively produced chitin metabolizing enzymes, chitin deacetyalse and chitosanase of *M. anisopliae* in the biological control of *H. armigera*. It has been demonstrated that CDA plays an important role in initiating the process of pathogenesis wherein the insect cuticular chitin was softened with the aid of CDA, thereby facilitating the entry of the fungus into the insect. Also, production of CDA indicated the conversion of *M. anisopliae* cell wall chitin to chitosan that in turn could play an important role in defense mechanism of *M. anisopliae* in an attempt to protect hydrolysis of its cell wall chitin in presence of insect chitinases (chapter IV B). Thus, one of the feasible alternatives would be the isolation and identification of insect pathogens that secrete high levels of chitin metabolizing enzymes, particularly CDA. Chitin hydrolyzing enzymes in *M. verrucaria* have also been identified during the course of this work. *M. verrucaria* , a deuteromycetous and a reported mycoparasite of *Cochliobolus sativus* exhibited complete mycolytic and cuticle degrading enzyme complexes. The soil application of this enzyme preparation significantly reduced the incidence of *S. rolfsii* in peanuts. The CDE preparation of *M. verrucaria* also played an important role in hydrolysis of *H. armigera* cuticle. When the mycolytic enzyme complex of *M. verrucaria* was supplemented with chitin metabolism inhibitors, the efficacy of ME preparation in the control of proliferation of *S. rolfsii* increased. However, the effect of

chitin synthesis of inhibitors either singly or in combination with CDE preparation in hydrolysis of insect cuticular chitin needs further studies. Also, seed dressing of *M. verrucaria* in control of *S. rolfsii*, could be a challenging approach for the effective control of *S. rolfsii* (chapter V). All these aspects will help in development and introduction of novel biocontrol strategies comprising of CDE/ME complex of *M. verrucaria* and chitin metabolism inhibitors, entomopathogenic fungi in conjunction with cuticle degrading enzymes, especially CDA spray, chitin metabolism inhibitors along with entomopathogenic fungi for implementation in integrated pest management programs and/ or organic farming package for effective control of insect pests in agriculture using chitin metabolism as a target.

However, extensive research studies have to be done with respect to large-scale production of conidia of entomopathogenic fungi, CDE/ ME complexes, stability of these preparations under laboratory and field conditions, use of molecular markers in determining the fate of applied fungus in soil, effect of these organisms on non-target organisms, cost-benefit studies and trans-sectorial issues to make the technology eco-friendly.

CHAPTER VII

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fluorescent than the intact chitin embedded in the overlay gel. Chitosan, in a separate overlay gel was subjected to a nitrous acid treatment which specifically depolymerizes chitosan while leaving chitin intact. Hydrolysis of chitosan by nitrous acid followed by calcoflour staining yielded dark (non-fluorescent) bands in the fluorescent chitin containing gel.

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LIST OF PUBLICATIONS/PRESENTATIONS

List Of Publications

1. Deepak B. Salunke, Braja G. Hazra, Vandana S. Pore, Manoj Kumar Bhat, **Pallavi B. Nahar**, Mukund V. Deshpande (2004) 'New Steroidal Dimers with Antifungal and Antiproliferative Activity ', *Journal of Medicinal Chemistry*, **47**: 591-1594

2. Deshpande, M. V., Chandele, A.G., **Nahar, P**., Hadapad, A., Patil, G., Ghormade, V., Keller, S., and Tuor,U (2003) 'Entomopathogenic fungi: Mycoinsecticides useful against lepidopteran pest in pulses', *IOBC/WPRS Bull*, **26**: 27-30

3. **P. Nahar**, V. Ghormade and M.V. Deshpande (2004) 'The extracellular constitutive production of chitin deacetylase in *Metarhizium anisopliae* : Possible edge to entomo-pathogenic fungi in the biocontrol of insect pest' , *Journal of Invertebrate Pathology*, **85**: 80-88
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4. **P. Nahar**, M. Kulye, P. Yadav, M. Hassani, U. Tuor, S. Keller and M.V. Deshpande (2003) 'Comparative evaluation of indigenous fungal isolates, *Metarhizium anisopliae* M34412, *Beauveria bassiana* B3301 and *Nomuraea rileyi* N812 for the control of *Helicoverpa armigera* (Hüb.) on chickpea', *Journal of Mycology and Plant Pathology,* **33**: 372-377

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6**. P. Nahar** , A.S. Patil , G.W. Gooday, V. Ghormade and M.V. Deshpande (2001) 'The constitutive production of chitin deacetylase and chitosanase activities in *Metarhizium anisopliae*: Possible role in the biocontrol of insect pest', *Chitin Enzymology*, pp.511-517 , Ancona ,Italy

7. R.S. Patil , A.M. Deshpande , A.A. Natu, **P. Nahar**, M. Chitnis , V. Ghormade, R.S. Laxman, S. Rokade and M.V. Deshpande (2001) 'Biocontrol of root infecting plant patogenic fungus , *Sclerotium rolfsii* using mycolytic enzymes and cell wall chitin metabolism inhibitors singly and in combination', *Journal of Biological Control* , **15**: 157-164

Presentations

1. **'Microbial control of pests: Entomopathogenic fungi as mycoinsecticides'**., Presented at National symposium on recent advances in management of plant diseases, technology development and applications, organised by Indian phytopathological society, New Delhi, and Western zone, Maharashtra, Pune, held at Modern College of Arts, Science and Commerce, Pune on December 20-21, 2003.

2. '**Comparative evaluation of indigenous fungal isolates, *Metarhizium anisopliae* M34412, *Beauveria bassiana* B3301 and *Nomuraea rileyi* N812 for the control of *Helicoverpa armigera* (Hüb.) on chickpea'**., Presented for the best paper award in the P.R.Verma award competition for Ph.D. students in Annual conference of society of mycology and plant pathology, held at Durgapura, Jaipur, October 8-10, 2003.

3. **'Optimization studies for the large scale production of infective propagules of entomopathogenic fungi'**., Presented in symposium on biocontrol based pest management for crop protection in the current millennium organized by Indian Society for the advancement of insect science and Society for Biocontrol advancement at Punjab Agricultural University, Punjab during July 18-19, 2001.

Articles in Newspapers/Popular Research

Pallavi Nahar, **Anil Lachke and Mukund Deshpande** "Jaiv–niyantran mein **kavakon ka sahayog " a paper in Hindi.**