RAPD ANALYSES OF ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* (METSCH.) SOROKIN (DEUTEROMYCOTINA: HYPHOMYCETES) AND ITS BIOEFFICACY ON *DYSDERCUS CINGULATUS* (FAB.) (HEMIPTERA: PYRRHOCORIDAE)

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CERTIFICATE

This thesis entitled "**RAPD analyses of entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes) and its bioefficacy on *Dysdercus cingulatus* (Fab.) (Hemiptera: Pyrrhocoridae)**" submitted by **J. Francis Borgio (Reg. No. 2093)** for the award of the degree of Doctor of Philosophy in Microbiology **of** Manonmaniam Sundaranar University is a record of bonafide research work done by him and it has not been submitted for the award of any degree, diploma, associateship, followship of any University / Institution.

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(K. SAHAYARAJ)

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CHAPTER – 1. GENERAL INTRODUCTION

"We must learn to listen and evaluate all possible alternatives...always looking for a safer, more effective way to control pest problems" - Stephen Leonard Tvedten

1.1. Agriculture in India

Agriculture is the most important economic activity in developing countries like India. Continued agricultural growth is a necessity, not an option, for developing countries. Agriculture has always been India's most important economic sector and it has most variable climatic regions owing to its geographic features. Over twenty two percent of the rural households depend on agriculture as the principal means of livelihood. Some of the major agricultural products in India include cash crops such as cotton and sugarcane, commercial crops like groundnut and other oilseeds, food grains, rubber, tea, coffee etc (David, 2008).

Tamil Nadu is one of the most urbanised states of India, it is still a rural land; agriculture is the mainstay of life for about three-quarters of the rural population. The percentage of area under crop cultivation in Tamil Nadu is 42.02 million hectares (TIDCO, 2003). The principal economically important crops in Tamil Nadu are cotton, sugarcane, oilseeds, coffee, tea rubber, and chillies. The food crops include rice, maize, jowar, bajra, ragi, and pulses (Anonymous, 2007b). The yield-rate of cotton per hectare is 324 kilograms (TIDCO, 2003).

1.2. Cotton

Cotton (*Gossypium* spp.) has been cultivated in the Indus valley for more than 5000 years. It is one of the most important commercial fiber crops playing a key role in the economic, political and social affairs of India. Cotton is cultivated in about 60 countries of the world, among which 10 countries, *viz.* Russia, USA, China, India, Brazil, Pakistan,

Turkey, Egypt, Mexico and Sudan account for nearly 85% of the total production. In India, *Gossypium hirsutum* L., cv. Crema from the Malvaceae is grown on large scale in Maharashtra, Gujarat, Karnataka, Madhya Pradesh, Punjab, Rajasthan, Haryana, Tamil Nadu and Uttar Pradesh nearly 8 million/ha. India ranks fourth in total global cotton production. This crop is severely attack by a number of pests (FAO, 2002). It includes 17 wild and 3 cultivated species of India such as *G. arboretum, G. herbaceum* and *G. barbadense* (FAO, 2002). It is chiefly grown for its fibre, which is used in the manufacture of cloth for the mankind. It is also used for several other purposes like making threads and extracting oil from the cotton seed. Cotton seed cake after extraction of the oil is good organic manure. Cotton seed, seed cake, cotton linters and pulp obtained during oil extraction and cotton meal are good feed for cattle.

1.2.1. Cotton pests

Cotton is a pure cash crop. Insect pests are the major constraints in cotton production. Pests are dynamic in nature and the cotton pests' complex changes with the agro-ecosystems (FAO, 2002). As many as 130 species of insects, besides, few mite species are known to attack cotton crop in the field (Rao *et al.,* 2003). Among the various pests of cotton, *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae), *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), *Dysdercus cingulatus* (Fab.) (Heteroptera: Pyrrhocoridae), *Aphis craccivora* Koch (Homoptera: Aphididae), *Mylabris pustulata* Thunb., *M. indica* (Faust) (Coleoptera: Meloidae), *Pericallia ricini* (Fab.) (Lepidoptera: Arctiidae) and *Oxycarenus hyalinipennis* (Costa) (Hemiptera: Lygaeidae) (Rao *et al.,* 2003; David and Ananthakrishnan, 2004) were found to be the most serious pests in India. Other pests of cotton include pink bollworm, thrips, cotton leafhopper *Amrasca biguttula* (Ishida) (Homoptera: Cicadellidae), cotton whitefly, *Bemisia tabaci* (Genn.) (Homoptera:

Aleyrodidae), boll weevil, tarnished plant, bug bollworm, tobacco budworm, beet armyworm, spider mites, cotton aphid and fall armyworm (Suasa-ard, 2002; Rao *et al.,* 2003; Bambawale *et al.,* 2006; Sen, 2006).

1.2.1.1. *Dysdercus cingulatus* (Fab.) (Hemiptera: Pyrrhocoridae)

D. cingulatus is a serious pest of cotton (Leakey and Peery, 1966; David and Kumaraswami, 1978), which infests cotton in all the cotton growing regions (David and Ananthakrishnan, 2004). The adults and nymphs suck plant-gap and greatly impair the vitality of the plant. They also feed on the seeds and lower their oil-content. Due to the excreta of this insect, the lint is spoilt. Red cotton bug (David and Ananthakrishnan 2004) cotton stainer, red seed bug and Hong Kong stink bug (Mohn, 2005) are the common names for *D. cingulatus*. They are called cotton stainers because their red bodies get crushed along with the cotton they eat when it is harvested, and these stains are difficult to remove (David and Ananthakrishnan 2004). The taxonomic position of *D. cingulatus* is as follows (El-Sayed, 2006)**.**

Kingdom: Animalia

Sub kingdom: Invertebrata

Phylum: Arthropoda

Class: Insecta

Sub class: Pterygota

Infra class: Paraneoptera

Order: Hemiptera

Family: Pyrrhocoridae

Genus*: Dysdercus*

Species*: cingulatus*

The pest is active from October to February. Apart from cotton, *D. cingulatus* can infest maize, pearl millet, wheat (Flint and van den Bosch, 1981), ipomea and hibiscus (Mohn, 2005) and groundnut (Nandagopal and Gunathilagaraj, 2008).

1.2.1.2. *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae)

S. litura is widely distributed throughout Asia and the Pacific islands (Wightman and Rao, 1993; Mantinez and Van Emden, 1999). It is commonly called as common cutworm (Catindig and Heong, 2003) and oriental leaf worm moth (Mohn, 2005). In recent years, *S. litura* have emerged as important pest in India (Rao *et al.,* 2007). The insect pest is polyphagous and has about 360 host species. These include cotton, cruciferous vegetables, cucurbits, groundnut, maize, potatoes, rice, soybean, tea and tobacco (Moussa *et al.,* 1960; Panchabbani and Nethrabbaniraj, 1987; Dhin *et al.,* 1992; Singh and Jalali, 1997; Catindig and Heong, 2003; Ghumare and Mukherjee, 2003; Chaudhari *et al.,* 2004; Rao *et al.,* 2007). The management of this pest using chemical insecticides is unsuccessful because of it's insecticide resistance (Ramakrishnan *et al*., 1984; Dhingra and Sarup, 1990; Kranthi *et al*., 2002; Patil *et al*., 2002; Kodandaram and Dhingra, 2007). The occurrence of this pest is sporadic and difficult to predict (Schreiner, 2000). Prabhuraj and Patil (2002) reported that management of this pest with chemical insecticides is also problematic, due to its nocturnal feeding habit and mobility.

1.2.1.3. *Aphis craccivora* Koch (Homoptera: Aphididae)

A. craccivora is one of the important sucking pests of cotton and other leguminous crops throughout India (Wightman and Rao *et al*., 1993; Sridhar and Mobto, 2000; David and Ananthakrishnan, 2004). It causes yield losses by feeding on the phloem sap and through the transmission of viral diseases (Alegbeja, 1997). Aphids appear on crops soon after they are planted and disperse readily. They are found primarily on the growing points

of the host plant, flowers and developing bean pods (David and Ananthakrishnan, 2004).

1.2.1.4. *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae)

Helicoverpa armigera is posing a potential threat for successful cultivation of several economically important crops especially cotton in India, China, Pakistan and Thailand (Wadyalkar *et al*., 2003). It is commonly called as cotton bollworm. It is an active pest throughout the year. They completely ate away the internal content of fruiting bodies resulting in serious loss (Gopalakrishnan and Narayanan, 1988; Ramanujam *et al.,* 2002; Nahar *et al.,* 2004; David and Ananthakrishnan, 2004). *H. armigera* is resistant to insecticides such as deltamethrin, endosulfan, fenvalerate, monocrotophos, phosalone and quinalphos (Manikandan,1997 and 1998; Das, 2002; Yadav *et al*., 2006).

1.2.1.5. *Mylabris pustulata* Thunb. (Coleoptera: Meloidae)

M. pustulata is a common insect pest in India on most of the field crops (Ness and Sastawa, 2000; Khan *et al.,* 2005). It consumes the food by eating the flowers of cotton, by which it lowers the production of cotton and directly cause inevitable economic loss to the farmers in cotton growing region (Kalaiyarasan and Kalyanasundaram, 2003; David and Ananthakrishnan, 2004; Nandagopal and Gunathilagaraj, 2008).

1.2.1.6. *Mylabris indica* (Faust) (Coleoptera: Meloidae)

M. indica is also an important pest of cotton in Southern part of India (Ambrose, 1999). It eats the flowers of cotton, by the way it cause severe economic loss to the farmers (Kalaiyarasan and Kalyanasundaram, 2003; David and Ananthakrishnan, 2004).

1.2.1.7. *Pericallia ricini* (Fab.) (Lepidoptera: Arctiidae)

P. ricini commonly known as “hairy caterpillar” or wooly bear is the major pest of castor, gingelly, cotton, country bean, brinjal, drum stick, coccina, banana, calotropis, sunflower, oleander, tea, sweat potato and pumpkin (Kalaiyarasan and Kalyanasundaram,

2003; David and Ananthakrishnan, 2004; Gloviana *et al.,* 2004; Mala and Muthalagi, 2008).

1.2.1.8. *Oxycarenus hyalinipennis* Costa (Hemiptera: Lygaeidae)

O. hyalinipennis is known as "dusky cotton bug". In India, it is found in all cotton growing regions along with the red cotton bug. Both adult and nymphs suck the sap from immature seeds and stain the lint. The seed do not ripen and thus get damaged (Nasr *et al.,* 1992; Kalaiyarasan and Kalyanasundaram, 2003; David and Ananthakrishnan, 2004).

1.3. Non-IPM

Out of total quantity of pesticides used in India, 54% is used on cotton, 17% on rice and 13% on vegetables and fruits. However, cotton, rice and vegetables occupy 5, 24 and 3% of total cropped area in the country respectively (FAO, 2002; Devi, 2007). Suicide among farmers is growing tremendously at a rate of 3.9% annually worldwide. In India, it is highest in Andhra Pradesh followed by Maharashtra, Madhya Pradesh, Goa, Tamil Nadu, Kerala, West Bengal and Puducherry and 60% of the suicides are by pesticide consumption (Anonymous, 2007a). Single and simple solution should be practiced to overcome this problem in the present scenario. The availability of safer and natural products like biopesticides should be increased for ecological sustainability and alternatives to pesticides.

1.4. IPM

The farmers in a desperate mood resorted to increased and higher doses/cocktail mixtures of pesticides. The higher dose of pesticides coupled with repeated uses increased the production cost of cotton cultivation, which struck disaster to cotton growers. Unable to repay the loans raised, a large number of cotton farmers in Andhra Pradesh, Karnataka and Punjab committed suicide during 2000-07 (FAO, 2002; Anonymous, 2007a). The

overdose of pesticide application on food crops also resulted in pest residues in food and food commodities. The residues find their way in the human bodies through consumption of such commodities contaminated with higher level of pesticide residues. Many instances of bio-magnification of pesticide residues in human tissues and products of animal origin have been documented to focus the vulnerability of human beings to toxic chemicals (FAO, 2002; David, 2008). The above facts clearly indicate that a possible solution for this is to adopt Integrated Pest Management (IPM) where biopesticides place an important role.

IPM can be used as an alternative of chemical pesticides since they are harmless to human being, mammals and plants. IPM is the application of an interconnected set of principles and methods to minimize problems caused by insects, diseases, weeds and other agricultural pests (Rao *et al.,* 2003). The goal of the IPM is not to eradicate the pests but to control or manage the pest population. Traditional agriculture practiced in Indian villages till 1960s was in harmony with the nature. It preserved the diverse life forms with the agro-ecosystems and promoted the conservation of bio-diversity within farming systems. Among the biopesticides (4.5%), the market share of the microbial pesticides is about 3%, while in India only 1% biopesticides of the total agrochemical scenario are being used. IPM includes cultural, physical, mechanical and biological control strategies to manage the insect pests in a compatible manner (David, 2008).

1.4.1. Entomopathogens in Biological control programme

Biological control is an important module in IPM (Noris *et al*., 2002). Entomopathogens are living organisms, utilised in regulating insect pests population. Biocontrol plays a major role in the cotton pest management programme. In several situations, parasitoids (trichogrammatids, encyolids, tochinids and others), predators (coccinelids, lacewings, reduviids, spiders) and entomopathogenic microorganisms

(bacteria, fungi, virus, protozoa and nematode) have been used to keep the population of pests below the damaging level (Balaji and Hemavathi, 2007).

The organisms used in microbial insecticides are essentially nontoxic and nonpathogenic to wildlife, humans, and other organisms not closely related to the target pest (Kunimi, 2007). The toxic action of most microbial insecticides is specific to a single group or species of insects and this specificity means that most microbial insecticides do not directly affect beneficial insects including predators or parasites (Balaji and Hemavathi, 2007). If necessary, most microbial insecticides can be used in conjunction with synthetic chemical insecticides because in most cases the microbial product is not deactivated or damaged by residues of conventional insecticides. The pathogenic microorganisms can provide control during subsequent pest generations or seasons (Balaji and Hemavathi, 2007).

1.4.1.1. Bacteria and virus

Bacterial pesticide includes the endospore forming (*Bacillus* spp.) and non-endospore forming (*Pseudomonas* spp.) bacteria. Most effective and regularly utilized bacterial pesticide is *Bacillus thuringiensis* (Berliner) (Bt) the ariquitous, gram positive; dendric endospore forming bacterium is used worldwide as biological pesticides in commercial agriculture, forest pest management and mosquito control (Santhanam *et al.,* 1994; Panda *et al.,* 1999; Reddy *et al.,* 2001; Dutta, 2004; Balaji and Hemavathi, 2007). Most commercial *Bt* products contain the protein toxin and spores (Weinzierl and Henn, 2004). *Bt* offers immense potential for eco-friendly pest management strategy in cotton pest management in India among the microbial pesticides (Kunimi, 2007). Large number of commercially available *Bt* formulations (Dispal, Halt, Biolep, Bioasp, Delfin WG, Biopit and Spicturin) has been evaluated against many field crop pests in India (Jayaraj,

2001; Rao *et al.,* 2003). Both DNA and RNA viruses have the potential to control the crop pest. The entomopathogenic DNA viruses particularly, the baculoviruses such as Nuclear Polyhedrosis Viruses (NPV) and Granulosis Viruses (GV) have great potential in the microbial control of agricultural pests (Jayaraj, 2001). Among the RNA viruses, CPV (Cytoplasmic Polyhedrosis Viruses) is wildly used viral pesticide. NPV has been found to be effective in the control of many insect pests of cotton. In India and Thailand, the NPVs have been tested against important cotton pests like the gram pod borer *H. armigera* (Rabindra and Jayaraj, 1986; Jayaraj *et al.,* 1994; Rao *et al.,* 2007; Saxena, 2008), tobacco army worm *S. litura* (Jayaraj *et al*., 1981; Rao *et al.,* 2007; Saxena, 2008; Rabindra and Balasubramaniam, 1980; Saxena, 2008) and *Spillosoma obliqua* (Walker) (Lepidoptera: Arctidae) (Purwar and Sachan, 2006). Most of the viruses that have been studied for use as potential insecticides are nuclear polyhedrosis viruses (NPVs), in which numerous virus particles are "packaged" together in a crystalline envelope within insect cell nuclei, or granulosis viruses (GVs), in which one or two virus particles are surrounded by a granular or capsule like protein crystal found in the host cell nucleus (Weinzierl and Henn, 2004; Saxena, 2008). The Granulosis Viruses has been used for the control of sugarcane early shoot borer, *Chilo infuscatellus* (Snell). (Easwaramoorthy and Jayaraj, 1987; Easwaramoorthy and Cory, 1990; Cherry *et al.,* 1999; Weinzierl and Henn, 2004; David, 2008). The viruses that cause these outbreaks are very specific, usually acting against only a single insect genus or even a single species.

1.4.1.2. Fungi

In biological control, entomopathogenic fungi (Zimmerman, 1993; Milner and Staples, 1995; Borgio and Sahayaraj, 2007; Sahayaraj and Borgio, 2007; Balaji and Hemavathi, 2007) play an important role. Entomopathogenic fungi, act as important

natural control agents that limit insect populations (Kunimi, 2007). Most of the species that cause insect diseases spread by means of asexual spores called conidia. Entomopathogenic fungi enter into the hosts by direct penetration of the cuticle that function as a barrier against most microbial attack. Consequently, fungal entomopathogens have higher potential for biological control of sucking and defoliator insect pests and all that are difficult to combat with synthetic insecticides (Kang *et al.,* 1999; Ramanujam *et al.,* 2002; Nirmala *et al.,* 2006; Mohi-Un-Din *et al.*, 2006; Kunimi, 2007).

More than 750 species of fungi mostly deuteromycetes and entomophthorales are pathogenic to insects (Mc Coy *et al.*, 1988; Charnley, 1989; Tanada and Kaya, 1993; Padmaja, 2005; Borgio and Sahayaraj, 2007; Sahayaraj and Borgio, 2008). *Beauveria bassiana* (Bals.) Vuill.*, Metarhizium anisopliae* (Metsch.) Sorokin*, Nomuraea rileyi* (F.) Samson*, Paecilomyces fumosoroseus* (Brown and Smith)*, P. farinosus* (Holm ex SF Gray)*, Entomophthora* sp., *Fusarium* sp., *Aspergillus* sp. (Rabindra, 2002) are the major entomopathogens used as fungal pesticides. The fungus frequently emerges from the insects body to produce spore which spread by the wind, rain or contact with other insects (Hoffmann and Frodsham, 1993; Tanada and Kaya, 1993; Padmaja, 2005; Nirmala *et al.,* 2006; Mohi-Un-Din *at al.*, 2006; Scholte *et al.,* 2006; Borgio and Sahayaraj, 2007; Sahayaraj and Borgio, 2007). Dispel and dammar (*B. bassiana*), kalichakra (*M. anisopliae*) and verticel (*Verticillium lecanii*) are the commercial products available in Indian market to manage Lepidoptera and Homoptera pests (Jayaraj, 2001; Balaji and Hemavathi, 2007). Several commercial products of *M. anisopliae* are available for insect control in different agricultural operations such as Bio-Green and Bio-Cane granules for control of soil grubs of pasture and sugar cane in Australia, Green Muscle for control of locusts in Africa, Ago Biocontrol for control of various pests of ornamental crops in South America, and BioPath

for control of cockroaches in the United States (Sallam *et al*., 2007).

1.4.1.2.1. *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes)

Metarhizium is one of the most common entomopathogenic fungi, with a worldwide distribution, categorised as a green muscardine fungus due to the green color of the sporulating colonies. The species is soil-borne and infects predominantly soil-dwelling insects (Sallam *et al.*, 2007). Taxonomy of *Metarhizium* is not straightforward. The current classification of the taxon is mainly based on the morphology of conidia and conidiogenous cells. Some authors combine them with biochemical and molecular characteristics (Riba *et al.*, 1986), and/ or host pathogenicity, cold-activity and sporulation color (Yip *et al.*, 1992; Rath *et al.*, 1995; Scholte *et al.,* 2004). Driver *et al*. (2000) used 10 different clades, based on molecular data. *M. anisopliae* consists of 4 varieties (Driver *et al.*, 2000), two of which are considered important, these being *M. anisopliae* var. *acridum* (previously designated *M. flavoviride*) found mainly on Homoptera and *M. anisopliae* var. *anisopliae* (Metsch.) Sorokin, the latter being the best known of the two species. *M. anisopliae* has a large host-range, including arachnids and five orders of insects (Boucias and Pendland, 1998; Borgio and Sahayaraj, 2007; Sahayaraj and Borgio, 2008), comprising over 200 species (Scholte *et al.,* 2004).

M. anisopliae has several characteristics that make it interesting as a microbial cotton pests control agent. It causes high mortality to cotton pests under laboratory conditions, the fungus can be grown in massive amounts on inexpensive artificial media, and conidia can be stored easily, make this fungus a very promising control agent (Scholte *et al.,* 2004). This *M. anisopliae* is resistant to the chemical pesticides like endosulfan, monocrotophas and fenvalerate and also fungicides (Rao, *et al.,* 2003; Sallam *et al.*, 2007; Luz *et al.,* 2007). Zimmermann (1993) claims that because of absence of toxicological or

pathological symptoms in birds, fish, mice, rats, and guinea pigs after exposure to conidia of the fungus, *M. anisopliae* was safer microbial pesticides. Also Strasser *et al.* (2000) conclude from their risk-assessment study that the fungus poses no obvious risk to humans, or the environment.

1.5. Nature of infection

Bacteria, viruses, rickettsia and protozoa enter into the insect body along with food (ingested microbes). Nematode and fungi enter into the insect body by penetrating through the integument (Rao, *et al.,* 2003; Jarrold *et al.,* 2007). Although infection through the digestive tract is also possible (Goettel and Inglis 1997; Jarrold *et al.,* 2007) within the class Deuteromycetes a morphological group of fungi known as Hyphomycetes exists. These are filamentous fungi that reproduce by conidia generally formed aerially on conidiophores arising from the substrate. Many genera of entomopathogenic fungi occur in this group of fungi and they have widest host ranges. Under favourable conditions, the conidium germinates into a short germ tube, which gives out small swelling called appressoria (Rao, *et al.,* 2003). The appressorium attach to the cuticle, germinate, and penetrate the cuticle (Shah and Pell, 2003; Jarrold *et al.,* 2007). Once it reaches the haemocoel, the mycelium spread throughout the host, forming hyphal bodies called blastospores. Death of the insect is often due to a combination of the action of fungal toxins, physical obstruction of blood circulation, nutrient depletion and/or invasion of organs. After the host has died, hyphae usually emerge from the cadaver and, under suitable abiotic conditions; conidia are produced on the exterior of the host. These are then dispersed by wind or water (Goettel and Inglis 1997; Purwar and Sachan, 2006; Jarrold *et al.,* 2007).

1.6. Low cost mass multiplication

Efficient mass production techniques are prerequisite for successful field applications of entomopathogenic fungi. An efficient and economic large-scale production, the formulation of fungal propagules into a product with an adequate shelf life, and a suitable application strategy are fundamental for the successful development of a mycoinsecticide. The development of a good mycoinsecticide relies on the biological properties of the isolate (Gao *et al.,* 2007). One of the properties that need to be considered in selecting an isolate as a potential microbial biological control agent after laboratory virulence and field performance tests is its productivity (Jenkins *et al*., 1998). According to Feng *et al*. (1994) and Gitonga (1996) rapid radial growth and high sporulation capacity *in vitro* and *in vivo* are important attributes of a good strain. The selection of strains with good growth and sporulation capacity on nutritionally poor media is important for mass production in an industrial setup (Feng *et al*., 1994; Gao *et al.,* 2007; Luz *et al.,* 2007). Commercial media such as PDB (Sharma *et al.*,1998; Wadyalkar *et al.*, 2003; Bruck *et al*., 2005; Nirmala *et al.*, 2005) has been used by insect pathologists for large scale production of conidia of *M. anisopliae*. Rice was an ideal natural substrate for the mass production of entomopathogenic fungi (Mendonca, 1992; Ibrahim and Low, 1993; Milner *et al.*, 1993; Cheery *et al*., 1999; Sharma *et al.,* 2002; Bharati *et al*., 2007). Rice wash water (Jenkins *et al.*, 1998; Patel *et al.*, 1990) and coconut water (Dangar *et al.*, 1991) were other cheaper natural substrates for mass multiplication of *M. anisopliae.*

1.7. DNA Polymorphism necessity

Different types of molecular techniques have been used to study genetic diversity of entomopathogenic fungi, for instance, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), simple sequences repeat (SSR or micro satellites) analysis and internal transcribed spacer (ITS)-rDNA sequence RFLP

analysis (Velasquez *et al.,* 2007) are used for studies of closely related isolates. Among the techniques, RAPD has given workers the opportunity to screen an almost unlimited number of highly polymorphic markers which can be successfully used to assess genetic diversity and phylogeny within a species (Williams *et al.*, 1990; Velasquez *et al.,* 2007) and among related species. RAPD can be used as a tool to understand and identify the species (Rao, *et al.,* 2003). The technique of Random Amplification of polymorphic DNA is performed using arbitrary sequence oligonucleotide primers on a genomic DNA template (Williams *et al.*, 1990). The main advantages of RAPD technology are its simplicity of use, reduced running time and lower cost. Moreover, it does not make use of radioactive probes and requires only small amounts of DNA (15-25ng) (Velasquez *et al.,* 2007). To find out variation in virulence on pathogenicity among pathogens of various geographical distributions, this method is useful (Rao, *et al.,* 2003).

1.8. Objectives of the study

In Indian agriculture, the use of microbial insecticides is still in infant stage. There is a need to take up systematic surveys to identify naturally occurring entomopathogenic fungi under varied agro ecological conditions and to study the dynamics of the diseases in order to identify potential pathogens in India (Purwar and Sachan, 2006) against insect pests of crop plants. With all the above views in mind, the purpose of this present study is proposed to examine the potential of different *Metarhizium anisopliae* isolates in controlling cotton pests and its DNA polymorphism. The objectives of the present study are six folds.

1. To isolate *M. anisopliae* from agro and forest ecosystem in Tamil Nadu, India.
2. To determine the pathogenicity of *M. anisopliae* isolates and standard MTCC892 against 7 cotton pests under laboratory conditions.

3. To find out the root of infection and haematological effects of *M. anisopliae* isolate on *Dysdercus cingulatus.*
4. To screen the low cost liquid media for mass production *M. anisopliae* isolates.
5. To evaluate the DNA polymorphism among *M. anisopliae* isolates standard MTCC892 using RAPD markers.

CHAPTER – 2. DISTRIBUTION OF *METARHIZIUM ANISOPLIAE* IN TAMIL NADU

2.1. Introduction

2.1.1. Tamil Nadu

Tamil Nadu is one of the important agriculture zone among the 28 states of India. It lies on the eastern coast of the southern Indian Peninsula bordered by Puducherry, Kerala, Karnataka and Andhra Pradesh. It is bound by the Eastern Ghats in the north; the Nilgiri, the Aanaimalai Hills, and Palakkad on the west; Bay of Bengal in the east and Indian Ocean in the south. It is classified into seven agro-climatic zones: north-east, north-west, west, southern, high rainfall, high altitude hilly, and Cauvery Delta (the most fertile agricultural zone). The mean maximum temperatures that the state experiences in the plains and hills are 43.0°C and 32.3°C respectively while the minimum temperatures are 13.1°C and 3.0°C respectively (Anonymous, 2007b; Anonymous, 2007c).

2.1.2. Soil type in Tamil Nadu

Five different types of soil areas are present in various districts of Tamil Nadu includes red loam (Parts of Kancheepuram, Cuddalore, Salem, Dharmapuri, Coimbatore, Tiruchirappalli, Thanjavur, Ramanathapuram, Madurai, Tirunelveli, Sivagangai, Thoothukudi, Virudhunagar, Dindigul and The Nilgiris Districts), laterite soil (Parts of The Nilgiris District), black soil (Parts of Kancheepuram, Cuddalore, Vellore, Thiruvannamalai, Salem, Dharmapuri, Madurai, Ramanathapuram, Tirunelveli, Sivagangai, Thoothukudi, The Nilgiris, Virudhunagar and Dindigul Districts), sandy coastal alluvium (On the Coasts in the districts of Ramanathapuram, Thanjavur, Nagapattinam, Cuddalore, Kancheepuram and Kanniyakumari) and red sandy soil (Small Patches in the districts of Coimbatore and The Nilgiris) (Chaudhary, 2007).

2.1.3. Soil as a habitat of *Metarhizium*

Soil is the main reservoir of infective propagules of many entomopathogenic fungi (Vänninen, 1995; Milner *et al.,* 1998a; Abebe, 2002) including *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes) (Rath *et al*., 1992; Brownbridge *et al*., 1993; Vänninen, 1995). The most virulent isolates are usually those, which cause natural epizootics in a particular host (Milner, 2000a, b). However, it is the best to search in the environment of the target pest for suitable isolation of *M. anisopliae* (Milner *et al*., 1998a, 1998b; Purwar and Sachan, 2006). Brownbridge *et al.* (1993) believes that indigenous strains should be preferably used, because they are already well adapted to survive and multiply in their native environment. Moreover, using indigenous strains avoids the introduction of exotic species that might disrupt the ecosystem.

2.1.4. Taxonomy of *Metarhizium*

A recent taxonomic revision of the genus *Metarhizium* by Driver *et al*. (2000), using internally transcribed spacer ribosomal DNA (rDNA) sequence data from 123 isolates, resulted in the description of ten distinct clades of *Metarhizium*. They recognised four clades within *M. anisopliae*. Two among the four correspond with *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *majus*. Driver *et al*. (2000) described the other two as new varieties such as *M. anisopliae* var. *lepidiotum* Driver and Milner and *M. anisopliae* var. *acridum* Driver and Milner. Prior to 1999, *M. anisopliae* var. *acridum* was referred as *M. flavoviride*, *Metarhizium album, M. flavoviride* var. *flavoviride* and *M. flavoviride* var. *minus, M. flavoviride* var. *nova-zealandicum* Driver and Milner and *M. flavoviride* var. *pemphigum* Driver and Milner (Abebe, 2002). These taxa are probably the most intensively studied entomopathogenic fungi. However relatively little information is known about their

diversity in the environment (Hughes *et al.,* 2004). In Pondicherry, the distribution of *M. anisopliae* is diverse ranging from paddy, cotton and groundnut fields (Ambethgar, 2002).

2.1.5. Growth factors

Many environmental factors, such as temperature, moisture, soil type, light and natural as well as artificial antagonists, either directly or indirectly affect both the persistence and survival of entomopathogenic fungi in terrestrial habitats (Roberts and Campbell, 1977; Fuxa, 1987; Mc Coy *et al.,* 1988; McCammon and Rath, 1994; Hughes *et al.,* 2004). To compensate for this natural mortality, many species produce copious quantities of infective spores or form modified adaptive structures such as resistant hyphae, chlamydospores, resting spores, sclerotia, and macrocyclic conidia to maximize survival (Pendland, 1982). Fungi do persist in nature and frequently cause catastrophic decline in insect populations (Fuxa, 1987; Tanada and Kaya, 1993). Ambethgar (2002) reported the distribution of *M. anisopliae* is diverse ranging from paddy, cotton and groundnut fields in Pondicherry. But no reports were available regarding the distribution of *M. anisopliae* in Tamil Nadu. With these concepts in mind, the present study was undertaken to investigate the prevalence and diversity of the fungal entomopathogen, *M. anisopliae* associated with and in the vicinity of the soils of various agricultural fields, forests and insect cadavers throughout Tamil Nadu state.

2.2. Materials and methods

2.2.1. Study area

Soil samples were collected from different agriculture field and forest ecosystems of all districts of Tamil Nadu, India (Figure 1).

2.2.2. Collection of samples

Forty-three soil samples and 10 cadavers of insects were collected from Tamil

Nadu. About 100 gm of soil in a sample site at the depths of 2 cm (Ignoffo *et al.*, 1977 and Storey *et al.*, 1989) was collected using a sterilised stainless steel spatula in sterile plastic bags (10 cm x 15 cm). The collections were carried out from September 2004 to August 2006. All the samples were brought to the Crop Protection Research Centre, Department of Advanced Zoology and Biotechnology, St. Xavier's College, Palayamkottai, Tamil Nadu, India and stored in the refrigerator (LG, Brooklgn, New York, USA) at 4^{o}C for microbial analyses. *Metarhizium anisopliae* MTCC892 was purchased from the Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology, Chandigarh, India and considered as standard throughout the experiment.

2.2.3. Preparation of potato dextrose agar media (PDA)

Two hundred grams of potatoes were cleaned and peeled off. The peeled potatoes were cut into small thin pieces, weighed and rinsed rapidly under running water. Thereafter the rinsed potatoes were boiled in 1 litre of distilled water until it was fully boiled. The boiled potatoes were crushed using pestle and mortar and this was filtered through a muslin cloth. Twenty grams of mycological agar and 10 gm of dextrose (Himedia, Mumbai) were added to the filtrate and were stirred until the dextrose dissolved completely. The suspension was diluted with tap water and was made upto 1 litre. The final medium was autoclaved at 121^{0}C / 15 lbs for 15 minutes. After cooling, chloramphenicol (1g/litre) was added in order to suppress the bacterial growth.

2.2.4. Isolation and Enumeration of Total Fungal Population (TFP) of soil and insects

Soil samples collected from each site were manually ground using a mortar and a pestle under aseptic conditions. One gram of the ground soil sample was taken in a test tube containing sterile distilled water (10 ml). Each test tube was agitated using a vortex mixture for 15 seconds. The suspension was diluted thrice according to ten fold dilution

series (10^{-1} to 10^{-7}). From each diluted resultant preparation, 0.1ml was transferred onto separate PDA plates and were spreaded using a sterile L-shaped glass rod. The seeded plates were then incubated at 26°C ± 2°C and were observed for fungal growth after 7 days. After eminent growth, the fungi that were suspected to be *Metarhizium* spp. were selected to obtain pure cultures of the suspected entomopathogen. Fungal growth was assessed to quantify the number of colony forming units. Cadavers of insects that might have died due to of pathogenic infection were collected from different fields in Tirunelveli districts of Tamil Nadu. One gram of dead cadaver was ground in a test tube containing 10ml of sterile distilled water. The samples were serially diluted and spread plated on Poatato dextrose agar for TFP analyses of insect samples.

2.2.5. Sub-culture and identification of *M. anisopliae*

The selected fungi, which were suspected to be *Metarhizium* spp. were sub-cultured to obtain pure cultures of the entomopathogen. Spores were selected from the initial cultures using a sterile loop and were streaked on PDA plates. The inoculated plates were then incubated at 26°C ± 2°C in an incubator (Kemi BOD incubator, Kerala) for 7 days. Spores growing on these secondary cultures were again sub-cultured for 7 days using the same media to obtain pure cultures. The identification of *Metarhizium* was carried out based on their cultural characteristics, colonial morphology, sporulating structures and nutritional requirements (Matsushima, 1975; Domsch, *et al.,* 1980; Matsushima, 1993). The description of Pathogenic Fungi and Bacteria according to CMI (Commonwealth Mycological Institute) was also used to identify the insect pathogenic fungi *M. anisopliae* (Barnett and Hunter, 2004).

2.2.6. Storage of *M. anisopliae* isolates

Stocks of pure cultures of each fungal isolate were grown on sterile PDA slants

with cotton plugs. The test tubes were kept at 26°C ± 2°C in an incubator for 7 days with the cotton plug and were further incubated until sporulation. The slope cultures were then stored at 4°C in a refrigerator. These were maintained as mother cultures and were revived for every 3 months until all the experiments were completed. Working cultures (on PDA plates) were prepared from these slopes for each isolate and were rejected when older than 15 days.

2.3. Results

2.3.1. Distribution of *Metarhizium anisopliae* isolates in soil

Forty-three soil samples were collected from 13 cotton [*Gossypium hirsutum* Linn. (Malvaceae)], 12 paddy [*Oryza sativa* Linn. (Poaceae)], 3 coconut [*Cocos nucifera* Linn. (Arecaceae)], 2 tomato [*Lycopersium esculentum* (Mill.) (Solanaceae)] fields one each from groundnut [*Arachis hypogeae* (Leguminaceae)], coriander [*Coriandrum sativum* (L.) (Apiaceae)], jackfruit [*Artocarpus heterophyllus* (Lam.) (Moraceae)], maize [*Zea mays* Linn. (Poaceae)], potato [*Solanum tuberosum* Linn. (Solanaceae)], black gram [*Vigna mungo* Linn. Hepper (Fabaceae)] and castor [*Ricinus communis* Linn. (Euphorbiaceae)] fields and 5 samples from forests [Manimuthar, Courtalam, Kalakkadu (Tirunelveli district), KRR Nagar (Theni district) and Pachippara (Kanyakumari district)] from Tamil Nadu (Table 2. 1 and Figure 1).

The isolates obtained were identified based on their colonial morphology and sporulating structures and was confirmed by the description of Pathogenic Fungi and Bacteria according to CMI (Commonwealth Mycological Institute). Identified *M. anisopliae* isolates were named as CPRC series (Crop Protection Research Centre). Standard *M. anisopliae* was used as reference and also for comparative identification. The field survey reveals that the number of total fungal population was found to be highest and

lowest in the samples 11 (S11) (1.8±0.25 x 10^7) and 18 (S18) (1.6±1.12 x 10^2) respectively. Among the forty-three samples analysed, 25 samples (58.14%) contained *M. anisopliae* strains (20.93,11.63, 11.62, 4.65, 2.32, 2.32 and 2.32% of cotton, paddy, forest, coconut, tomato, groundnut and carrot respectively).

The incidence of *M. anisopliae* colonies were the most abundant (21 colonies) in the soil sample S14 (Table 2. 1) which was collected from the cotton field of Vilupuram District, followed by 12 colonies from the cotton field samples of Thoothukudy district. Among the cotton field soil analysed in the study, 69.23% contained *M. anisopliae* colonies. Though the highest total fungal population (1.9±1.50 x 10^5) was recorded in the paddy field of Trichy district, soil samples of Trichy district did not contain *M. anisopliae*. The occurrence of *M. anisopliae* colonies among other paddy fields was recorded as 41.67%. However among the cotton fields, the total fungal population (4.8±1.13 x 10^4) was most predominant in Thoothukudy district. *M. anisopliae* strain was absent in the soil samples collected in Trichy, Madurai, Virudunagar, Kanchipuram, Tiruvallur, Dharmapuri, Salem, Namakkal, Chennai, Cuddalore, Pondicherry, Sivagangai, Dindhugal, Ramanathapuram and Karur Districts (Table 2.1).

All the five soil samples collected from the forests contained *M. anisopliae* isolates, Courtalam of Tirunelveli (S56) and KRR Nagar of Theni (S42) district contained 7 colonies, followed by the Pachippara of Kanniyakumari (2 colonies in S53). Among the five forest samples, the total fungal population was maximum in the forest soil samples of Courtalam, Tirunelveli district (1.2±0.56 x 10^5).

2. 3. 2. Distribution in insects

The body of ten insects [*Dysdercus cingulatus* (Fab.) (Heteroptera: Pyrrhocoridae), *D. koenigii* (Fab.) (Hemiptera: Pyrrhocoridae), *Oxycarenus hyalinipennis* Costa

(Hemiptera: Lygaeidae), *Rhynocoris marginatus* (Fab.), *Rhynocoris fuscipes* (Fab.) (Hemiptera: Reduviidae), *Mylabris pustulata* Thunb. (Coleoptera: Meloidae), *Mylabris indica* (Faust) (Coleoptera: Meloidae), *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae), *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), and *Pericallia ricini* (Fab.) (Lepidoptera: Arctiidae)] were analysed for the TFP. The TFP was highest in *P. ricini* ($5.0\pm1.15 \times 10^3$) (Table 2.2).

2.4. Discussion

Metarhizium anisopliae is a ubiquitous component of the mycoflora of the soil (Milner *et al.,* 1998a). In the present study, from the 43 soil samples collected from different fields, only 58.14% of the soil sample contains *M. anisopliae* isolates of by plating method. Similarly, Hughes *et al.* (2004) obtained 58 isolates of *Metarhizium* from soil samples, either by "plating" or "live baiting" method. But Milner (1992) used ''*Galleria* bait method'' (Bedding and Akhurst, 1975) for the isolation of *M. anisopliae*. Zimmermann (1992) isolated *M. anisopliae* from 42% of soils in the Darmstadt region of Germany, while Hokkanen and Zimmermann (1986) isolated 17% of this fungus from agro forestry soils in Finland. In the present study, *M. anisopliae* was isolated from the upper 2cm soil layer whereas Hughes *et al.* (2004) isolated the same fungi at a depth of 5cm soil layer. If we had collected the soil from more than 2cm, there would have been a chance of getting more *M. anisopliae* isolates.

The isolation rate of *M. anisopliae* in the present study was considerably more than that of the observations by Bedding and Akhurst (1975), Hokkanen and Zimmermann (1986), Rath *et al.* (1992), Milner (1992), Zimmermann (1992), Roddam and Rath (1997), Parker *et al*. (2003) Hughes *et al.* (2004) and Purwar and Sachan (2006). Possible reasons for the great success in isolating the entomopathogenic fungi during this present study

might have been due to the use of new media and the fact that most samples were collected from wet soils and in irrigated zones. In the soils sampled, *M. anisopliae* appeared to dominate the other entomopathogenic mycoflora (Hughes *et al.,* 2004). It was also reported that *M. anisopliae* was more often isolated from loamy soil, relative to other soil types (Rath *et al*., 1992, Steenberg, 1995). Its abundance was related with high rainfall (Milner *et al*., 1998a; Rath *et al*., 1992; Prior, 1992; Steenberg, 1995; Vänninen, 1995) too. *M. anisopliae* is considered to be thermophilic as it is rare in cold areas (Vänninen, 1995).

The results also showed that all the forest soil samples tested contain *M. anisopliae* isolates (100%) as reported by Harney and Widden (1991) and Brownbridge *et al*. (1993). They suggested that forest soil was a natural reservoir of a broad spectrum of entomopathogenic fungi. In India, Ambethgar (2002) recorded the distribution of entomopathogenic fungi from Tamil Nadu and Pondicherry. The study was based on 9-year field surveys in paddy, pulses, groundnut, cotton and cashew conducted in Tamil Nadu and Pondicherry revealed the occurrence of 16 species of entomogenous fungi including 11 of Deuteromycotina *viz*., *Aspergillus flavus* Link., *Aspergillus niger* Van Tiegh., *Beauveria bassiana* (Bals.) Vuill, *Cladosporium* sp., *Fusarium pallidoroseum* (Cooke) Sacc., *Fusarium* sp., *Metarhizium anisopliae* (Mets.) Sorokin, *Metarhizium flavoviridae* var minus Rombach, *Metarhizium* sp., *Nomuraea rileyi* (Farlow) Samson and *Verticillium lecanii* (Zimm.) Viegas and 5 of Zygomycotina. This study correlates with the present study in the fact that in Tamil Nadu, paddy, cotton and groundnut fields has a favorable environment that could support the growth of *M. anisopliae.*

The occurrence of *M. anisopliae* was highest in the soil sample collected from the cotton field of Vilupuram District, followed by those in the cotton field samples of Thoothukudy district. *M. anisopliae* was also noticeable for the 45% of sites sampled by

Hughes *et al.* (2004). The results of Rath *et al.* (1992), Vänninen (1995), Chandler *et al.* (1997), Klingen *et al.* (2002) and Ali-Shtayeh *et al.* (2002) were controversy with the present study. They reported lower abundances (<5 and 30%) of the entomopathogenic fungi in agricultural habitats of their study.

The present work reveals a greater likelihood in finding the insect pathogenic fungi in both arable fields of farmed soils as well as in forest soils. Least difference was found in their distribution based on the various types of soils. In general the occurrence of insect pathogenic fungi is influenced by the isolation methods (bait insect, incubation temperature, depth of soil sample, etc.). Without analysing each factor in detail and modeling the systems, it is difficult to highlight which factors might be important for the distribution of fungi. However, it appears that forest and cotton farmed soils might be a more suitable habitat for insect pathogenic fungi.

For practical reasons, however, it is often best to search in the environment of the target pest for suitable strains of entomopathogenic fungi, and simultaneously testing isolates from other hosts (Milner *et al*., 1998a). A very good example for the "old associations" approach is the *M. anisopliae* var. *acridum* strain that had been isolated from grasshoppers and *M. anisopliae* has utilized against many locust and grasshopper species in sub-Saharan Africa (Lomer *et al*., 2001) till today. The effects of geographical location, climatic conditions, habitat type, soil conditions and pesticide use have determined the occurrence and distribution of insect pathogenic fungi (Vänninen, 1995; Mietkiewski *et al.,* 1997; Chandler *et al.,* 1997; Klingen *et al.,* 2002; Hughes *et al.,* 2004). Comparisons between the occurrence of insect pathogenic fungi in organically versus conventionally farmed soil has so far only been undertaken on a minor scale (von Kleespies *et al.,* 1989). The present study therefore was designed with an objective to compare the abundance of

insect pathogenic fungi in agricultural soil with those of the soil in the forests.

Milner (1992) reported that *M. anisopliae* was common over a wide range of habitats. He isolated *M. anisopliae* from 53% of agricultural soils in warm and wet climatic zones of Australia. Vänninen (1995) found that *M. anisopliae* was isolated less frequently as latitude increased and concluded that the lower isolate rate of entomogenous fungi in Finland was due in part to both the lesser abundance of insect species and the colder temperatures in the sub arctic region.

2.5. Conclusion

The present study and the discussion proceeded clearly indicate that *M. anisopliae* distributed throughout Tamil Nadu. *M. anisopliae* could survive in the tomato, coconut, paddy, groundnut, potato, carrot and cotton fields as well as in all the forest soils prevailing in Tamil Nadu. Further studies are needed to check the biological control potentials of all the isolates against economically important pests.

CHAPTER – 3: BIOLOGICAL CONTROL POTENTIAL

3.1. Introduction

In India, cotton [*Gossypium hirsutum* Linn. (Malvaceae)] is farmed as a cash crop on large scale in Maharashtra, Gujarat, Karnataka, Madhya Pradesh, Punjab, Rajasthan, Haryana, Tamil Nadu and Uttar Pradesh. Insect pests are one of the chief restraints in cotton cultivation. As many as 130 species of insects, besides few mite species, are known to attack cotton crops in the field (Rao *et al*., 2003). Among the various pests of cotton, *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae), *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), *Dysdercus cingulatus* (Fab.) (Hemiptera: Pyrrhocoridae), *Aphis craccivora* Koch (Homoptera: Aphididae), *Mylabris pustulata* Thunb. (Coleoptera: Meloidae), *M. indica* (Faust) (Coleoptera: Meloidae), *Pericallia ricini* (Fab.) (Lepidoptera: Arctiidae) and *Oxycarenus hyalinipennis* Costa (Hemiptera: Lygaeidae) (Rao *et al*., 2003; David and Ananthakrishnan, 2004) were found to be the most serious pests in India.

D. cingulatus commonly called as "Red cotton bug" or "cotton stainer" or "red seed bug", is a serious pest of cotton (Leakey and Perry, 1966; David and Kumareswari, 1978; Waterhouse, 1998; Mohan *et al.,* 2005), which infests cotton in all the cotton growing regions of India, Bangladesh, China, Japan, New Guinea, eastern Australia and New Caledonia (Waterhouse, 1998; David and Ananthakrishnan, 2004). The other principle host plants of *D. cingulatus* belong to the families Malvaceae and Bombacaceae and include kapok, okra and rosella (Jack and Sands, 1922; Dressner, 1955). It was also recorded in wheat (Srivastava and Gupta, 1971), pearl millet, *Pennisetum glavicum* (Ahmad, 1979) and groundnut (Nandagopal and Gunathilagaraj, 2008). The sap removed

and the fungus introduced into the punctures caused staining of the lint, giving rise to one of its common names, “cotton stainer”. Chemical pesticides like benzophenylureas (Chakraborti and Chatterjee, 1999), monocrotopas and DDT (David and Ananthakrishnan, 2004) were used to manage this pest.

A. craccivora is notorious, cosmopolitan and phytophagous sucking pest of cotton and other leguminous crops throughout India, southeastern Europe and widespread throughout warm temperate, subtropical and tropical regions of the world (Wightman and Rao, 1993; Sridhar and Mobto, 2000; David and Ananthakrishnan, 2004; Hsu *et al.,* 2005). It is found primarily on the growing points of the host plants, including tips, flowers and developing bean pods (David and Ananthakrishnan, 2004). It is one of the most successful insect groups of all times owing to their intricate life-cycle, polymorphism and their ability to reproduce both sexually and asexually (Dixon, 1998; Nirmala *et al*., 2006; Sahayaraj, 2008). Hsu *et al.* (2005) reported the pesticidal resistant ability of *A. craccivora* against the pesticides, phenthoate, benfuracarb, carbosulfan, pirimicarb, bifenthrin, cyfluthrin, deltamethrin, flucythrinate and imidacloprid under laboratory conditions.

The “tobacco caterpillar”, *S. litura* is a widely distributed and polyphagous pest affecting several crops worldwide causing extensive loss of agricultural output (Mehrotra, 1989; Guo *et al.,* 2007). This insect pest has about 360 host species. They include cotton, cruciferous vegetables, cucurbits, groundnut, maize, potatoes, rice, soybean, tea and tobacco etc (Moussa *et al.,* 1960; Panchabbani and Nethrabbaniraj, 1987; Dhin *et al.,* 1992; Singh and Jalali, 1997; Catindig and Heong, 2003; Ghumare and Mukherjee, 2003; Chaudhari *et al.,* 2004; Sahayaraj and John, 2004; Kodandaram and

Dhingra, 2007; Saxena, 2008). Management of this pest using synthetic chemicals has been relatively abortive because of its insecticidal resistance (Mehrotra, 1989; Guo *et al.,* 2007; Pandey, 2007; Kodandaram and Dhingra, 2007).

H. armigera is a hindrance for the successful cultivation of several economically important crops especially cotton in Australia, China, India, Pakistan, South Africa, Taiwan and Thailand (Wadyalkar *et al*., 2003; Bency *et al*., 2005; Dolinski and Lacey, 2007). It is ordinarily called as "cotton bollworm". In India, it is known as the "gram pod borer" as it is a serious pest on pulses. The larvae feed on leaves and fruiting bodies. They completely feed away the internal contents of fruiting bodies resulting in serious loss (Gopalakrishnan and Narayanan, 1988; Ramanujam *et al.,* 2002; Nahar *et al.,* 2004; David and Ananthakrishnan, 2004). *H. armigera* is resistant to insecticides such as against deltamethrin, endosulfan, fenvalerate, monocrotophos, phosalone and quinalphos (Manikandan,1997 and 1998; Das, 2002; Yadav *et al*., 2006).

O. hyalinipennis is known as "dusky cotton bug". In India, it is found in all cotton growing regions co-inhabiting along with the red cotton bug. Both adult and nymphs suck the sap from immature seeds and also stain the lint. The seeds do not ripen and thus get damaged (Nasr *et al.,* 1992; Abd-El-Naby *et al.,* 1993; David and Ananthakrishnan, 2004; Brambila, 2007). *O. hyalinipennis* was reported from Turks and Caicos Islands of Providenciales and North Caicos in West Indies (Slater and Baranowski, 1994), Africa, some countries in South America, Central America, Caribbean and Egypt (Brambila, 2007).

P. ricini usually known as "hairy caterpillar" or "wooly bear" is a major pest of castor, gingelly, cotton, country bean, brinjal, drum stick, banana, calotropis, sunflower,

oleander, tea, sweat potato and pumpkin (David and Ananthakrishnan, 2004; Gloviana *et al.,* 2004; Mala and Muthalagi, 2008). *M. pustulata* is a common insect pest in India on most of the field crops (Ness and Sastawa, 2000; Khan *et al*., 2005; Nandagopal and Gunathilagaraj, 2008).

Chemical pesticides were phytotoxic (Miranpuri and Khachatourians, 1996), caused resistance, outbreak and resurgence of the insect-pests, pollution hazards and disruption of the eco-balance (Chakraborti and Chatterjee, 1999; Yadav *et al.,* 2006). Due to the ineffectiveness, inherent health and environmental hazards of chemical pesticides, search for less disruptive and economically friendlier control strategies has increased over the recent years (Gauchan *et al*., 1998; Ericsson *et al.,* 2007; Dong *et al.,* 2007). One such possible alternative pest control option in biological control where entomopathogenic fungi (Zimmerman, 1993; Milner and Staples, 1995; Sahayaraj and Borgio, 2008; Saxena, 2008) play an important role. More than 750 species of fungi are known to be pathogenic to insects (Mc Coy *et al*., 1988; Charnley, 1989; Tanada and Kaya, 1993; Purwar and Sachan, 2006; Kenis *et al.,* 2008; Posada-Flórez, 2008). Fuxa (1987), Stolz (1999), Purwar and Sachan (2006), Dolinski and Lacey (2007), Thomas and Read (2007) and Posada-Flórez (2008) discussed that the use of entomopathogenic fungi does not contaminate ground and surface water supplies nor cause other environmental problems commonly associated with broad-spectrum chemical pesticides.

The biocontrol agent, *M. anisopliae* is an extensively studied, cosmopolitan, filamentous fungus, which is a key regulatory organism of insect populations (Inglis *et al*., 2001; Kassa *et al*., 2004; David, 2008). It is a member of the Hyphomycetes class of fungi and is categorised as a “green muscardine fungus” due to the green color of the

sporulating colonies (Stolz, 1999). Elie Metchnikoff first used *M. anisopliae* against wheat grain beetle, *Anisoplia austriaca* (Herbst) (Coleoptera: Scarabaeidae) in 1989 (Rath *et al*., 1992). It was later used to control the sugar beet curculio, *Cleonus punctiventris*. *M. anisopliae* is recognised pathogen of more than 200 insect species, including several major pests (Roberts and Hajek, 1992; Rao *et al.,* 2007). It is used as a biological agent against many insect pests particularly *H. armigera* (Gopalakrishnan and Narayanan, 1998; Ramaniujam *et al*., 2002; Nahar *et al*., 2004; Abed *et al*., 2005); *S. litura* (Ramanujam *et al*., 2002; Anonymous, 2004); *D. cingulatus* (Borgio and Sahayaraj, 2007), aphids in general (Butt *et al*., 1995; Chandler, 1997) and *A. craccivora* in particular (Ibrahim and Nugroho, 2005; Nirmala *et al*., 2006), green stink bug *Nezara viridula* (L.) (Hemiptera: Pentatomidae) (Borges *et al*., 1993; Sosa-Gomez *et al*., 1997; Daniel *et al*., 1998), chinch bugs of the genus *Blissus* (Samuels *et al*., 2002), plant bug, *Lygus lineolaris* Palisot de Beauvois (Hemiptera: Miridae) (Liu *et al*., 2003) and subterranean burrower bug, *Cyrtomenus bergi* Froeschner (Hemiptera: Cydnidae) (Jaramillo *et al*., 2005; Jaramillo and Borgemeister, 2006). Although *M. anisopliae* have a very wide host range (Veen, 1968; Brady, 1979a, 1979b; Milner and Jenkins, 1996; Scholte *et al*., 2006), individual isolates can be considerably host-specific (Goettel *et al*., 1990, Rath, 2000; Ericsson *et al.,* 2007; Dong *et al.,* 2007). No previous reports were available about the virulence of *M. anisopliae* against *M. pustulata*, *P. ricini* and *O. hyalinipennis*. It is a recurrent paradigm in the literature that there are fungal isolates or genotypes adapted for pathogenesis toward certain species or taxa of insects (Sneath and Sokal, 1973; Bridge *et al*., 1993; Fegan *et al*., 1993; Leal *et al*., 1997; Neuveglise *et al*.,

1994; Tigano-Milani *et al*., 1995; Bridge *et al*., 1997; Leal *et al*., 1997; Purwar and Sachan, 2006; Kunimi, 2007).

Because *M. anisopliae* offers an environmentally safe alternative to chemical pesticides, it is of growing interest in the control of agriculturally important pests (Purwar and Sachan, 2006) and more virulent strains are being sought for commercial biological control applications. Even though few studies considering the virulence of *M. anisopliae* against *S. litura* and *H. armigera* (Ramanujam *et al.,* 2002; Nahar *et al.,* 2004; Anonymous, 2004) were conducted separately, no report was available for the impact of a single fungal isolate against all the above-mentioned pests. Since these pests now appears to have evolved as an economically crucial pest in Asia and also in other countries, too little is known about the effective enemies of the related species (Waterhouse, 1998; Purwar and Sachan, 2006), the present study was designed with the main objective to evaluate the efficacy of the most virulent indigenous *M. anisopliae* isolate against the insect pests such as *O. hyalinipennis, A. craccivora, M. pustulata, D. cingulatus* adults and also against the various stages (II, III and IV instars) of *P. ricini, S. litura* and *H. armigera* under laboratory conditions using different spore doses by dermal toxicity method.

3.2. Materials and Methods

3.2.1. Sources of pests

Adults and nymphs of *Dysdercus cingulatus* were originally collected from the cotton fields in different parts of Tamil Nadu, India to initiate a laboratory population in Crop Protection Research Centre, St. Xavier's College, Palayamkottai, TN, India. They were reared on potted cotton plants and maintained in the laboratory at 26 ± 2^0C, 75%

relative humidity (RH) and 16 hrs of light and 8 hrs of dark photoperiod. Colonies for this trial were also maintained in 30 x 15 cm plastic containers with soaked cottonseeds as food. They were covered with muslin cloth to prevent the escape of the nymph. The rearing room was maintained as the above-mentioned conditions. Colony size was maintained at approximately 300 – 500 adults and 2000 – 4000 nymphs throughout the bioassays. Adult females and males (>6 hrs) emerged from the laboratory culture was used for the present study.

Life stages of *Pericallia ricini, Oxycarenus hyalinipennis*, *Spodoptera litura, Helicoverpa armigera, Aphis craccivora* and *Mylabris pustulata* were collected from the cotton fields in and around Palayamkottai, Tirunelveli District, Tamil Nadu, India and maintained on cotton. Laboratory reared II, III and IV instars of *P. ricini, H. armigera* and *S. litura* respectively and adults of *O. hyalinipennis, A. craccivora* and *M. pustulata* (>1 day) were used in the present study.

3.2.2. Cultivation of the isolates and preparation of conidial suspension

All the *Metarhizium anisopliae* isolates obtained from the previous chapter was used samples and also the reference culture were used in these assays. Conidia were harvested directly from the fungal cultures grown on PDA at 26 ± 2^0C for in an incubator for 1 week by scraping the sporulating colonies and suspended in 0.1% of castor oil in 1ml of sterile distilled water. The conidial suspension was filtered through two layers of cheesecloth to eliminate mycelial fragments. The stock solution was diluted and conidial counts were made using an improved Neubauer haemocytometer. The concentrations of the diluted stock solutions of all the isolates *viz*., CPRC1, CPRC2, CPRC3, CPRC4, CPRC5, CPRC6, CPRC7, CPRC8, CPRC9, CPRC10, CPRC11, CPRC12, CPRC13,

CPRC14, CPRC15, CPRC16, CPRC17, CPRC19, CPRC20, CPRC21, CPRC22 and the standard were 3.5 x 10^7, 3.6 x 10^7, 7.3 x 10^7, 2.9 x 10^7, 2.6 x 10^7, 1.7 x 10^7, 3.5 x 10^7, 2.4 x 10^7, 2.6 x 10^7, 3.4 x 10^7, 1.5 x 10^7, 1.4 x 10^8, 6.3 x 10^7, 1.1 x 10^8, 2.3 x 10^7, 3.2 x 10^8, 1.7 x 10^8, 2.8 x 10^7, 3.5 x 10^8, 4.8 x 10^7, 1.1 x 10^7, 6.6 x 10^7 and 1.9 x 10^7 conidia/ml respectively. The conidial suspensions were diluted using an eppendorf micropipette. Four spore concentrations of 10^7, 10^6, 10^5 and 10^4 spores/ml were prepared for each isolate from the diluted stock cultures.

3.2.3. Laboratory bioassay on *D. cingulatus* eggs

3.2.3.1. Soil preparation

Bioassays were performed on the soil collected from the farmers' cotton field in Mannarpuram, Nanguneri agricultural zone, Tirunelveli district, TN, India. Previously, the soil (1000–1500 g) was autoclaved at 121 °C for 1 h, left overnight for the germination of any spores if present and was again autoclaved for 30 min. The soil was then placed in a drying oven at 70 °C for 12 hrs. The sterile soil was stored in a sterile container until use.

3.2.3.2. Bioassay

10^7, 10^6, 10^5 and 10^4 conidial spores/ml for each isolate were prepared for the bioassay as mentioned above (section 4.2.2.). 0.1% of castor oil was used as the wetting agent. For each assay, 20 laboratory laid eggs of *D. cingulatus* (>6 hours) placed on the sterile soil in plastic container (250 ml) and sprayed with respective conidial suspension. The sprayer was held 15cm above the container and was operated for each spray lasted for 15‑20s, followed by about 5 min deposition. In bioassay of each isolate, a spray of 0.1% of castor oil was also included as a blank control. Treated eggs were maintained in

incubators at 26 ± 2^0C, 85% relative humidity and 16 hrs of light and 8 hrs of dark photoperiod. Five replications were maintained for each isolate and control. Egg colour change, incubation period, hatchability were recorded till no more eggs hatched in all treatments. The unhatched eggs were finally transferred into moist Petri dishes for 2 to 3 days if fungal outgrowths were not evident, and eggs were individually examined under a microscope for the verification of fungal infection. Eggs with outgrowths of the fungal pathogen and unhatched were recorded as dead eggs which killed by the fungal pathogen.

3.2.4. Bioefficacy of selected isolates on *D. cingulatus* instars

Laboratory emerged three first instar *D. cingulatus* nymph was placed on a 500ml container and sprayed with 1 ml conidial suspensions of selected isolates such as CPRC16 (5.8 x 10^5, 4.9 x 10^6, 2.1 x 10^7 and 2.8 x 10^8), CPRC18 (1.4 x 10^5, 1.5 x 10^6, 1.4 x 10^7 and 1.9 x 10^8) and MTCC892 (1.9 x 10^5, 1.3 x 10^6, 3.0 x 10^7 and 4.4 x 10^8) separately using a hand sprayer. The nymphs were then allowed to feed water soaked cotton seeds. The above protocol was also followed for second, third, fourth and the fifth instars and adults of *D. cingulatus.* Six replications were maintained for each concentration. The control insects were treated with sterile distilled water containing 0.1% of castor oil. All the treated and untreated insects were incubated at 26 ± 2^0C for 1 week with a 16:8 hrs (light: dark) photoperiod and the diet was changed every day and mortality was recorded daily till 120 hours. In order to confirm that death, dead insects were transferred to Petri dishes lined with moist cotton to encourage fungal growth and sporulation of *M. anisopliae*. The mycelial growth on the insects was recorded for every 24 hrs till 120 hours.

3.2.5. Bioassay on *D. cingulatus* adults

3.2.5.1. Contact toxicity

Three *D. cingulatus* adults were randomly selected and placed in containers (500ml capacity). Inoculations were sprayed with each suspension separately using a hand sprayer with a nozzle size of 0.3mm in diameter (Amway product, U.S.A). 0.1% of castor oil was used as the wetting agent. Control category was sprayed with distilled water plus 0.1% castor oil. Excess conidial suspensions were removed by placing inoculated larvae onto filter paper. Six replications were maintained for each treatment. Ten minutes after the treatment, insects were transferred and housed on a clean plastic container (500 ml) with water soaked cotton seeds and incubated under laboratory conditions (26± 2^0C, 75% RH and 16:8h D: L). Dead larvae were recorded daily until 120 hours. The mycelial growths on the insects were also recorded under the microscope (10x).

3.2.5.2. Direct plate assay

For direct plate assay, 10 red cotton bugs (5 males + 5 females) were infected by letting them to walk over the 7 days old sporulating *M. anisopliae* isolates for 10 min. The infected adults were then placed on a plastic container containing water soaked cotton seeds and maintained under above said laboratory conditions. Mortality of the adults, their fecundity, and hatchability and survival of the first instar nymphs and their mortality were recorded for every 24 hours till 120 hours.

3.2.5.3. Sapling bioassay

For sapling bioassay tests, three-liter plastic pots (30cm diameter, 13.5cm height) filled with sandy soil and cotton plant was cultivated. Adult *D. cingulatus* was released

into 20-day-old cotton saplings, allowed for acclimatisation for a day then *M. anisopliae* (CPRC 16, CPRC 18 and standard) at: 10^5, 10^6, 10^7 and 10^8 concentrations was sprayed at a height of 25cm using hand sprayer. Then the sapling in the pot was covered with chimney glass. The uncovered top portion of the chimney glass was covered by a mosquito net (1cm with 3mm mesh). The mycelial growth on the insects and mortality were recorded for every 24 hrs till 120 hours. The percentage of infection among the live and dead adults were calculated using the following formulae

$$\text{Infection among live insects } (\%) = \frac{\text{Number of infected organisms}}{\text{Total number of organisms released on sapling}} \text{ X } 100$$

$$\text{Infection among dead cadavers } (\%) = \frac{\text{Number of infected organisms}}{\text{Total number of dead organisms.}} \text{ X } 100$$

3.2.6. Bioefficacy of selected isolates on 6 cotton pests

Pure stock cultures of the most virulent isolate of *M. anisopliae* CPRC16 was selected for this study (1.6×10^7 conidia/ml). Four spore concentrations of 1.7×10^4, 2.6×10^5, 1.9×10^6 and 1.6×10^7 spores/ml were prepared from the stock culture as mentioned in section 3.2.2**.** It was sprayed onto the insects (II, III and IV instars of *P. ricini, H. armigera* and *S. litura* and adults of *O. hyalinipennis, A. craccivora* and *M. pustulata*) and larval mortality were recorded every 24 hours till 120 hours. Death due to mycosis was inferred by observing the mummification of cadavers. The cadavers were placed on wet cotton in Petri plates and observed for mycelial growth and sporulation in the next 48 h to confirm death due to infection by fungi.

3.2.7. Statistical analysis

Finney's (1971) formula was used to calculate the corrected mortality. From the corrected mortality data, the probability integral of chi-square distribution and LC_{50} were calculated in order to differentiate the difference in their efficacy among the isolates. Mortality data obtained in these experiments were subjected to correlation analysis using STATISTICA/w 5.0. software. A one-sample 't' test analysis was also performed to determine if the mortality due to direct plate exposure of each isolate was significantly different from zero (P = 0.05). The mean values were calculated using STATVIEW software. LSD (Least Significant Difference) 0.05 was calculated to determine any significant difference in growth of the cultures. Cluster analysis of the isolates based on their radial growth during 10 days for three successive generations was performed using the average linkage UPGMA algorithm in the statistical software package using STATISTICA/w 5.0.

3.3. Results

3.3.1. Laboratory bioassay on *Dysdercus cingulatus* eggs

Eggs treated with *Metarhizium anisopliae* became shriveled and turned dark yellow to brown (Plate 3.1). Seven days after fungal inoculation sporulation was first observed on egg surfaces. The attacked eggs were totally covered with a white mycelial cover. *In-vitro* assays on the eggs color changes, incubation period, mortality, hatchability and appearance of mycelial growth on the eggs are reported in table 3.1. The number of days needed for the eggs color change was longest in the isolates CPRC4 (8.3 days) followed by CPRC22 (8.2 days) and CPRC18 (7.7 days). The incubation period for the treated eggs to hatch was longest in the isolates CPRC4 (9.2 days) followed by

CPRC22 (8.7 days) and CPRC18 (8.7 days). The highest mortality (96.67%) was noticed among the eggs treated with the isolates CPRC6, CPRC11 and CPRC16. Mycelial emergence on the surface of the treated eggs after 7 days of incubation was confirmed microscopically. Only those eggs showing external mycelial growth were considered as unhatched eggs due to the fungal infection. For instance CPRC16 showed maximum percent (70.00%) mycelial growth, followed by CPRC22 and CPRC18.

3.3.2. Bioefficacy of selected isolates on *D. cingulatus* instars

The most virulent *M. anisopliae* isolates (CPRC16 and CPRC18) were chosen based upon the biological control potential on *D. cingulatus* adults and they were used to evaluate their bioefficacy on nymphal stages of LC_{50} values, lower and upper fiducial limits, slope, intercept and chi-square values of each isolate are tabulated in the table 3.2**.** The mortality was directly proportional to the concentration of *M. anisopliae* spore concentrations tested. The percentage of mortality of the first, second, third, fourth and fifth instars, and adults of *D. cingulatus* are presented in Figure 3.1. The highest mortality was observed in third instar nymphs treated with CPRC16 at 10^8 concentration. Lowest LC_{50} (2.25×10^5) was recorded in *D. cingulatus* II instars treated with CPRC16. As observed for the adults CPRC16 isolate caused the most significant mortality (Chi-square = 28.85611; df=2; $p < 0.0001$) on the II instar, reaching fiducial limits from 2.13×10^5 conidia/ml to 2.27×10^5 conidia/ml (Table 3.2).

3.3.3. *D. cingulatus* adults

3.3.3.1. Contact bioassay

Bioassays were carried out to determine lethal concentrations LC_{50} of 22 *Metarhizium anisopliae* isolates along with standard against *D. cingulatus* adults. LC_{50}

values shows that isolates CPRC16 (1.26 x 10^5 conidia/ml) and CPRC17 (3.66 x 10^8 conidia/ml) were the most and least virulent isolates respectively against *D. cingulatus* (Table 3.3). LC_{50} values coincided with both upper lower fiducial limits. The most virulent isolate CPRC 16 was 2904.76 and 13.36 times more efficient than CPRC 16 and standard respectively. CPRC 16 also caused the most significant mortality (Chi-square = 31.95032; df = 1; $p < 0.0001$) on the adults, reaching corrected mortality percentages from 46.15 to 99.99 (Figure 3.2.). Efficiencies were statistically distinguishable between all the isolates (Table 3.3). The mortality of the isolates CPRC 4, CPRC 11, CPRC 13, CPRC 17 and CPRC 20 were insignificant ($P > 0.05$). Remaining isolates were significant ($P < 0.05$). After 73 hours, highest sporulation (11 cadavers) was observed on the cadavers treated with *M. anisopliae* CPRC18 (Figure 3.3) followed by CPRC16, CPRC22, CPRC4 and CPRC7, CPRC9 and MTCC 892, CPRC10, CPRC8, CPRC12 and CPRC1. Plate 3.2. shows *D. cingulatus* adult shriveled on ventral side and dark brown colouration on dorsal side after treatment with *M. anisopliae* CPRC16 after 5 days of incubation.

3.3.3.2. Direct plate assay

The biological control efficacy of *M. anisopliae* under direct plate assay method on *D. cingulatus* adult's mortality, fecundity and hatchability are presented in tables 3.4 and 3.5. From the results, it was very clear that mortality caused by CPRC16 was significantly greatest (91.66%) (Mean = 53.330, df = 4, $P < 0.05$), followed by CPRC21 (79.17%) (Mean = 35.000, df = 4, $P < 0.05$). It was also clear that CPRC2, 4, 5, 6, 9, 11, 15, 17, 18 and MTCC892 were insignificant at 5% by "t" test. The efficacy of the isolates to grow and sporulate on the cadavers also varied. Adults that survived after infection,

produced fewer eggs when compared to the control (Table 3.5). Maximum numbers of eggs (413) were laid by control categories and the fecundity was completely arrested by CPRC1, CPRC20 and CPRC22. Though CPRC16 caused higher mortality, its live *D.cingulatus* produced more number of eggs (163eggs/female). The incubation period of the eggs were also extended to 9 days in the eggs produced by the adults treated with CPRC19 while compared to control (5 days). Most of the eggs produced by the adults treated with CPRC1, 2, 3, 4, 9, 10, 11, 12, 14, 16, 17, 20, 21 and 22 failed to hatch. Among the emerged nymphs, the survival period was ranged from 1 (CPRC8) to 10 days (CPRC8). Survival rate was highly positively correlated with the incubation period. Both the standard and CPRC6 caused 100% mortality followed by CPRC19, CPRC5, 7 etc.

3.3.3.3. Sapling bioassay

Using sapling experiment, the efficacy of the two most virulent isolates (CPRC 16 and CPRC 18) and the standard strain were tested against *D. cingulatus* adults. All the isolates were highly infective to *D. cingulatus* adults at all dosages tested (Table 3.6). From the LC_{50} value, it was very clear that isolate CPRC18 (2.27×10^5 spore/ml) of *M. anisopliae* was the most virulent against *D. cingulatus* adults (Table 3.6) and statistically significant (Chi-square = 19.34137; df=1; $p < 0.0001$). The same isolate, yielded the highest corrected mortality (86.67%) confirming its maximum efficacy among the three isolates (Figure 3.4). Plate 3.3 demonstrates the mycelial growth of the isolates on the leaf attached to dead *D. cingulatus*. Maximum number of adults showed mycelial development after treatment at the concentration of 10^7 spores/ml, was those treated with the isolate CPRC18 (100%) followed by CPRC16 (75%) (fig 3.5). The highest

percentage of infection among the live (80.00%) and dead (92.30%) adults was observed at 10^8 spores/ml with the isolate CPRC18 (Table 3.7).

3.3.4. Bioefficacy of selected isolates on 6 pests

Impact of the most virulent isolate CPRC16 was used to evaluate its efficacy against the adults of *Aphis craccivora*, *Oxycarenus hyalinipennis* and *Mylabris pustulata* and II, III and IV instars of *Helicoverpa armigera, Pericallia ricini* and *Spodoptera litura* is presented in table 3.8. Percentage mortality caused by CPRC16 on 6 pests is presented in figure 3.6. Irrespective of the pests tested, the corrected mortality varied from pest to pest. The isolate caused maximum corrected mortality on *M. pustulata* adults (95.83%) followed by *H. armigera* third and fourth instar (93.33%) and *S. litura* second instar (93.33%) at the concentration of 1.6 x 10^7 conidia/ml (fig 3.6). The LC_{50} values were ranged from $1.62x10^4$ to $1.75x10^6$ spores/ml. The isolate had lowest LC_{50} values on the II instar larvae of *P. ricini* (Chi-square = 7.50341; df=1; $p < 0.05$) followed by *A. craccivora* adults (Chi-square = 14.75082; df=1; $p < 0.05$) and the III instar of *S. litura* (Chi-square = 7.70020; df=1; $p < 0.05$). Among the pests tested, mycelial growth was observed only on 20.0, 26.6 and 46.6% adults of *A. craccivora* treated with 2.6 x 10^5, 1.9 x 10^6 and 1.6 x 10^7 conidia/ml respectively after 4 days of treatment. Correlations mortality of *M. anisopliae* isolate against 6 insects pests are presented in table 3.9.

3.4. Discussion

Entomopathogenic fungi are ecologically considered as fungi that grow either inside the insect bodies or on the surface of their exoskeleton, which eventually cause the death of the host insect (Lee *et al*., 2005). Entomopathogenic fungi enter into the hosts by direct penetration of the cuticle, that functions as a barrier against most microbial attack.

Consequently, fungal entomopathogens have higher potential for biological control of sucking and defoliator insect pests and all that are difficult to combat with synthetic insecticides (Kang *et al.,* 1999; Ramanujam *et al.,* 2002; Nirmala *et al.,* 2006; Mohi-Un-Din *et al.*, 2006; Saxena, 2008). Diverse toxic metabolites have been described in several fungal biological control agents including species of *Metarhizium, Beauveria, Paecilomyces* (Vey *et al*., 2001; Purwar and Sachan, 2006, Saxena, 2008). Some of these metabolites have been found to display antibiotic, fungicidal or insecticidal properties against insect pests and diseases (Kershaw *et al*., 1999; Vey *et al*., 2001).

3.4.1. Bioassay on *Dysdercus cingulatus* eggs

Based on our *in-situ* soil bioassay, using 22 isolates and standard strain of *Metarhizium anisopliae* had shown potentials as a biological control agent against *D. cingulatus* eggs. The bioassays of fungal biocontrol agents on eggs of pests are different from those dealing with immature or adult pests (Alves *et al*., 2002; Shaw *et al*., 2002). First, the eggs are too small to be moved by hand without injury to conduct the bioassay. To minimise artifacts with their hatch rates, all eggs used in this study were laboratory laid on the soil in laboratory. Second, the eggs were inoculated by fungal spray in a way similar to a fungal formulation applied in the field or greenhouse. This ensured that the eggs were exposed to fungal infection as naturally as possible, which is a concern in bioassays of fungal agents against insect pests (Lacey *et al*., 1999; Wraight *et al*., 1998). Third, perhaps the largest difference was the way to obtain counts of dead eggs attributed to fungal infection after spray. Unlike active immature and adult stages, the eggs are immobile, making it difficult to determine their mortality status. Slight changes in egg morphology such as shape and color were not sufficient to judge the deaths based on our

experience. Thus, the unhatched eggs observed on a given day after spray could not be classified as dead or alive until fungal outgrowths were visible or they hatched.

In this study, the number of days taken for the change in color of the eggs and the incubation period for the treated eggs was longest in the isolate CPRC4 (8.33 and 9.16 days). The highest mortality (96.67%) was noticed among the eggs treated with the isolate CPRC16 at 3.2 x 10^8 spores/ml. Pirali-Kheirabadi *et al*. (2007) studied the virulence of 3 native strains of *M. anisopliae* on *Rhipicephalus annulatus* life stages. Their results revealed that egg hatchability decreased (89.1%) at 10^7 conidia/ml of *M. anisopliae* (IRAN 437 C). Perhaps more attention should be given to the isolate CPRC16 due to its better ovicidal activity. The ability of *M. anisopliae* to infect *D. cingulatus* eggs or life stages has not been reported earlier in the literature. Recently Borgio and Sahayaraj (2007) reported the ovicidal effect of *M. anisopliae* on *D. cingulatus* eggs. However, studies on the efficacy of *M. anisopliae* isolates against the eggs of *Deois flavopicta* (Stal) (Homoptera: Cercopidae) (de Faria *et al*., 1995), *Anoplophora glabripennis* Motschulsky (Coleoptera: Cerambycidae) (Hajek *et al*., 2007), *Tetranychus cinnabarinus* Boisduval (Acaridae: Tetranychidae) (Shi and Feng, 2004), *Bemisia* sp. (Barbieri *et al*., 2005) and ticks (Gindin *et al*., 2002) were available in the literature. Newly laid eggs of some insects have also been reported to be more susceptible to *M. anisopliae* than aged eggs (Zimmermann, 1982; Villecorta, 1983; Maniania, 1991). Any application of an entomopathogenic fungus in the field would be targeted at the onset of egg hatch says Bruck *et al.* (2005). This study shows that the eggs of *D. cingulatus* were highly susceptible to infection by all the isolates of *M. anisopliae,* indicating a possible new approach to the control of red cotton bug in particular and sucking pests in general.

3.4.2. Bioefficacy of selected isolates on *D. cingulatus* instars

Crops are often infested with several different stages of the pest. For the successful introduction of an integrated pest management (IPM) programme, information is needed about the most susceptible stage of the pest species (Ferron, 1985; Cuthbertson *et al*., 2005). In the present study, different developmental stages of the tested pests varied in their susceptibility to infection of *M. anisopliae.* Among the life stages of *D. cingulatus*, the second instars were the most susceptible to infection by the isolate CPRC16 than the adults. This observation concurs with those of Bero´n and Diaz (2005), who reported that larval stages of the white grub, *Cyclocephala signaticollis* Burmeister (Coleoptera: Scarabaeidae) were more sensitive to infection by *M. anisopliae* than adults. In this research, the mortality in all stages was dose-dependent one. Similar dose-dependent mortality responses at different developmental stages have been reported by several authors (Feng *et al*., 1985; Ferron, 1985; Poprawski *et al*., 1985; Fransen *et al*., 1987; Bero´n and Diaz, 2005; Pirali-Kheirabadi, 2007). This is of importance in the control of this quarantine species where it is desirable to infect the pest species at the earliest life-stage possible resulting in a rapid effect. Studies should be developed to improve the level of control to that required in commercial IPM systems, by optimisation of application and post-application conditions and other means. Further work is required to raise the pest mortality to commercially acceptable levels.

3.4.3. Contact bioassay on *D. cingulatus* adults

Laboratory screening of isolates is an essential step in identifying virulent strains prior to field use. The approach to screening adopted in this study proved a robust mechanism that has been used successfully by many other workers earlier (Adane *et al*.,

1996; Moino *et al*., 1998; Kassa *et al*., 2002; Borgio and Sahayaraj, 2007; Dong *et al*., 2007). *M. anisopliae* have wide host ranges, although it has been recognised as a species contain a diverse assemblage of genotypes and probably comprise 'species complexes'. Within these species, individual isolates can exhibit substantially restricted host range (Inglis *et al*., 2001; Ibrahim and Nugroho, 2005; Mohi-Un-Din *et al*., 2006). The present assay proved that the 22 isolates of *M. anisopliae* from different districts of Tamil Nadu, varied greatly in their virulence to few locally available pests. Moreover this is the first study to evaluate the susceptibility of *D. cingulatus* to indigenous *M. anisopliae* isolates from Tamil Nadu. Of the 22 isolates tested in this study, all isolates were infective to life stages of *D. cingulatus* by contact assay method, the single dose time–mortality bioassay demonstrated differences in LC_{50} values between all the isolates indicating the distinctive efficacy of each isolate. The present study support the findings of Zimmerman (1993), Gindin *et al*. (2001), Ibrahim and Nugroho (2005) and Mohi-Un-Din *et al*. (2006) in the concept that, the rate of infection varies from strain to strain. The isolates CPRC16 and CPRC18 were most virulent than the other isolates as well as the standard strain.

Reports were not available about the impact of *M. anisopliae* on *D. cingulatus*, although there are studies on the efficacy of *M. anisopliae* isolates from other locations against other insects. The green muscardine soil inhabiting fungus, *M. anisopliae* was reported to infect a wide range of insect hosts, including Orthoptera, Coleoptera, Lepidoptera, Hemiptera, Hymenoptera, and also soil living Arachnida (Abebe, 2002; Purwar and Sachan, 2006; Patrick, 2007). CPRC16 and 18 were the two best isolates identified in this study were as virulent or more than isolates and tested by Luz *et al*. (1998), Lawrence and Khan (2002) and Ansari *et al*. (2004). Ansari *et al*. (2004) selected

a highly virulent fungal isolate, *M. anisopliae* CLO 53 obtained from *Hoplia philanthus* (Füessly) (Coleoptera: Scarabaeidae) in Belgium, for controlling *H. philanthus*. In the present study, the possible causes of poor efficacy of some isolates *viz*., CPRC 6, 17 and 21 may be due to the weak pathogenicity of fungal isolates (Mazodze and Zvoutete, 1999). Mietkiewski *et al*. (1994) found maximal mortality in *Galleria melonella* (L.) treated with *M. anisopliae* at 30°C that is more or less similar to the temperature incubated in the present findings.

Isolate CPRC 16 caused the most significant mortality (46.15 to 99.99%) in *D. cingulatus* adults. Different isolates of *M. anisopliae* were effective against *Chilo sacchariphagus* Boj. (Lepidoptera: Pyralidae) (Mazodze and Zvoutete, 1999; Easwaramoorthy *et al.*, 2001; Shaw *et al.,* 2002), *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae) (Sun *et al.,* 2002), *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) (Batta, 2005), *Boophilus microplus* (Canestrini) (Acaridae: Ixodidae) (Alonso-Díaz *et al*., 2007).

In the present study, highest sporulation (91.67%) was observed from the *D. cingulatus* treated with CPRC18 followed by CPRC16, CPRC22, CPRC4 and CPRC7, CPRC9 and MTCC 892, CPRC10, CPRC8, CPRC12 and CPRC1 isolates. However, it was also reported that insect cuticle has antifungal properties; body exudates or defensive secretions may play a role in lower in sporulation rates (Rosengaus *et al*., 2000). An interesting continuation of this research would be to compare the capability of isolates with quick and total sporulation versus those with high virulence (Fuxa and Tanada, 1987; Sun *et al*., 2001). One way of improving *M. anisopliae* efficacy could be to combine the spores with suitable synthetic insecticides as suggested by Hiromori and

Nishigaki (2001). Additional studies are needed to focus on conditions which increase the efficacy and to understand the role of secondary recycling in extending the period of impact. Formulation of conidia may be one route to increasing efficacy and persistence. Further, mass production and field efficacy studies are essential before recommending these isolates as biological control agents.

3.4.4. Direct plate assay

All the tested isolates of *M. anisopliae* and standard under direct plate assay method on *D. cingulatus* adults caused high mortality, reduced fecundity and hatchability. Exposure of *R. annulatus* to *M. anisopliae* reduced egg laying prior to death and caused 70–98% mortality of treated eggs and the fungal pathogens was also shown to reduce the weight of the eggs laid and the egg laying capacity of adult female ticks (Gindin *et al*., 2002). Fungi and their metabolites can affect several traits of insect biology, such as survival, development, fecundity, feeding activity (Wright *et al*., 1982; Mulè *et al*., 1992; Seyoum *et al*., 1994; Thomas *et al.,* 1997; Amiri *et al*., 1999; Arthurs and Thomas, 2000; Blanford and Thomas, 2001; Ekesi *et al*., 2001; Ganassi *et al*., 2004; Purwar and Sachan, 2006; Jarrold *et al.,* 2007; Kenis *et al.,* 2008) reduce egg mass and hatch rate in ticks (Kaaya *et al.*, 1996) and also affect egg fertility and longevity (Ekesi and Maniania, 2000). In the present research, egg hatchability and adult longevity decreased significantly in *D. cingulatus* that survived post infection when compared to the control. Pirali-Kheirabadi *et al.* (2007) indicated that the egg laying and hatching capacity of engorged tick females; *R. annulatus* exposed to *M. anisopliae* (IRAN 437 C) were significantly reduced.

Between the tested isolates, the isolate CPRC16 caused maximum mortality (91.66%) on *D. cingulatus* adults. Studies on the mortality of various pests by direct plate assay using *M. anisopliae* have been reported on *N. viridula, Piezodorus guildinii* (Westwood) (Hemiptera: Pentatomidae), and *Euschistus heros* Fabr. (Heteroptera: Pentatomidae) (Sosa-gomaz and Moscadi, 1998), *Ceratitis capitata* Weidemann (Diptera: Tephritidae), *C. cosyra* Walker (Diptera: Tephritidae) *and C. fasciventris* Bezzi (Diptera: Tephritidae) (Maniania and Odulaja, 1998; Dimbi *et al*., 2003), *Diatraea saccharalis* (Fabricius) (Lepidoptera: Pyralidae) (Destefano *et al*., 2004), *A. glabripennis* (Hajek *et al*., 2007) and *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae) (Santos *et al*., 2007). In the microbial control of insect pests, secondary effects of the pathogen infection in addition to the mortality caused by the target isolates may play an important role in the management of the pest says Falcon (1985). Egg production in insects is also known to be influenced by the diet protein content (Engelmann, 1984) and cotton seeds is known to be high in protein is an important source of egg production. In this experiment, both control and experimental category insects were provided with same cotton seeds. The results obtained in the present study indicated that true differences in egg production were by fungal infection and not by insect diet. Sikura *et al.* (1972) reported that *Beauveria bassiana* (Balsamo) Vuillemin caused infection in larvae of Colorado potato beetle, which induced histological and cytological injuries to the ovaries, thus preventing follicle development or causing their degeneration and thereby reducing fecundity.

Entomopathogenic fungi are being developed worldwide for the control of many pests of agricultural importance (Ferron, 1985). This study portrays *M. anisopliae* as a promising biological control agent for *D. cingulatus.* Environmental safety and

ecosystem stability considerations lead to the conclusion that the use of native isolates in a microbial control program is preferable (Lockwood, 1993). Field-testing of these isolates will ultimately determine its place in the red cotton bug management programme. Good coordination and awareness of all stakeholders, the government, private sectors and farmers will determine the success of such non-pesticide method development.

3.4.5. Sapling Bioassay

Entomopathogenic fungi have shown their great potential to control insect pests both in the field and on sapling assay (Inglis *et al*., 2001). Sapling experiment revels that the isolate CPRC18 of *M. anisopliae* that proved to be the most virulent against *D. cingulatus* adults. Sahayaraj and Borgio (2008) has already reported the impact of *M. anisopliae* on *D. cingulatus* on sapling. There are studies on the efficacy of *M. anisopliae* isolates from other locations against other insects on sapling. For instance Ansari *et al*. (2004) and Maniania *et al*. (2001) have proved that *M. anisopliae* to be effective in laboratory and on sapling against third instar larvae of *H. philanthus* and *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) respectively.

In the current investigation, mycelial growth of the isolates was prominently observed on the treated dead *D. cingulatus* adults. The fungus emerges from the death host and sporulation or conidiogenesis usually occur on the outside of the cadavers (Shah and Pell, 2003). The conidia of *M. anisopliae* are hydrophobic and are passively dispersed from infected cadavers (Shah and Pell, 2003). Secondary infection from the conidia on the cadavers occurs when key are carried on wind currents or by co-occurring insects (Shah and Pell, 2003). Very interestingly we have observed a live insect infected followed by fungal growth on the dorsal surface. The same type of observation that

conidia of *Entomophthora thripidum* Samson, Ramakers and Oswald (Zygomycetes: Entomophthoracea) on the fly *Strongwellsea castrans* Batko and Weiser are discharged while the host insect is still alive (Steinhaus, 1964; Shah and Pell, 2003).

In our present investigation we have also observed the cadavers, which are attached to the upper foliage of the cotton leaf, and fungal growth covered the entire *D. cingulatus.* With entomophthorelean species, cadavers are attached to foliage by fungal rhizoids, which emerge through the ventral surface of mouthparts of the cadavers (Shah and Pell, 2003). Specialized attachment structures ensure that the fungus remains in the environment of now host for further transmission (Shah and Pell, 2003).

3.4.6. Bioefficacy of selected isolates on 6 cotton pests

M. anisopliae could infect a wide range of insect pest belonging to Orthoptera, Coleoptera, Lepidoptera, Hemiptera, Hymenoptera, and also soil dwelling arachinids (Abebe, 2002; Purwar and Sachan, 2006). However, it is safer to human beings and domestic animals (Zimmerman, 1993). In the present study, all the tested *M. anisopliae* isolates including standard strain exhibited mortality against *Aphis craccivora, Oxycarenus hyalinipennis, Mylabris pustulata, Pericallia ricini*, *Spodoptera litura* and *Helicoverpa armigera*. The pathogenicity of the isolate on all the pests were considered significantly different from one another, as none of the fiducial limits overlapped. Thus this study reveals that the fungus is also able to reach and affect other related harmful pests in the cotton field. The present study has strengthened the pathogenicity reports of *M. anisopliae* against sucking pests such as aphids of many agricultural crops world-wide (Butt *et al.,* 1995; Miranpuri and Khachatourians, 1996; Chandler, 1997; Ibrahim and Nugroho, 2005; Sahayaraj, 2008).

In-vitro bioassay of *M. anisopliae* CPRC16 against the six tested cotton pests reveals that, it was most pathogenic to II instar of *P. ricini* (LC_{50} = 1.62 x 10^4 conidia/ml) than the remaining pests. However, studies about the impact of *M. anisopliae* spores on *P. ricini*, *O. hyalinipennis* and *M. pustulata* were not available in the literature. Hence, the present study is considered to be the first report. In cotton *M. pustulata* and *P. ricini* and *O. hyalinipennis* are the minor and major pests in India (David and Ananthakrishnan, 2004). Gloviana and their co-workers (Gloviana *et al.*, 2004) reported the pathogenicity of entomopathogens such as *Bacillus thuringiensis* sub sp. *kurstaki* and *B. bassiana* to the larvae of *P. ricini.* Recent reports of Elumalai *et al.* (2006) on *P. ricini* reveals that fractions (2000ppm) of *Hyptis suaveolens* (Lamiaceae) and *Melochia chorcorifolia* (Sterculiaceae) caused 77.2 and 63.51% mortality respectively.

Previously Nasr *et al.* (1992) and Khan *et al.* (2005) reported the microbial control of *O. hyalinipennis* and *M. pustulata* respectively. The infection dynamics of *B. bassiana* on *M. pustulata* was also studied by Devi and Rao (2006). Ramanujam *et al.* (2002) and Anonymous (2004) reported the disease causing ability of *M. anisopliae* to *S. litura*. Devi *et al*. (2003) and Srisukchayakul *et al*. (2005) identified the virulent isolates of *N. rileyi* for the management of *S. litura.*

Several reports were available about the biocontrol of *H. armigera* using *M. anisopliae*. In the present study, *M. anisopliae* exhibited promising toxicity levels against II - IV instar larvae of *H. armigera.* It was recorded that later instars were being more susceptible. The study also revealed the larval mortality to be extended up to 93.33% when sprayed with fungal suspension of 1.6 x 10^7 spores/ml. Wadyalkar *et al*. (2003) reported the virulence of *M. anisopliae* against I - IV instar larvae of *H. armigera* and

their study revealed that the larval mortality extent was to 10.00, 90.00, 76.67 and 56.67% respectively on 8^{th} day with fungal suspension of 10^8 conidia/ml. Cent percent larval mortality up to IV instar larvae of *H. armigera* with 1.8 x 10^9 conidia/ml suspension was reported by Gopalakrishnan and Narayanan (1988). Nahar *et al*. (2004) evaluated the impact of *B. bassiana*, *M. anisopliae* and *N. rileyi* on *H. armigera* in pigeon pea field. Kencharaddi and Jayaramaiah (1997) reported the LC_{50} value of *M. anisopilae* against 1^{st}, 3^{rd} and 4^{th} instars larvae of *H. armigera* was 6.07 x 10^4, 6.15 x 10^5 and 1.10 x 10^8 spores/ml respectively. Kencharaddi and Jayaramaiah (1997), Sandhu *et al.* (2001), Ramanujam *et al.* (2002) and Nahar *et al*. (2004) reported the pathogenicity of *M. anisopliae* against *H. armigera.* In addition in India, Verghese *et at.* (1997) pointed out the failure of *Bacillus thuringiensis* (Bt) and Nuclear Polyhedrosis Virus (HaNPV) formulations against this pest. They also urge the fellow entomologists that popularise environmentally safe alternative method to manage *H. armigera*.

Many researchers suspected that the rapid killing ability of *M. anisopliae* to its host could be caused not only through direct physical invasion of the hyphae, but also possible due to some enzymatic mechanisms of glucosidase and acid trehalase which extra cellular hydrolysis of trehalose occurs in host haemolymph during fungal infection (Xia *et al.,* 2002) and also cuticle degrading enzymes. Whereas in sap-sucking insects, fungal entomopathogens enter along with the plant sap and cause high mortality which are difficult to combat with synthetic insecticides (Kang *et al.,* 1999). The positive results obtained are a stimulus to further studies to identify, characterize and possibly increase in the field application of fungi producing bioactive metabolites and to evaluate the effects on target and non-target organisms. Since there is no indication regarding the host range

of the entomopathogens, more work is required to determine host specificity of these fungus.

3.5. Conclusion

The present study has proved that the isolates CPRC 16 and CPRC18 of *Metarhizium anisopliae* could act as biocontrol agent against both defoliators and sucking pests and have the potential against the eggs, instars and adults of *Dysdercus cingulatus* and also for 6 other pests of agricultural importance under laboratory conditions as well as on the saplings. In developing *M. anisopliae* as a commercial control product, the environmental conditions under which the isolates are most pathogenic should be studied in the laboratory, as the information gained would ultimately translate into recommendations for its optimal performance in the field. Fungal characteristics such as spore germination and hyphal growth rates and immunological responses reflected due to treatment must also be evaluated so that the further development of the most appropriate strain can proceed.

CHAPTER – 4: ROUTE OF INFECTION AND HAEMATOLOGY

4.1. Introduction

Insects are infected when getting into contacted with fungal conidiospores (Stolz, 1999; Scholte *et al.,* 2004; Wang *et al*., 2005). After attaching to the insect's body (Bateman *et al.*, 1996), conidia penetrate the cuticle (Clarkson and Charnley, 1996; Charnley *et al.,* 1997; Wang and Leger, 2005) with the help of enzymatic degradation and pressure of the germ tube (Bateman, 1998; Ferron, 1981; Starnes *et al.*, 1993; Pirali-Kheirabadi *et al*., 2007). Similarly *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes), infect its host with spores that adhere and germinate to form a series of infection structures during penetration (Wang *et al*., 2002; Wang and Leger, 2005). Structures and processes for the invasion of insect tissues are similar to plant pathogens, including the formation of germ tubes, appresoria and penetration pegs (Samson *et al*., 1988). The infection may also take place via the respiratory system-spiracles (Burges and Hussey, 1971). The fungus germinates and penetrates into the respirical siphon, blocking the breathing mechanism (Lacey *et al.,* 1988) of the insects. Plugging of the spiracles usually leads to death before significant invasion of the haemocoel has occurred, so hyphal body formation is minimal. Invasion of the insect body and circulatory system (haemolymph) occurs once the fungus has passed through the cuticle of the external insect skeleton.

After infection, yeast-like hyphal bodies (blastospores) (Samson *et al.,* 1988) are produced and spread throughout the haemocoel (Ferron, 1981; Flexner *et al.*, 1986), take nutrients and lead to the death of the host by physiological starvation about 3–7 days after infection depending on species and their size (Boucias and Pendland, 1998; Stolz, 1999;

Pirali-Kheirabadi *et al*., 2007). However, comparatively little is known about the development of the fungus in the haemolymph, in particular assimilation of nutrients. On death of the insect host, the fungus emerges from the dead host and sporulation or conidiogenesis usually occurs on the outside of the cadaver (Whitten and Oakeshott, 1991; Starnes *et al.*, 1993; Shah and Pell, 2003), which can subsequently infect other susceptible hosts (Bateman *et al.*, 1996). Because the normal route of infection is through the cuticle, fungi are especially suitable microbial control agents for sucking insects of Hemiptera (Dolinski and Lacey, 2007).

Recently, Destruxins (dtx) A, B, D, E, and E-diol were identified from this fungi (Seger *et al.,* 2006) and was predicted that this might depress the cellular immune reaction. Saxena and Tikku (1990) performed studies to show deformity in haemocytes due to application of plumbagin, a plant-based phytochemical. Circulating haemocytes play important roles in defense mechanisms against microorganisms in the haemocoel. Cellular defenses refer to haemocyte-mediated responses such as phagocytosis, nodulation, and encapsulation (Schmidt *et al*., 2001). Histopathological studies of beetles tissues infected by *M. anisopliae* suggest that toxins like Destruxins A, B, D, E, and E-diol, Swainsinone, and Cytochalasin C kill the host by inciting degeneration of the host tissues due to loss of the structural integrity of membranes and then dehydration of cells by fluid loss (Ferron, 1981; Strasser *et al.,* 2000; Scholte *et al.,* 2004; Seger *et al.,* 2006). With these facts in mind, an experiment was conducted to examine the route of penetration and distribution of *M. anisopliae* at various parts of *Dysdercus cingulatus* (Fab.) (Hemiptera: Pyrrhocoridae). Moreover haemocyte count and haemolymph quantity were also performed.

4.2. Materials and Methods

4.2.1. Re-isolation of *Metarhizium anisopliae*

The insect cadavers that had fungal sporulation on the surface after 72 hours of treatment with 10^7 conidia/ml of *M. anisopliae* CPRC18 were considered for analysis to confirm that the death of the insects was due to the pathogenicity by *M. anisopliae*. The water washed dead insects were immersed individually thrice in 0.1% (v/v) sodium hypochloride solution, 0.1% (v/v) Tween 80 solution, 0.85% (w/v) sodium chloride solution separately and then rinsed finally in sterile distilled water with gentle agitation to remove non-attached fungi. The antennae, rostrum, head (after removal of antennae and rostrum), tergal plate, sternal plate, leg, haemolymph, muscle, fat body, foregut, midgut, hindgut and testis were individually dissected and separated aseptically. Each body part was ground separately using pestle and mortar along with 1ml distilled water. From each crushed suspension, 0.1ml was transferred using 100 µl sterile micropipettes, onto separate potato dextrose agar (PDA) plates and spread using a sterile, L-shaped glass rod. The body parts of the untreated insects were also plated individually as control. The plates were then incubated at 26°C ± 2°C in an incubator and observed for total fungal population after 7 days. After eminent growth was observed, the fungal colonies suspected to be *Metarhizium* spp. were sub-cultured to obtain pure cultures of the suspected entomopathogen.

4.2.2. Haemosomic index

Twenty *D. cingulatus* adults treated with CPRC18 isolate at concentrations of 2.8 x 10^5,3.1 x 10^6,1.9 x 10^7 and 2.3 x 10^8 conidia/ml were used in this study. Ten live adults were selected randomly from each concentration after 24, 48, 72 and 96 hours of

infection and their initial weights were recorded individually. Base of the antenna was amputated using fine sterile scissors and the oozing out haemolymph was collected in an eppendorf tube. The final weight of the individual insects was recorded after the haemolymph collection. Untreated control adults were also weighed before and after haemolymph collection. The haemosomic index was calculated using the following formula (Jones, 1962):

$$\text{Haemosomic index (\%)} = \frac{\text{Initial weight of the animal - Final weight of the animal}}{\text{Initial weight of the animal}} \times 100$$

4.2.3. Total haemocyte count

Haemolymph collected in the previous experiment was swabbed with 70% ethanol and allowed to air dry. In another experimentation, haemolymph was directly drawn into the WBC pipette up to 0.5 marking for the total haemocyte count (THC). Then it was diluted with an acidified physiological saline (NaCl - 4.65 g; KCl - 0.5 g; $CaCl_2$ - 0.11 g; Gentian violet - 0.005 g and acetic acid 0.125 ml and make up into 100 ml with double distilled water). The diluting fluid was drawn into the pipette up to mark 1.1 giving 20 times dilution. The inner surface of the WBC pipette was rinsed several times with diluting fluid before drawing the haemolymph. At least one minute of gentle stirring was required for complete dispersion of the cells to prevent agglutination and plasma clot formation. Neubauer haemocytometer was loaded immediately with the haemolymph taken in WBC pipette. The cells in all the four 1.0 mm squares were counted. The haemocyte population per mm^3 of haemolymph was calculated (Jones, 1962) as follows.

$$\text{Total Haemocyte count (cells/mm}^3\text{)} = \frac{\text{Haemocytes in four 1 mm}^3\text{ X depth factor X dilution factor}}{\text{Number of squares counted}}$$

Where, Depth factor = 10; Dilution factor = 20

THC was determined in 10 individuals from both control and *M. anisopliae* treated experimental *D. cingulatus* separately .

4.2.4. Enumeration of *M. anisopliae* in haemolymph

The haemolymph (0.1ml) from each concentration was transferred using 100 µl sterile micropipettes, onto separate potato dextrose agar plates and spread using a sterile, L-shaped glass rod for every 24 hours till 96 hours. The haemolymph of the untreated insects were also plated individually as control. The plates were then incubated at 26°C ± 2°C in an incubator and observed for total fungal population after 7 days. After eminent growth was observed, the fungal colonies that were suspected to be *M. anisopliae* were counted.

4.2.5. Cuticle mounting

1. *D. cingulatus* adults treated with CPRC16 were longitudinally opened on its lateral side.
2. The dissected abdomen was heated in 15% KOH over low flame until the cuticle appeared transparent.
3. Then it was fixed on clean glass slides.
4. It was spread evenly using a dissection needle to differentiate the upper and the lower cuticle using dissection microscope.

5. Cuticle was were mounted temporarily in a drop of 15% KOH and covered with a coverslip and examined using an inverted microscope (MOTIC M210, Motic China Group Co., Ltd. China) to observe the changes in the cuticle and also in the spiracles after *M. anisopliae* treatment.

4.2.6. Statistical analysis

Correlation analysis was performed for haemosomic indices at various time intervals and total haemocyte counts during different days after treatment of *D. cingulatus* adults was performed using STATISTICA/w 5.0. software.

4.3. Results

4.3.1. Re-isolation

Metarhizium anisopliae colonies in various parts of *Dysdercus cingulatus* adults after 72 hours is presented in figure 4.1. The fungal population in haemolymph was found to be maximum (13 colonies) followed by leg (8 colonies) and tergum (7 colonies). The antennae, rostrum and testis were free from *M. anisopliae* infection.

4.3.2. Haemosomic index

The haemosomic index (HSI) and percentage of haemolymph reduction of *D. cingulatus* adults is presented in table 4.1. The HSI decreased gradually as the period of incubation after treatment increased. Highest mean HSI was recorded in the control (20.28 ± 0.92), while in treated categories, maximum reduction in haemosomic index was observed after 96 hours of treatment at a concentration of 2.8×10^5 conidia/ml (4.53 ± 0.52). HSI was nil after 96 hours of *M. anisopliae* treatment (2.3×10^8 conidia/ml) which indicates the complete reduction of total haemolymph (Table 4.1). The percentage of total haemolymph reduction in *D. cingulatus* adults treated with *M. anisopliae* at different time

intervals reveals that least reduction (11.29%) of haemolymph was observed after 24 hours of treatment at 2.8 x 10^5 spores/ml. Haemolymph reduction was absolute (100.00 %) in the insects treated with 2.3x 10^8 conidia/ml after 96 hours of treatment. Highest correlation (0.99) was observed between HSI at 72 hours and 96 hours of treatment.

4.3.3. THC

The total haemocyte count (cells/ mm^3) in *D. cingulatus* adults at various time intervals after the treatment of *M. anisopliae* is shown in table 4.2. The haemocyte count decreased suddenly after 24 hours of treatment. However THC count increased to some extent after 48 hours followed by gradual decrease at 72 and 96 hours of treatment. Maximum number of haemocytes (11300 ± 93.0 cells/ mm^3) was observed after 96 hours of treatment in the control *D. cingulatus* adults. But in the case of treated adults, the count was highest after 48 hours (1387 ± 62.0 cells/ mm^3) at 2.8 x 10^5 conidia/ml. At 96 hours the count was least (223 ± 10.0 cells/ mm^3) at a concentration of 2.3 x 10^8 spores/ml. Highest percentage of total haemocyte count reduction was recorded in the *D. cingulatus* adults treated with *M. anisopliae* (99.12 %) at 2.3 x 10^8 spores/ml after 96 hours of treatment (Figure 4.2). Highest correlation (0.99) was observed among the THC at all the incubation periods except among 48 hours and 96 hours.

4.3.4. Enumeration of *M. anisopliae* in haemolymph

The total number of *M. anisopliae* colonies in haemolymph of *D. cingulatus* adults at different time intervals was differed (Figure 4.3). The *M. anisopliae* population in haemolymph was found to be maximum (63 colonies) at a concentration of 1.9 x 10^7 conidia/ml followed by 3.1 x 10^6 conidia/ml (48 colonies) after 96 hours of treatment.

4.3.5. Cuticle mounting

Whole mount of both the sternal (dorsal) and tergal (ventral) abdominal segments of *D. cingulatus* adults examined under an inverted microscope are shown in figure 4.4. The observations showed the following: conidia adherence to the host cuticle and germinate after 24 hrs post-infection (plate 4.1d), falling down of bristles was recorded in 24 hrs (plate 4.1d), the entry of spores through the spiracles (plate 4.2b), three sacs were recorded on the dorsal side of the abdomen (plate 4.3; 4.4; 4.5) and *M. anisopliae* were trapped at the base of the bristles (plate 4.1e) of sternum of the *D. cingulatus* (plate 4.1f). Within 24–48 h post-infection showed, trapping of the spores nearer to the spiracle and trachea (plate 4.6b, c and d). Moreover, colonization and emergence of the fungus to cuticular surface (plate 4.2d and e; 4.6c and d) within 72–96 hrs post-infection was also recorded.

4.4. Discussion

Conidial adhesion to host surface and integument penetration are the initial events during the pathogenic process (Askary *et al.,* 1999). McCauley *et al.* (1968) reported that infection sites varied within host species and most infections occur in the membranous inter-segmental regions. *Metarhizium anisopliae* conidia readily adhere to the surface of most insects (Arruda *et al*., 2005). *M. anisopliae* generally enters into the insects through spiracles and pores in the sense organs. Once inside the insect, the fungus produces a lateral extension of hyphae, which eventually proliferate and consume the internal contents of the insect. Hyphal growth continues until the insect is filled with mycelia. When the internal contents have been consumed, the fungus breaks through the cuticle and sporulates, which makes the insect appear "fuzzy." *M. anisopliae* can release spores

(conidia) under low humidity conditions (<50%). In addition, *M. anisopliae* can obtain nutrition from the lipids on the cuticle. The fungus can also produce secondary metabolites, such as destruxin, which have insecticidal properties on moth and fly larvae (Cloyd, 2005; Purwar and Sachan, 2006). The observations of the present study also revealed that *M. anisopliae* conidia adhered to all the body parts of *D. cingulatus* (except antennae, rostrum and testis). However, the degree of preference was maximum at the legs. Similar findings were also reported by Vestergaard *et al.* (1999). They reported that *M. anisopliae* conidia were capable to bind to cuticle of adult *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae), but were frequently trapped by the setae on the wings and legs. Other studies with *M. anisopliae* (Goettel *et al.*, 1989; Sosa-Gomez *et al.,* 1997) showed that the cuticle regions of *Nezara viridula* (L.) (Hemiptera: Pentatomidae) contain a large number of setae where conidial adhesion was maximum. Polar *et al.* (2005) have indicated that oil formulations can be more effective than water-based formulations. In the present investigation also the use of castor oil might have lead to the attachment of *M. anisopliae* to *D.cingulatus.* A high propensity for attaching to the cuticle of insects may increase infectivity (Jeffs and Khachatourians, 1997; Altre *et al.,* 1999).

In the presence of nutrients and water, conidia of *M. anisopliae* form germ tubes. The germ tube continues undifferentiated hyphal growth on a soft surface or if nutrient quality and quantity is not conducive to differentiation. On a host cuticle, however, apical elongation terminates and the germ tube swells distally to form an appressorium, a major site of adhesion and for production of enzymes that help breach host cuticle and establish a nutritional relationship with the host (Wang and Leger, 2005). The entomopathogenic

fungus *M. anisopliae,* produces variety of hydrolytic enzymes such as proteases (Campos *et al*., 2005), chitinases and lipases on the host cuticle during the infection process (Clarkson *et al*., 1998; Krieger de Moraes *et al*., 2003; Pinto *et al*., 1997; St. Leger *et al*., 1987, 1996a, b; Tiago *et al*., 2002). *M. anisopliae* produce a complex mixture of chitinolytic enzymes during the growth on insect cuticle (St. Leger *et al*., 1996b). Since many such enzymes have been detected and isolated from different strains of *M. anisopliae*, the fungus is considered as a model organism for the study of genes and mechanisms involved in insect pathogenesis (St. Leger *et al*., 1996a, b; Leal *et al*., 1997; Bidochka *et al*., 2001). Previous work showed that *M. anisopliae* infects Elateridae larvae (McCauley *et al.*, 1968) and the conidia germinated within 24–48 hrs after post-infection. In the current investigation, conidial germination was observed on the surface of the host within a period of 24–48 hrs. However, other reports reveal that *M. anisopliae* conidia germinated within 12–18 hrs in *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae) (Gunnarson, 1988), 24 hrs in *F. occidentalis* (Vestergaard *et al.*, 1999), 40 hrs in *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae) (St. Leger *et al*., 1996a and b; Vestergaard *et al.,* 1999). Vestergaard *et al.* (1999) studied the infection of *F. occidentalis* by *M. anisopliae* and reported that colonisation was observed after 72 h post-infection. But in the present work, colonisation of *M. anisopliae* in trachea was observed after 24 h might indicate that rapid colonisation of host would lead to quicker mortality of the host.

The microscopic examination revealed that the route of infection of *D. cingulatus* by *M. anisopliae* was through the spiracles and root of the bristles since spores of the fungi were observed on them. Spores nearer to the spiracle and also at different areas of

trachea suggest that this fungus could enter into the host through the respiratory tract. This result corroborates with Burges and Hussey (1971) who suggested that insect infection due to fungi might also take place via the respiratory system. Several authors (Hajek and St. Leger, 1994; Boucias and Pendland, 1998; Vilcinskas and Götz, 1999; Kurtz and Franz, 2003; Seger *et al.,* 2006) have reported that entomopathogenic fungi are characterized by their ability to replicate internally after entering into the insect, either by penetrating the cuticle using a combination of physical forces and the secretion of enzymes, or in some cases by entering the haemocoel through the insect gut after ingestion. Our study reveled that there is no possibility of entry of *M. anisopliae* into *D. cingulatus* through the food because cotton seeds soaked in sterile water were provided for *D. cingulatus*.

In the current work, the entry of *M. anisopliae* into host was also observed through dorsal sac1, sac2, and sac3 of *D. cingulatus* which might be another possible route of infection. Once it enters into the body, the growth of *M. anisopliae* may be confined largely to the haemolymph prior to host death (Clarkson and Charnley 1996; Charnley *et al.,* 1997). Comparatively little is known about the development of the fungus in the haemolymph. Samson *et al.* (1988) reported that the invasion of the insect body and circulatory system (haemolymph) occurs once the fungus has passed through the cuticle of the external insect skeleton. The observation of *M. anisopliae* colonies in the haemolymph in this study suggests that the haemolymph of *D. cingulatus* could enhance the multiplication of *M. anisopliae*. Peveling and Demba (1997) have also recorded yeast-like cells found in the haemolymph of hosts *in vivo* when treated with *M. anisopliae.*

After entering into the haemocoel, *M. anisopliae* normally kills the host within few days, due to tissue penetration and nutrient depletion (Boucias and Pendland, 1998) leads to physiological starvation (Stolz, 1999). The present study also suggests that the death of the host might be due to nutrient depletion in the host after treatment with *M. anisopliae* by the reduction in the quantity of haemolymph of *D.cingulatus.* Hassan *et al.* (1989) showed that germination of *M. anisopliae* is initiated by water but progress to the first overt stage of germination (swelling) is depended on an exogenous nutrient. Within the haemocoel the main cellular response of the insect is a multi-haemocytic encapsulation of the fungal element following initial recognition of the fungus by the haemocytes (Huxham *et al.,* 1989).

Best and common bio-assay method of invertebrate immunity is blood-cell counts (Carton and Nappi, 2001; Kurtz and Franz, 2003). The current investigation highlights that immune response of *D. cingulatus* could be disturbed due to treatment of *M. anisopliae* since there were significant reductions in the total haemocyte counts in *D. cingulatus*. The results also reveal that the immunity of *D. cingulatus* gradually diminished from 24 to 96 hrs and finally lead to the death of the pest. It was proposed that the spores in the insect haemolymph reduce the effectiveness of the cellular defenses and not being as antigenic as the mycelium (Charnley, 1989). Recently, destruxin (dtx) A, B, D, E, and E-diol were identified from this fungi (Seger *et al.,* 2006) and was predicted that this might depress the cellular immune reaction of hosts. Saxena and Tikku (1990) studies to showed the deformity in haemocytes due to the application of plumbagin, a phytochemical.

In addition, the immune system of the host and the fungal response is likely to be another important factor governing the pathogenicity of *M. anisopliae* (Moorhouse, 1993). Many researchers hypothesized that the rapid killing ability of *M. anisopliae* on its host could be due to some enzymatic mechanisms of glucosidase, protease and acid trehalase which extra cellular hydrolysis of trehalose occurs in host haemolymph during fungal infection (Xia *et al.,* 2002).

4.5. Conclusion

The infection process in *Dysdercus cingulatus* by *Metarhizium anisopliae* observed in the present work involved the following events: (i) conidia adherence to the host cuticle and germination after 24 hrs post-infection accompanied by falling of bristles (ii) penetration into host through spiracles, root of bristles, haemolymph and three dorsal sacs within 24–48 hrs post-infection; (iii) colonisation of *M. anisopliae* occurred in both trachea and dorsal sacs within 24–48 hrs post-infection; (iv) cuticle colonisation and emergence of the fungus to cuticular surface within72–96 hrs after infection and (v) complete reduction in haemocyte count within 96 hrs. Morphological alterations of *M. anisopliae* during penetration of host, the identification of enzymes that solubilise the cuticle of *D. cingulatus* during the key step of host penetration, identification of sequence tags expressed by *M. anisopliae* during the infection process and other immune responses exhibited by the host to the fungi has to be further studied in the future. Studies on the ability of spore production by all the above isolates are also essential to mass-produce the organisms in large scale.

CHAPTER – 5: SCREENING OF LOW COST MEDIA FOR MASS PRODUCTION OF *METARHIZIUM ANISOPLIAE*

5.1. Introduction

Entomopathogenic fungi are being used worldwide for the control of many pests of agricultural importance (Ferron, 1985; Mehrotra, 1989; Nasr *et al.,* 1992; Waterhouse, 1998; Slater and Baranowski, 1994; Wraight *et al.,* 2001; Rao *et al*., 2003; Mohan *et al.,* 2005; Guo *et al.,* 2007; van Lenteren *et al.*, 2008; Rosell *et al*., 2008). Their mass production is easy and does not require high-input technology (Prior, 1988). Efficient mass production techniques are a prerequisite for successful field applications of entomopathogenic fungi (Purwar and Sachan, 2006; Shah *et al*., 2007). An efficient and economic large-scale production, the formulation of fungal propagules into a product with an adequate shelf life, and a suitable application strategy are fundamental for the successful development of a mycoinsecticide (Abebe, 2002; Shah *et al*., 2005; Sun and Liu, 2006; Sallam *et al*., 2007).

The main advantages of using *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes) as biocontrol agent are: they are easy to store and apply, highly virulent (Toledo *et al.,* 2007; Rosell *et al*., 2008), could be used in an inundative manner (Alves and Pereira, 1998), effective by direct contact with insect cuticle (Roberts and Humber, 1981; Prior and Gatehead, 1989), mass-produced cheaply with considerable shelf-life (Khetan, 2001), safer to birds, fish, or mammals (Zimmermann, 1993), non-target organisms (Hokkanen and Lynch, 1995; Rosell *et al*., 2008), worldwide distribution (Scholte *et al*., 2003), availability of numerous strains (St. Leger *et al.,* 1992) and *in vitro* mass-culture (Jackson *et al*., 2000; Shah *et al*., 2007).

In addition, *M. anisopliae* has been successfully used in the field (Fernandes and Alves, 1991; Milner and Staples, 1995; Gitonga, 1996; Purwar and Sachan, 2006; Rosell *et al*., 2008) for the control of numerous insect pests. Several commercial products of *M. anisopliae* are available for insect control in different agricultural operations such as Bio-Green and Bio-Cane granules for control of soil grubs of pasture and sugar cane in Australia, Green Muscle for control of locusts in Africa, Ago Biocontrol for control of various pests of ornamental crops in South America, and BioPath for control of cockroaches in United States (Shah and Goettel 1999; Wraight *et al.,* 2001; Sun *et al.*, 2002; Batta, 2003; Liu *et al*., 2003; Shah *et al*., 2005; Sun and Liu, 2006; Sallam *et al*., 2007) and EPA was the registered isolate in US (Cook *et al*., 1996; Rath, 2000). The type of spores used in these formulations mainly depends on the mode of mass production (Auld, 1992; Goettel and Roberts, 1992). Surface culture was generally promotes the development of conidia, the natural propagules, while sub-merged culture favors the development of blastospores (Peveling and Demba, 1997).

Fungal conidiation is determined by two measurements, spore numbers per colony or per gram mycelium fresh weight. For practical purpose, the cost for application of a fungal formulation beyond $5x10^{13}$ conidia/ha could be too high to be accepted for mass production and formulation of fungal conidia (Bradley *et al*., 1992; Feng *et al*., 1994; Jaronski, 1997). It has been shown that alternative nutritional components can significantly influence growth and sporulation of fungi *M. anisopliae* (Leite *et al*., 2003; Sun and Liu, 2006). This provides opportunities to find the most effective and commercially available ingredients, through systematically screening of carbon

requirements, to facilitate the mass production of a potential high-virulence biocontrol isolate (Sun and Liu, 2006).

Liquid fermentation is easier from a technical point of view, but blastospores are generally considered less virulent and persistent than conidia (Mc Coy *et al*., 1988; Goettel and Roberts, 1992). Several attempts have been made throughout the world to mass-produce the entomopathogenic fungi by using both solid and liquid media (Bharati *et al*., 2007). Many researchers have extensively studied the mass production of *M. anisopliae* using different grains, synthetic broth and non-synthetic naturally available solid and liquid media (Ibrahim and Low, 1993; Sharma *et al*., 2002; Wadyalkar *et al*., 2003; Namasivayam, 2005). Production of conidia on artificial media facilitates the mass production and therefore enhances an isolates commercial potential (Liu *et al*., 2003). For *M. anisopliae* to be commercially viable, mass production has to be done on artificial substrates. This requires an understanding of the relationship between nutrition and virulence, which currently remains obscure (Shah *et al*., 2005).

M. anisopliae can develop independent of their host, which presents the opportunity for large-scale production of these fungi on liquid or solid media, which makes the use of entomopathogenic fungi as biocontrol agents technically and economically viable (Grimm, 2001). Indigenous cereals tested as substrate for growing *M. anisopliae* yielded 10 times less than rice (Mamuye, 1999). Because rice and other cereals also serve as food, mass production of fungi on waste by-products should be advisable and developed. Moreover, experiences in countries like Brazil, Costa Rica and Philippines have shown that *M. anisopliae* can be mass-produced in small local facilities to serve local needs. These production systems provide *Metarhizium* spp. mainly for the

control of cash crop pests. Therefore, the challenge is to develop a strain that is both virulent and robust (Abebe, 2002). Following an extensive development phase an isolate of *M. anisopliae* var. *acridum*, IMI 330189, has been mass-produced and commercialized under the product name `Green Muscle'. This product has been shown to be a good alternative to organophosphate pesticides says Inglis *et al.* (2000).

The selection of *M. anisopliae* strains with good growth and sporulation capacity on nutritionally poor media was important for mass production in an industrial setup (Feng *et al*., 1994). Second, good growth and sporulation on artificial substrates determines the economics of industrial mass production (Feng *et al*., 1994). Culture media must not only maximise spore yield, but also enhance qualities such as desiccation tolerance, stability in dry preparation and virulence.

Commercial media such as PDB (Sharma *et al.*,1998; Wadyalkar *et al.*, 2003; Bruck *et al*., 2005; Nirmala *et al.*, 2005) has been widely used by insect pathologists for large scale production of *M. anisopliae* conidia. Rice was an ideal natural substrate for the mass production of entomopathogenic fungi (Mendonca, 1992; Ibrahim and Low, 1993; Milner *et al*., 1993; Cheery *et al*., 1999; Sharma *et al.,* 2002; Bharati *et al*., 2007). Rice wash water (Jenkins *et al.*, 1998; Patel *et al*., 1990) and coconut water (Dangar *et al.*, 1991) were other cheaper natural substrates used for the mass multiplication of *M. anisopliae*.

In the present study, non synthetic liquid media such as rice wash water, fish waste, rice boiled water, coconut water and a commercial media, PDB were used to assess the spore producing capability, mycelial dry weight (MDW) and the quantity of media utilized by the most virulent isolates CPRC16, CPRC18 and also MTCC892.

5.2. Materials and Methods

5.2.1. Fungal cultures

Two fungal isolates of *Metarhizium anisopliae* CPRC16 and CPRC18 and MTCC892 obtained from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology, Chandigarh, India were evaluated. These cultures were stored on potato dextrose agar (PDA) (Hi-Media, Mumbai, India) at 4^0C. Working cultures were grown on PDA slopes at 28ºC ± 2ºC for 7 days, and the cultures were stored at 4^0C for up to 6 weeks. Single spore colonies were transferred to PDA and these first sub-cultures were used in subsequent studies.

5.2.2. Conidial inoculum preparation

20 ml of PDA medium was dispensed in a 250 ml conical flask and autoclaved at 121^0C for 20 min. After cooling, the medium was poured onto a sterile Petri plate. Upon solidification of the medium, 0.2 ml of spore suspension of the fungal culture was inoculated. The plate was gently rotated to achieve even distribution of spores throughout the medium and left at room temperature (28ºC ± 2ºC) for 7 days, then they were stored at 4^0C until use. To harvest the conidia from the agar surface in the flask, 1 ml 0.01% sterile Tween 80 solution was added, shaken thoroughly to prepare a proper suspension. Prepared suspensions were then passed through a double-layered muslin cloth. The conidial counts were made from serially diluted conidial suspensions using improved Neubauer haemocytometer. The suspensions of 6.8×10^7, 6.1×10^7 and 6.5×10^7 conidia/ml for CPRC16, CPRC18 and MTCC892 respectively were set as the amount of inoculums in each mass multiplication.

5.2.3. Culture media

5.2.3.1. Synthetic and non-synthetic liquid household waste media

A synthetic liquid medium, Potato Dextrose Broth (PDB) (Hi-Media, Mumbai, India) and three non-synthetic liquid media *viz*., rice wash water, rice boiled water and coconut water collected from households were used in this study. Fifty ml of each broth was filled into 250 ml conical flasks. The flasks were plugged with cotton wool bungs and were covered with gauze. PDB and non-synthetic media were autoclaved at 121ºC (15 psi) for 15 minutes and 40 minutes (Arzumanov *et al*., 2005) respectively.

5.2.3.2. Dry fish waste water

Another non-synthetic liquid media, namely dry fish waste water was prepared by boiling 100g of solid dry fish waste in 200 ml distilled water for 30 minutes. It was then filtered through double-layered muslin cloth and the filtrate was named as dry fish waste water (DFWW). Fifty ml of the DFWW was taken in three 250 ml conical flasks. To the second and the third flask, 1 and 2 % of dextrose respectively were added to improve the spore producing capacity of fungi. The flasks were plugged with cotton wool bungs and covered with gauze and autoclaved at 121ºC (15 psi) for 20 minutes and cooled.

5.2.3. Inoculation

After cooling, each flask was inoculated with 0.5 ml of conidial suspension (6.8 x 10^7, 6.1 x 10^7 and 6.5 x 10^7 conidia/ml of the isolates CPRC16, CPRC18 and MTCC892 respectively) and used for fungal culture. Three replications for each medium was maintained separately and uninoculated control were also maintained for each media. The flasks containing different media were incubated in a BOD incubator (Kemi, India) with periodical shaking at 28ºC ± 2ºC for 25 days in dark.

5.2.4. Growth parameters

To estimate the spore production (5^{th}, 10^{th}, 15^{th}, 20^{th} and 25^{th} day), 50ml of the media was mixed thoroughly with 0.01% sterile Tween 80 solution, filtered through a sieve with pore size of 75-microns and the volume made up to 100ml. Suspensions were serially diluted and spore count was determined using a haemocytometer and expressed as number per ml of broth

To quantify the mycelial dry weight of *M. anisopliae* on the same days, cultures in broth conditions were filtered through Whatman No. 1 filter paper. The fungal mats were oven-dried at 50-60ºC until constant weight was achieved. The dry weight of the mycelium was weighed using a monopan balance (Dhona, Mumbai, India) (Nirmala *et al.,* 2005).

Fifty ml of each sterile PDB, rice wash water, rice boiled water, coconut water and fishery wastewater was measured separately using a 100 ml graduated measuring cylinder accurately. There were three replications were maintained for each medium. The inoculated flasks were incubated at 28ºC ± 2ºC for 25 days in darkness with periodical shaking. The quantity of media utilized by *M. anisopliae* isolates and standard were recorded on 5^{th}, 10^{th}, 15^{th}, 20^{th} and 25^{th} day using a 100 ml graduated measuring cylinder. The difference in the quantity of media before and after growth was considered as the quantity of media consumed by the fungi.

$$\text{Cost of production (Rs./L)} = \frac{\text{Weight of fungi (g)}}{\text{Amount of media consumed (L)}} \text{ x cost of the media (Rs./L)}$$

5.2.5. Colonial radial growth of isolates for three successive generations

The growth rate of mycelia of all the isolates in terms of diameter of the fungal mat (cm.) was assessed on PDA plates. The fungal disc of each isolate measuring 0.5cm before sporulation was inoculated at the centre of individual Petri plates (9cm diameter) and were incubated at 26°C ± 2°C in an incubator for 10 days. The colony diameter (cm) was measured for every 24 hours until 240 hours. The Petri dishes containing the first generation colonies were stored at 4°C as inoculum to evaluate the radial growth of the isolates for the next generations. The same procedure was adopted for the next two generations. Three replications were maintained for each isolate (Onofre *et al.,* 2001; Lord and Howard, 2004).

5.2.6. Statistical analysis

The spore counts were first subjected to log transformation and the data were then analysed by means of one-way- and two-way-ANOVA. Comparison of means was carried out using Turkey test and the significance was expressed at 5%. A correlation analysis between the mycelial dry weight of CPRC16, CPRC18 and MTCC892 on different media was also performed using STATISTICA/w 5.0. software. The data on the radial growth for the isolates as well as the standard during three successive generations was also subjected to Two Way ANOVA.

5.3. Results

5.3.1. Spore production in PDB

Spore production of CPRC16, CPRC18 and the standard strain in PDB cultures during 5^{th}, 10^{th}, 15^{th}, 20^{th} and 25^{th} day of incubation is presented in table 5.1. CPRC18 significantly ($F = 139.1132$; $p<0.05$) produced maximum sporulation on 20^{th} day

($6.13X10^8$ conidia/ml) followed by MTCC892 ($4.17X10^8$ conidia/ml) ($F = 226.2001$; $p<0.05$) on the 20th day; and CPRC18 on 25th day ($3.73X10^8$ conidia/ml).

5.3.2. Media consumption and biomass production

The isolates as well as the standard strain exhibited remarkable differences in the quantity of media utilised during their multiplication (Table 5.2). The results clearly indicate that the quantity of media gradually decreased when the incubation periods increased (Table 5.2). Media consumption was maximum by the isolate CPRC18 (Mean=12.17±0.44 ml/50ml; $P<0.05$) on the 25th day of incubation followed by CPRC18 (Mean=11.33±1.20 ml/50ml; $P<0.05$) on the 20th day and MTCC892 on the 25th day (Table 5.2).

The biomass production of the isolates and the standard strain in terms of mycelial dry weight (mg) per 50ml PDB medium during the observation periods of incubation is presented in table 5.3. Biomass production was invariably high in the isolate CPRC16 on the 20th day (Mean=106.37 ± 2.92 mg/50ml) followed by CPRC18 on the 20th day (Mean=104.37 ± 24.03 mg/50ml) and CPRC16 on the 25th day (Mean=100.20 ± 5.87 mg/50ml) (Table 5.3). The production cost of CPRC16 using PDB at the 25th day was lowest (Rs. 72.99 / litre) compared with CPRC18 and standard (refer Table 5.2).

5.3.3. Non-synthetic liquid media

5.3.3.1. Rice wash water and rice boiled water

In rice wash water, significantly highest spore production was recorded significantly ($F = 614.437$; $p<0.05$) in CPRC16 on 25th day, followed by MTCC892 on 20th day and CPRC18 on 25th day (Table 5.1).

Media consumption was significantly eminent in rice wash water by CPRC18 (Mean=11.67±0.33 ml/50ml; P<0.05) on 25th day of incubation followed by CPRC16 as well as MTCC892 on the same day (Mean=11.33±0.33 ml/50ml; P<0.05) and in rice boiled water, the isolate CPRC18 consumed significantly more quantity of media than the others (Mean=22.5±9.12 ml/50ml; P<0.05) (Table 5.2).

The mycelial dry weight of isolates and standard in rice wash water and rice boiled water during the incubation period is represented in table 5.3. In rice wash water, biomass production was maximum in CPRC16 (Mean=117.07 ± 4.96 mg/50ml; P<0.05) followed by MTCC892 on the 20th day (Mean=110.77 ± 3.69 mg/50ml; P<0.05) and CPRC16 on the 25th day (Mean=100.23 ± 6.68 mg/50ml; P<0.05). As observed in rice wash water, CPRC16 produced maximum biomass on 20th day (Mean=169.67 ± 2.36 mg/50ml; P<0.05) followed by MTCC892 on 25th day (Mean=154.57 ± 4.99mg/50ml; P<0.05) and CPRC16 on the 25th day (Mean=154.53 ± 19.80 mg/50ml; P<0.05) in rice boiled water. The production cost of CPRC18 using RWW and RBW at the 25th day was lowest (Rs. 10.08 and 8.43 / litre) when compared with CPRC16 and standard (Table 5.2).

5.3.3.2. Coconut water

Between the two isolates and also the standard, highest spore production was significantly (F = 493.144; p<0.05) recorded in CPRC18 on 20th day, followed by CPRC16 on 25th day (Table 5.1). In general, the spore count gradually increased as the incubation days proceeded until 20th day. Least spore producing capacity was recorded in MTCC892 on the 5th day. Media consumption was significantly higher in the isolate CPRC16 (Mean=13.33±1.33 ml/50ml; P<0.05) on the 25th day of incubation followed by

MTCC892 on the same day (Mean = 13.33 ± 0.88 ml/50ml; P<0.05) and least consumption was observed in CPRC16 (Table 5.2).

The production cost of MTCC892 using CW at the 25th day was lowest (Rs. 635.31 / litre) when compared with CPRC16 and CPRC18 (Table 5.2). As observed for media consumption, coconut water supported maximum biomass production in CPRC16 (Mean=127.10 ± 4.75mg/50ml; P<0.05) (Table 5.3), followed by CPRC18 (Mean = 110.50 ± 10.68 mg/50ml; P<0.05) and MTCC892 (Mean = 105.86 ± 3.27; P<0.05) on the 25th day.

5.3.3.3. Dry fish wastewater

Spore production of CPRC16, CPRC18 isolates and standard strain in fishery wastewater, fishery wastewater with dextrose (FDI and FDII) during incubation period is represented in table 5.4. Among the 3 tested strains, CPRC18 produced maximum sporulation on the 25th day ($2.25x10^4$ ±$6.5x10^2$ conidia/ml), followed by CPRC16 on the 25th day ($2.15x10^4$ ±$1.5x10^2$ conidia/ml). Media consumption was also highest in the isolate CPRC18 (Mean=30.00±9.00 ml/50ml; P<0.05), followed by MTCC892 on the 25th day (Mean=30.00 ±0.00 ml/50ml; P<0.05) in FDII. Least medium was utilised by the isolate CPRC16 on the 5th day than the others in DFWW (Mean=3.5 ±0.50ml/50ml; P<0.05). Highest biomass was observed on the 25th day followed by CPRC16 on the 25th day (Mean = 410.00 ± 29.00 mg/50ml; P<0.05) (Table 5.4). Due to least spore producing capacity, DFWW was considered as unfit for mass production.

5.3.4. Colonial generation growth

Radial growth of *M. anisopliae* isolates was measured every day by measuring the diameter of each colony over a period of 10 days for three generation continuously. The

colony size was maximum in CPRC18G1 (1.567 cm), followed by CPRC15G2 (0.813 cm) and CPRC11G3, CPRC19G3 and CPRC20G3 (0.800cm) after 24 hrs of incubation during the first, second and third generations respectively. Comparatively, the isolates of the first generation had higher radial growth than the growth at second and third generations. Indeed, at first generation, the difference in radial growth among the isolates was more pronounced than the growth at second generation. The radial growth of the isolates during the third generation was relatively lower than the other two generations (Figure 5.1 - 5.3). The radial growth of all the isolates was also directly proportional to their incubation time up to 10 days. In the first generation, the isolate CPRC 18 had the most significant (F= 74.16031; $P < 0.0001$) difference in radial growth among the isolates when compared with the standard strain, MTCC892 (Table 5.5). However, the same isolate exhibited differences in their significance during the second (F= 30.79162; $P < 0.05$) and the third (F= 7.75334; $P < 0.05$) generations. A data matrix of 22 isolates as well as the standard strain and 2 variables (radial growth for 1 to 10 days for three successive generations) was used for this analysis. The isolates were separated into two main groups I and II based on their growth-rate: the isolates CPRC15 and CPRC18 with the highest growth-rate were clustered in group II and remaining isolates with medium and low growth-rate were grouped into I (Figure 5.4.). In the group II, the two isolates (CPRC15 and CPRC18) were sub-clustered into IIA and IIB. In group I, the isolates were separated into two sub clusters, IA and IB. The isolate, CPRC16 of sub-cluster IB was of medium growth-rate and all those of IA were of low growth-rate type (Figure 5.4.).

5.3.5. Correlation analysis

In the present study, the resultant matrix revealed strong positive correlation among all the media used ($r > 0.7$). Highest correlation (0.99) was observed between CPRC18 in coconut water and MTCC892 in PDB as well as between MTCC892 in rice wash water and rice MTCC892 in boiled water.

5.4. Discussion

Based upon the overall pathogenicity and virulence of the tested indigenous *Metarhizium anisopliae* isolates, the isolates CPRC16 and CPRC18 were selected for the mass production studies. Mazodze and Zvoutete (1999) postulated that only highly virulent strains would be mass cultured for field trials. Different isolates within the same species vary in their requirements for nutrition and water activity of the substrate for mycelial growth and conidiation (Johnpulle, 1938; Bartlett and Jaronski, 1988; Latge and Moletta, 1988; Kleespies and Zimmermann, 1992; Alves and Pereira, 1998; Inglis *et al*., 2000; Abebe, 2002; Shah *et al*., 2005; Rosell *et al*., 2008). Thus, a set of particular parameters is needed to identify the optimization of individual isolate production (Jenkins *et al*., 1998). Jenkins and Goettel (1997) reported those in industrial terms, liquid fermentation of microbial agents is more advantageous than solid-state fermentation, because the former method considerably lower the production costs. Hence the present study was designed to assess the utility value of both synthetic liquid media (PDB) and non-synthetic liquid media (rice wash water, rice boiled water, coconut water and fishery wastewater) for the mass production of *M. anisopliae.* The production cost of CPRC18 using RWW and RBW at the 25^{th} day was lowest (Rs. 10.08 and 8.43 / litre) when compared with CPRC16 and standard.

Good growth and sporulation on artificial substrates determines the economics of industrial mass production (Feng *et al*., 1994). Thus a commercial media, PDB was also included in the present investigation to compare the spore production with low cost media. The results of this study clearly revealed that both the isolates of *M. anisopliae* produced more number of spores on PDB medium indicating their innate property of heavy sporulation. This corroborates with the findings of Wadyalkar *et al.* (2003) and Nirmala *et al.* (2005) who mass multiplied *M. anisopliae* and found that PDB was a better media in terms of spore production, viability of conidia and its production cost. Sharma *et al.* (1998) reported that both 27^0 and 28^0C were the best temperatures for the optimum sporulation of *M. anisopliae.* The present study was also carried out at 27^0 C. Similar report on Mass multiplication of *M. anisopliae* carried out in darkness was as described by Bruck *et al*. (2005).

In the current investigation, all the isolates significantly produced higher mycelial dry weight in PDB after 25 days of incubation. Among the three strains tested, the isolate CPRC16 had significantly higher biomass production (106.37 mg/50ml) than others. Nirmala *et al*. (2005) have also tested four *M. anisopliae* isolates for biomass production in PDB and obtained a maximum biomass of 1.09 g/100ml. It was interesting to record here that the tested isolates invariably produced mycelial pellets. Mycelial pellets are the mass of mycelia that are considered as reproductive propagules like spores (Kleespies and Zimmermann, 1992; Nirmala *et al*., 2005).

Rice is an ideal substrate for the mass production of entomopathogenic fungi (Mendonca, 1992; Ibrahim and Low, 1993; Milner *et al*., 1993; Cheery *et al*., 1999; Sharma *et al.,* 2002; Bharati *et al*., 2007). This is probably due to a combination of

factors, including nutritional balance, worldwide availability, hydration properties and structural integrity even after colonisation of fungi (Jenkins *et al.*, 1998). But Mamuye (1999) reported white rice as an expensive food commodity for the mass production of fungi. In the present study, the house hold waste of rice *viz*. rice wash water as well as rice boiled water were chosen for the mass production of this important fungi. From this study, it was very clear that *M. anisopliae* isolates CPRC16 and CPRC18 produced highest number of spores in rice wash water ($5.16x10^8$ conidia/ml) and rice boiled water ($7.76x10^7$ conidia/ml). Patel *et al*. (1990) reported that the purified rice wash water gave the best spore count in *M. anisopliae*.

Entomopathogenic fungi require oxygen, water, organic and inorganic sources and elements such as minerals and growth factors are very essential in addition to potassium, magnesium and sulphur (Rath *et al*., 1992). Coconut water contains sugars, vitamins, minerals, proteins, amino acids and growth promoting factors (Shaw and Srivastava, 1963) and we selected it as a medium. In this study, the *M. anisopliae* isolate CPRC16 grew significantly faster, reaching the highest spore yield of $6.40x10^8 \pm 2.64x10^7$ spores/ml and similarly Dangar *et al.* (1991) mass-multiplied *M. anisopliae* in sterilized coconut water and harvested the fungal spores.

Attempts were also made to mass-produce the isolates in DFWW, fishery wastewater with 1% and 2% dextrose. Dextrose was added to enhance the sporulation rate. Nevertheless, the media yielded very low spore counts when compared to other media. Hence it could be concluded that fishery wastewater is not a suitable media for the mass multiplication of *M. anisopliae.* However, fishery wastewater with 2% dextrose

yielded very high biomass (421.00 mg/50ml in CPRC18). Reports were not available in the literature on the usage of fish wastes for the mass production of *M. anisopliae.*

For the first time, the radial measurements of indigenous *M. anisopliae* isolates from Tamil Nadu for three successive generations have been assessed on PDA. In this study, the colony size was maximum in the isolate CPRC16G1 (4.500 cm) on 10^{th} day on PDA after incubation at 27^0C during the first generation. Many authors have reported radial growth of M. anisopliae isolates from other locations. The radial growth of 4.07cm at 10 days was observed in SDA+Y medium by Liu *et al*. (1989) and Kulat *et al.* (2002). Nirmala *et al.* (2005) recorded the highest radial growth (4.55cm) of *M. anisopliae* cultured on potato dextrose agar (PDA) medium on 10^{th} day of incubation, which exactly correlate the current investigation. Radial growth of *M. anisopliae* was significantly higher in media supplemented with $NaNO_3$ (4.8cm) (Tamizharasi *et al.,* 2005).

In the present study, the isolates of the first generation had higher radial growth, than the growth at second and third generation. The colony size was maximum in the isolate CPRC18G1 (1.567 cm) after 24 hours on PDA after incubation at 27^0C during the first generation. The same isolate was found to be the most efficient against the eggs, second instar as well as the adults of *D. cingulatus.* Thus there may be correlation between the radial growth with the pathogenicity of the isolate. Also, there was significant reduction in their size during the second and the third generations indicating that the radial growth of the isolate might have gradually decreased as generation proceeds. Repeated in vitro culture can cause attenuation of fungal pathogens (Butt and Goettel, 2000; Wraight *et al*., 2001) and may lead to differences in virulence between

isolates. is in favour of this fact. Thus the present study suggests that isolates of first generation may be chosen for biocontrol to achieve maximum efficacy.

5.5. Conclusion

Thus from the present investigation it is very clear that the virulent isolate *Metarhizium anisopliae* CPRC 18 is the best isolate to be mass produced cheaply using RWW and RBW. Interestingly, the quantity of all the media decreased after the growth of the fungi and it was confirmed that decrease was due to the consumption of media by the fungi during their growth. But control showed no decrease in the media quantity. Amazingly, each isolate exhibited difference in the uptake of media for their growth. Maximum uptake was recorded during the growth of CPTC18 in fishery wastewater with 2% dextrose. Further studies are essential to find the differences among the strains using molecular techniques.

CHAPTER 6: GENETIC DIVERSITY OF *METARHIZIUM ANISOPLIAE* ISOLATES FROM TAMIL NADU

6.1. Introduction

Among the entomopathogenic fungi, *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes) has a wide spectrum of pathogenicity and infectivity against different species of pest insects (Freire *et al*., 2001; Bidochka *et al.,* 2002; Destefano *et al*., 2004; Entz *et al*., 2006; Velasquez *et al.,* 2007). While we select a microbial agent, its isolates must be formally identified on the sub-species level (Jenkins and Grzywacs, 2000). This will place the fungal isolate within a taxonomic rank, initially at the species level, but many fungal species are widely distributed in the environment and thus have an enormous number of particular strains making it difficult to be identified. Morphology-based identification methods have traditionally been used for detection of fungi. However, morphological characters have been shown to have only limited potential to distinguish between species of *Metarhizium* (Driver *et al*., 2000) and enzyme synthesis can vary significantly during growth, it often takes up to two months (Kim *et al.,* 1999).

PCR-based detection approaches have been introduced as an alternative to the time-consuming morphology-based method (Oh *et al*., 2004; Zhu *et al*., 2006; Velasquez *et al.,* 2007). Deficiencies in traditional microbial detection techniques have led to research into molecular methods with the selectivity and sensitivity required to distinguish non-native strains from indigenous populations (Clarkson, 1992; Bidochka *et al*., 1995; Khachatourians, 1996; Bidochka *et al*., 2000; Bielikova *et al.,* 2002; Destefano *et al*., 2004; Pe´ros *et al*., 2005; Velasquez *et al.,* 2007). Among the numerous techniques

developed to detect DNA polymorphisms by PCR, Random-amplified polymorphic DNA (RAPD) is one of the quickest and easiest methods to perform (Williams *et al*., 1990; Bidochka *et al*., 1995; Bogo *et al*., 1998; Freire *et al*., 2001; Destefano *et al*., 2004; Entz *et al*., 2006; Velasquez *et al.,* 2007).

The RAPD technique was described in 1990 (Williams *et al*., 1990). It is a modification of PCR (polymerase chain reaction) and allows revealing polymorphism within completely unknown samples without the need of probe hybridization or DNA sequencing. Only one short oligonucleotide primer (6–12 bases) is used for the reaction, and the sequence of primers is fully arbitrary. This technique involves the use of arbitrary GC-rich decamers as single primers (Ruiz-Dı´ez *et al*., 1997; Bidochka *et al*., 2000; Boucias *et al*., 2000; Devi *et al*., 2001; Destefano *et al*., 2004; Zhu *et al*., 2006;Velasquez *et al.,* 2007). The product of the reaction is a spectrum of DNA fragments differing from each other in length and nucleotide sequence. The total number of products and the length of each nucleotide depend on the template DNA and primer used and is specific for a particular combination.

The application of random-amplified polymorphic DNA (RAPD) markers is similar to those of other methods based on detection of DNA polymorphism. Since its development, the RAPD protocol has acquired a diversity of uses, such as: the establishment of the genetic similarity degree between individuals within a population (Anderson and Fairbanks, 1990; Bidochka *et al*., 1994; Bogo *et al*., 1998; Devi *et al*., 2001; Saal and Struss, 2005; Velasquez *et al.,* 2007), the construction of genetic maps as well as the localization of economically interesting genes (Williams *et al*., 1990), the production of a genomic fingerprint (Welsh and McClelland, 1990; Cobb and Clarkson,

1993; Fegan *et al*., 1993; Bidochka *et al*., 1994; Leal *et al*., 1994; Bidochka *et al*., 1995; Bidochka *et al*., 2001; Pe´ros *et al*., 2005; Velasquez *et al.,* 2007), strain identification (Lehmann *et al*., 1992), to study the genetic diversity along with the identification of fungi (Fani *et al*., 1993; Fegan *et al*., 1993; Henson and French, 1993; Jungehülsing and Tudzynski, 1997; McEwen *et al*., 2000; Bielikova *et al.,* 2002; Pe´ros *et al*., 2005; Velasquez *et al.,* 2007) and exhibit the ability to discriminate between entomopathogenic fungal isolates (Leal *et al.*, 1994; Bidochka *et al*, 1994; Fungaro *et al.*, 1996; Urtz and Rice, 1997; Jensen *et al.*, 2001; Freire *et al.*, 2001; Destefano *et al*., 2004; Saal and Struss, 2005; Velasquez *et al.,* 2007).

DNA-based molecular markers are almost unlimited in number and are not affected by the environment (Williams *et al*., 1990). The distinct patterns obtained for each individual can be taken as specific fingerprints that describe and identify them. Molecular markers are fast and safe alternatives to differentiate and characterise fungal isolates (Crowhurst *et al*., 1991; Guthrie *et al*., 1992; Cobb and Clarkson, 1993; Assigbetse *et al*., 1994; Otoya *et al*., 1995; Bogo *et al*., 1998; Boucias *et al*., 2000; Devi *et al*., 2001; Zhu *et al*., 2006; Velasquez *et al.,* 2007). It has been possible to correlate particular fungal genotypes defined by RAPD markers with particular pathogenicity groups (Goodwin and Annis, 1991; Cooke *et al*., 1996; Bidochka *et al*., 2000; Bidochka *et al*., 2002; Entz *et al*., 2006; Velasquez *et al.,* 2007). The RAPD characterisation would be useful for proprietary reasons when fungal isolates are introduced into new ecosystems for pest control. It ensures a means of detecting the introduced pathogens versus the native one. It will be useful for patenting purposes when the isolates are used in commercial formulations. The aim of the present study was to assess the genetic

variability of ten *M. anisopliae* isolates collected from different districts of Tamil Nadu by means of the RAPD technique.

6.2. Materials and Methods

6.2.1. Fungal strains

CPRC1, CPRC4, CPRC6, CPRC8, CPRC9, CPRC10, CPRC12, CPRC16 and CPRC18 *Metarhizium anisopliae* isolated from different parts of Tamil Nadu and standard were used in this study to analyze their molecular diversity. The isolates selected were the most virulent strains to *Dysdercus cingulatus* (Fab.) (Hemiptera: Pyrrhocoridae). All fungal isolates were propagated and maintained on PDA. Sources and geographical origins of *M. anisopliae* isolates were from tomato soil (Melapalayam, Tirunelveli), paddy soil (Seithunganallore, Tirunelveli), forest soil (Manimuthar, Tirunelveli), paddy soil (Cherinthal, Tiruvannamalai), cotton soil (Thuraimangalam, Perambalur), cotton soil (Ulundurpet, Viluppuram), coconut soil (Mailaduthurai, Nagapathinam), cotton soil (Melamnakkal, Thiruvarur) and potato soil (Kottamanthu, Nilgiri).

6.2.2. Mycelial preparation

Conical flasks with 100ml of PDB were inoculated separately with 10^7 conidia/ml of the standard and wild isolates were incubated at 27^0C for 72 hours with periodical shaking. The young mycelium was recovered by centrifugation at 8000 rpm for 3 minutes, washed with sterile distilled water and stored at -20^0C until used.

6.2.3. Genomic DNA extraction from *M. anisopliae*

The total genomic DNA of the samples was extracted from the young mycelium by a modified method described by Freire *et al.* (2001), Bielikova *et al.* (2002), Destefano *et al.* (2004) and Entz *et al.* (2006). The following steps were performed.

1. Hundred milligrams of the *M. anisopliae* mycelium grown in PDB for 72 hours were placed in a 1.5ml micro-centrifuge tube.
2. The mycelial biomass was frozen at –20^{o}C for 24 hours.
3. It was ground well with a sterile steel rod for 15 seconds.
4. Then 500 µl of lysis buffer [5 mM Tris HCl; 150 mM NaCl; 100 mM EDTA] was added to each sample.
5. 50 µl of 10 % SDS [1 g/ double distilled water 10ml] was added.
6. It was shaken gently for 30 seconds.
7. The tubes were closed and incubated at 37^{0}C for 30 minutes in an incubator.
8. 75 µl of 5 M NaCI [2.92 g/ double distilled water 10ml] was added and the contents were mixed gently for 5 seconds.
9. Add 60 µl of 10 % CTAB [0.7 M NaCl (4.0908 g/ double distilled water 100ml) = 10 ml, Cepyltrimethyl Ammonium Bromide = 1 g]
10. The contents were mixed again by gentle inversion for 10 seconds. Then the tubes were incubated at 65^{0}C for 20 minutes in water bath.
11. Samples were then centrifuged at 8000 rpm for 2 minutes and the supernatant was then transferred to a new marked tube.
12. An equal volume of buffer saturated phenol was added to the supernatant.
13. The contents were mixed gently by inverting the microfuge tube for 3 times and were spined at 8000 rpm for 3 minutes.
14. The top aqueous layer was transferred (some times it might be in lower or middle layer; care was taken to prevent careless mixing of the layers) into a fresh eppendorf.

15. Equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) [Phenol = 25 ml, Chloroform = 24 ml and Isoamylalcohol = 1 ml] was added and centrifuged at 8000 rpm for 3 minutes.

16. The top layer was transferred into a clean microfuge tube.

17. Equal volume of Chloroform: Isoamylalcohol (24:1) [Chloroform = 24 ml and Isoamylalcohol = 1 ml] was added.

18. The resulting suspension was centrifuged at 8000 rpm for 3 minutes.

19. The final aqueous (top) layer was carefully transferred into a clean eppendorf.

20. Add two volumes of cold ethanol and 0.1 M NaCl [0.5844 g/100 ml of double distilled water] and keep at -20^{o} C for 40 minutes.

21. Then the contents were centrifuged at 12000 rpm for 10 minutes.

22. Ethanol was poured off and the tubes left open to allow air-drying of the DNA-pellet.

23. Added 30 µl of TE buffer [10 mM Tris HCl = 0.1576 g, 1 mM EDTA = 0.0372 g in 100ml distilled water].

24. The isolated genomic DNA was run on an agarose gel (1 %) and was visualized under UV following staining with ethidium bromide.

25. The extracted DNA was stored at -20^{0}C for further studies.

6.2.3.1. Quantity and quality of DNA

The quantity of DNA present in each sample was calculated as follows:

Concentration of DNA sample (µg/ml) = A_{260} x 50 µg/ml x dilution factor

The absorbance of the DNA at 260 nm and 280 nm were measured in UV spectrophotometer (Elico ltd., Mumbai, India) using quartz cuvettes to check the purity of DNA.

$$\text{Quality of DNA} = \frac{A_{260}}{A_{280}}$$

The ratio (260/280) was maintained between 1.7 and 1.8 because ratio less than 1.7, indicates the presence of proteins in the sample (Kaufman *et al.,* 2000). In this case, the steps 15-30 were repeated to achieve pure DNA.

6.2.4. RAPD analysis

Genomic DNA from the *M. anisopliae* samples was amplified using RAPD protocol (Williams *et al.,* 1990). For each isolate, PCR-RAPD of the extracted DNA was performed in an eppendorf thermocycler (Mastercycler Gradients, Hamburg, Germany).

6.2.4.1. Random primers used

The random primers OPE-8 (5'-AACGGCGACA- 3'), OPE-13 (5'-AGGACTGCCA- 3') and OPE-16 (5'-GGTGAACGCT-3') (Genei, Bangalore, India) were used as described by Bidochka *et al.* (2001).

6.2.4.2. PCR conditions

6.2.4.2.1. Amplification reactions mixture of the RAPD

The RAPD reactions were performed in 25µl volumes of the reaction mix. All the chemicals except the primers and Taq polymerase were utilised from the Genei DNA Amplification Reagent Kit [KT03]; primer was dissolved in 100µl of TE buffer; Taq polymerase was diluted [1:2 Taq polymerase: Diluent buffer] in order to obtain 1Unit (Genei, Bangalore, India).

Genomic DNA (50 ng)	:	50ng
0.25mM dNTP mix (0.2mM)	:	1µl
1x Taq buffer with $MgCl_2$ (1x)	:	2.5µl
Primer (0.5µM)	:	1µl
Dis. H_2O (Nuclease free)	:	19.5µl
Taq polymerase (1Unit)	:	1µl

6.2.4.2.2. Temperature profile

Initial denaturation step	92°C for	5 minutes	
DNA denaturation	92°C for	1 minute	} 40 cycles
Primer annealing	39°C for	1 minute 30 seconds	
Primer extension	72°C for	2 minutes	
Final extension	72°C for	5 minutes	

Amplification products were analysed by electrophoresis (Biotech, Yarkad, TN, India) in 1.5% (TBE) agarose gels and detected by staining with Ethidium Bromide [Agarose 600mg + 30ml Dis. H_2O; Melted completely; Added 10ml TBE buffer + 7µl Ethidium Bromide, mixed well; Poured into the boat]. The gels were photographed and genetic diversity was analysed using the gel documentation system (Biotech, Yercaud, India).

6.2.3. Statistical analysis

The Advanced DGelDAS (Digital Del Documentation and Analysis software) version (Biotech, Yercaud, India) computer programme was used for data analyses. The

similarities of the lanes were matched with the selected reference lane and similarity matrix was generated. Based on this Similarity Matrix Values (SMV), a Distance Matrix (DM) was generated using Jaccard coefficients with similarity methods. Then the Phylogenetic tree (Dendogram) was constructed using UPGMA (Unweighted Pair-Group Method using Arithmetic Average). The bands of the standard DNA ladder were selected as reference lane and their molecular weight values were entered in a table to determine the molecular size and the Rf values of the unknown bands. The content percentages of the bands were calculated using densitometry tracing.

6.3. Results

RAPD fragment patterns were assessed with three primers in DNA from nine *Metarhizium anisopliae* isolates and the standard *M. anisopliae* MTCC892. Plate 6.1 shows the fragment patterns generated for three primers. On the basis of reproducible amplification fragment patterns for all the three primers, the *M. anisopliae* isolates were clearly distinguishable. The primers revealed a total of 157 repeatable bands with an average of 52.33 bands per primer for nine isolates and the standard. No bands were present in negative controls. No monomorphic band was observed among the 9 isolates and the standard MTCC892 with the 3 primers used because none of the band was common in all samples.

The sizes of the bands were between 136 and 3761 base pairs (Table 6.1, 6.2 and 6.3). CPRC8 and MTCC892 gave two bands with same molecular sizes (2932 and 3018 bp) when randomly amplified using the primer OPE8. Another band with 1645bp was common in three isolates CPRC1, CPRC16 and CPRC18 (Table 6.1). CPRC8 and CPRC18 gave one band with same molecular sizes (2309bp) with the primer OPE13.

Another band with 1926bp was common in two isolates CPRC1 and CPRC12. 1999bp was common for CPRC9 and CPRC16 and 1549bp was common for CPRC9 and CPRC12 (Table 6.2). CPRC6, CPRC12 and CPRC18 produced one band with same molecular size (1859bp) using the primer OPE16 (Table 6.3). There were significant differences in fragment patterns between all isolates and the standard, except the isolates CPRC9 and CPRC10 contained almost similar fragments with primer OPE8 (Plate 6.1). Almost all the isolates and the standard were difficult to categorize with primer OPE13 (Plate 6.1), since the RAPD bands were either unclear or irreproducible. The RAPD analysis with the primers, OPE16 demonstrated that isolates CPRC4 and CPRC6 were identical (Plate 6.1).

Three dendograms were constructed with the fragments generated from RAPD data by OPE8, OPE13 and OPE16 primers as shown in figure 6.1, 6.2 and 6.3 respectively. RAPD analyses of the nine isolates and standard with three OPE primers allowed complete separation of isolates. The isolates from different districts, showed similarity ranging from 70-92 (Figure 6.1), 33-67 (Figure 6.2) and 17-57% (Figure 6.3) obtained with primers OPE8, OPE13 and OPE16 respectively. The isolates from different districts showed dissimilarity and could be sub-classified into different groups at 92 (Figure 6.1), 67 (Figure 6.2) and 57% (Figure 6.3) similarity for OPE8, OPE13 and OPE16 primers respectively.

Cluster analysis based on the single linkage method evidenced two clades in the dendogram for all three primers (Figure 6.1, 6.2 and 6.3). Isolate (CPRC16) clustered in cluster number I (fig 6.1) with OPE8 primer. This isolate was from cotton field in Melamnakkal of Thiruvarur district and was the most virulent isolates to *Dysdercus*

cingulatus. Cluster number II was the largest clade with 9 isolates and it was sub-clustered into two clades: IIA and IIB. Only one isolate grouped in IIA at 90% similarity. Remaining 7 isolates and the standard grouped in IIB at 90% similarity (Figure 6.1).

In OPE13, the two moderate virulent isolates to *D. cingulatus* (CPRC8 and CPRC12) clustered in cluster number I (fig 6.2) and these isolates were obtained from the cotton field in Cherinthal, Tiruvannamalai and Mailaduthurai, Nagapathinam districts. Cluster number II was the largest clade with 8 isolates; it was sub-clustered into two clades: IIA and IIB. Four isolates grouped in IIA at 59% similarity. Remaining 3 isolates and the standard grouped in IIB with the same similarity (Figure 6.2). Two isolates (CPRC8 and CPRC9) clustered in cluster number I (fig 6.3) in OPE16 primer. These isolates were collected from cotton field in Cherinthal, Tiruvannamalai and Thuraimangalam, Perambalur districts and were moderately virulent isolates to *D. cingulatus*.

The content percentage of each band of randomly amplified products of MTCC892 and the 9 isolates obtained by densitometry tracing is represented in table 6.4. The highest content percentage of 24.41 (OPE8), 100 (OPE13) and 41.86 (OPE16) was observed in the first band of CPRC4, first bands of MTCC892, CPRC1 and CPRC4, as well as in the fifth band of MTCC892 respectively. The Rf values of each band of randomly amplified products of *M. anisopliae* isolates are presented in table 6.5. The Rf values were highest as 0.877 (OPE8), followed by 0.665 (OPE13) and 0.772 (OPE16) was observed in the ninth band of CPRC1, third band of CPRC18 and in the fifth band of CPRC6. All the isolates and the standard of *M. anisopliae* presented a ramification point which ranged from 0.70 to 0.92 (Figure 6.1), 0.33 to 0.67 (Figure 6.2) and 0.17 to 0.57

(Figure 6.3), for OPE8, OPE13 and OPE16 primers respectively which corresponds to a high variation degree. Both the CPRC9 and CPRC10 isolates presented the highest similarity index (about 80.0%) obtained from the RAPD using OPE8 primer. The CPRC8 and CPRC9, CPRC11 and CPRC6 and also CPRC6 and MTCC892 presented a ramification point around 0.34 (34.0%) with primer OPE16. The shortest genetic distance was 20.0% (CPRC4 and CPRC8). Considering all isolates used in this study, the mean genetic distance was 39.83%, demonstrating the great diversity among the 9 isolates and the standard *M. anisopliae* analysed.

6.4. Discussion

Though several fungi have been cited as being potential mycoinsecticide (Kenis *et al.,* 2008), only a few have been intensively investigated and examined at the molecular level (Hegedus and Khachatourians, 1996; Purwar and Sachan, 2006). Besides, once selected for development and utilization as a microbial control agent, the fungal isolate must remain stable and be clearly identifiable at the sub-species level. This level of identification is particularly important as it provides a mechanism for tracking the progress and fate of the agent in the environment, and to validate the purity and identity of the registered formulations and a standard reference that may be used to register or protect as an individual isolate (Jenkins and Grzywacz, 2000).

RAPD procedures have been developed for the detection and identification of several entomopathogenic fungi such as *M. anisopliae* (Cobb and Clarkson, 1993; Hegedus and Khachatourians, 1996; Leal *et al.,* 1994; Fungaro *et al.,* 1996; Freire *et al.,* 2001; Destefano *et al.,* 2004; Velasque *et al*., 2007), *M. flavoviride* Gams and Rozsypal (Inglis *et al.,* 1999), *Beauveria bassiana* (Balsamo) Vuillemin (Hegedus and

Khachatourians, 1996; Urtz and Rice, 1997; Devi *et al.,* 2001; Bidochka *et al.,* 2002); *Entomophaga grylli* Fresenius (Bidochka *et al.,* 1995); *Paecilomyces fumosoroseus* (Wize) Brown and Smith (Bielikova *et al.,* 2002); *Verticillium lecanii* (Zimmerman) Viegas (Zare *et al.,* 1999); *Nomuraea rileyi* (Farlow) Samson (Boucias *et al.,* 2000) and *Gliocladium catenulatum* Gilman and Abbott (Paavanen-Huhtala *et al.,* 2000). The current study also demonstrated that the nine *M. anisopliae* isolates and the standard *M. anisopliae* could be clearly differentiated using RAPD.

Samples of DNA from plants (Do and Adams, 1991) and fungi (Pfeifer and Khachatourians, 1993) may retain polysaccharides, which can cause problems during amplification (Hackman, 1974). The extraction protocol of the present study produced DNA with sufficient purity for direct PCR amplification and although this DNA produced a reduced amount of product in comparison with that produced when DNA from pure cultures was used. The amount of product was still sufficient for us to identify the fungal species. The method used in the current analyses was modified with special attention to its routine and easy use. This procedure enables precise characterisation and identification of individual strains and eliminates problems with lower reproducibility of RAPD patterns or problematic interpretation of complex banding patterns as reported by Williams *et al*. (1993), Hardys *et al*. (1992), Samec (1993), Backeljau *et al*. (1995), Tigano-Milani *et al*. (1995) and Mcdonald (1997). Optimisation of reaction conditions and selection of suitable primers overcame those disadvantages.

In the present study, the use of selected primers, OPE-8, OPE-13 and OPE-16 allowed to evaluate the genetic diversity among the *M. anisopliae* isolates sampled from different districts. The polymorphisms identified were extensive. No monomorphic band

was observed among the 9 isolates and the standard *M. anisopliae* with the 3 primers used (157 bands). Such complete polymorphism is in accord with the study of Junghans *et al*. (1998). Bidochka *et al.* (2001) also studied the habitat association in two genetic groups of *M. anisopliae* using the same primers for 83 isolates and recorded polymorphisms. Freire *et al.* (2001) analysed five mutants (MaE10, MaE27, MaE24, MaE41 and MaE49) of *M. anisopliae* along with wild strain E9 for DNA profile through the RAPD technique using 20 primers of the kit OPW and recorded 47 bands in all strains.

The 3 RAPD primers used in this study to analyse the 9 isolates of *M. anisopliae* and standard generated a total of 157 bands ranging from 136 and 3761 bp. Leal-Bertioli *et al.* (2000) used 20 RAPD primers used to analyse the 39 isolates of *M. anisopliae* which generated a total of 189 bands ranging from 200 bp to 1500bp. The current work has proved the genetic diversity among all the *M. anisopliae* isolates with a high level of genetic distance among them. Fegan *et al*. (1993) also analyzed a great genetic variability in 13 isolates of *M. anisopliae* var. *anisopliae* using RAPD. Fungaro *et al*. (1996) verified a great genetic diversity among them. In contrast to other studies, RAPD PCR analysis showed high genetic homogeneity among Brazilian *M. anisopliae var. acridum* isolates (Silveira *et al*., 1998).

In the RAPD analysis of *M. anisopliae* isolates, the clustering of the most virulent isolates reflected their similarity in virulence to *Dysdercus cingulatus*. The most virulent isolate CPRC16 fell into the clade I (Figure 6.1). In the study of Berretta *et al.* (1998) *Beauveria bassiana* isolates were highly virulent to *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae) and formed one group with 85% similarity. However, in the

current analyses, the clustering of all other succeeding most virulent isolates did not reflect their similarity according to their virulence and fell into different clusters and was grouped with least virulent isolates. Luz *et al.* (1998) found a similar situation in *B. bassiana* isolates tested against *Triatoma infestans* Klug (Hemiptera: Reduviidae). In the present study the most virulent isolate CPRC16 presented a ramification point 0.92. The isolates of *M. anisopliae* analysed by Freire *et al.* (2001) presented a ramification point around 0.81 (81.2% of similarity).

The RAPD analysis showed similarity among the *M. anisopliae* isolates, though they had been isolated from different districts of Tamil Nadu indicating that isolates of different geographical origin could be genetically diverse. Leal *et al.* (1994) also obtained only 60% similarity among the twenty *M. anisopliae* isolates, which corroborate with the present result. The RAPD molecular markers used in the current investigation allowed verification of DNA polymorphism among the nine *M. anisopliae* isolates the standard *M. anisopliae* MTCC892. This also confirms the capability of the RAPD technique to distinguish *M. anisopliae.*

6.5. Conclusion

The RAPD method used in this study offers an alternative approach for studying *Metarhizium anisopliae* strains and reduces the need for time-consuming conventional methods. Also, further increase in the number of primers is necessary to obtain precise genetic similarity degree between individuals within a population. Nevertheless, differentiation of fungal strains and species by RAPD certainly is an easy tool to detect polymorphism in a large number of samples. SSR (simple sequences repeat or microsatellites), Amplified fragment length polymorphism (AFLP) and ITS (internal

transcribed spacer) markers could be used in a complementary manner to identify specific isolates. SSR markers have the advantage of being more reliable than RAPD markers and therefore easier to use. PCR-RFLP may be advantageous over SSR markers to study the closely related isolates at the species level.

7. Summary

Metarhizium anisopliae (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes) was distributed in the tomato, coconut, paddy, groundnut, potato, carrot and cotton fields as well as in all the forest soils throughout Tamil Nadu except in the soil samples collected from Trichy, Madurai, Virudunagar, Kanchipuram, Tiruvallur, Dharmapuri, Salem, Namakkal, Chennai, Cuddalore, Pondicherry, Sivagangai, Dindhugal, Ramanathapuram and Karur Districts.

The isolates CPRC 16 and CPRC18 of *M. anisopliae* had the potential as biological control agents against the eggs, nymphal instars and adults of *Dysdercus cingulatus* (Fab.) (Hemiptera: Pyrrhocoridae) under laboratory conditions as well as on the saplings. Adults that survived after the *M. anisopliae* infection produced fewer eggs. The fecundity in *D. cingulatus* was completely arrested by those treated with CPRC1, CPRC20 and CPRC22. Eggs produced by the adults treated with CPRC1, 2, 3, 4, 9, 10, 11, 12, 14, 16, 17, 20, 21 and 22 failed to hatch. The incubation period for the treated eggs to hatch was prolonges. In laboratory sapling experiment, CPRC18 was the most virulent against *D. cingulatus* adults.

The pathogenic effect of indigenous *M. anisopliae* isolates from Tamil Nadu was also evaluated against *Aphis craccivora* Koch (Homoptera: Aphididae), *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), *Oxycarenus hyalinipennis* (Costa) (Hemiptera: Lygaeidae), *Pericallia ricini* (Fab.) (Lepidoptera: Arctiidae), *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) and *Mylabris pustulata* Thunb. (Coleoptera: Meloidae). CPRC16 had lowest LC_{50} values on the II instar larvae of *P. ricini.* After 73 hours of treatment, sporulation was observed on the cadavers treated with *M. anisopliae*.

M. anisopliae colonies were observed in haemolymph, leg, tergum, antennae, rostrum and testis of *D. cingulatus* adults after 72 hours of treatment. The haemosomic index decreased gradually as the period of incubation after treatment increased. The haemocyte count also decreased suddenly after 24 hours of treatment, but increased to some extent after 48 hours followed by gradual decrease at 72 and 96 hours of treatment. Cuticle mounting of sternal and tergal abdominal segments of treated *D. cingulatus* adults showed the penetration of fungus took place through spiracles, root of bristles, haemolymph, trachea and three dorsal sacs. It was clear that the virulent isolate CPRC18 was the best isolate to be mass produced cheaply using RWW and RBW.

The randomly amplified polymorphic DNA (RAPD) technique used to analyse the genetic variation of the nine *M. anisopliae* isolates using three primers produced distinguishable repeatable bands of sizes ranging between 136 and 3761 base pairs. Cluster analysis based on the single linkage method evidenced two clades in the dendogram for all three primers. The content percentage obtained by densitometry tracing was highest in the first band of CPRC4, first bands of MTCC892, CPRC1 and CPRC4, as well as in the fifth band of MTCC892. The Rf values were highest in the ninth band of CPRC1, third band of CPRC18 and in the fifth band of CPRC6. CPRC9 and CPRC10 presented the highest similarity index in the cluster obtained from RAPD using primer OPE8.

8. FUTURE AREA OF RESEARCH

1. Record more *Metarhizium anisopliae* strains from Tamil Nadu and test the effectiveness of the isolates against economically important crop pests. Moreover, research on the stability of propagules for storage and formulation, commercial viability and sustainability should be carried out.
2. Exact killing mechanism of *M. anisopliae* on specific host should be studied in detail.
3. SSR (simple sequences repeat or microsatellites), amplified fragment length polymorphism (AFLP) and ITS (internal transcribed spacer) markers could be used in a complementary manner to identify specific isolates more easily. Furthermore, Group I intron analysis of 28S rDNA may be used to study distantly related isolates at the genus level.

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