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1.1 General introduction

The last century has been witness to both the keynote augmentation of the chemical pesticides as well as its backlashes. The intensive use of the chemicals has shown to cause numerous deleterious effects on the environment and caused its toll on human safety and non-target hosts. Saying ‘no’ to the hazardous chemical pesticides has been explicitly talked about since a long time but somehow the real momentum for the cause is not yet a thorough practicality. The virtual torrent to fight back the ‘silent-killers’ has its basis in real-time studies and proven evidences. Frantic efforts are being (and should be) directed towards non-chemical and sustainable plant protection methodologies (Flexner et al., 2000). Cultural methods, though quite effective are handicapped by their slow and low efficacy. Under this management turmoil, all hopes in the present agricultural scenario are besetted upon the bio-control of insect pests (Lacey and Goettel, 1995). This method involves reduction in pest population through the activity of living organisms that occur naturally, by manipulation of environment or through the introduction of antagonists. Though the bio-management is not as rapid as the chemicals, but it is practically more feasible and sustainable than the chemical cure (McClintock et al., 2000). Several microorganisms are currently under consideration as control agents of insects, including viruses, bacteria, fungi and protozoa. Entomopathogenic fungi (EPFs) are used for the biological control of insect pests. Eilenberg et al. (2001) recognized four cardinal strategies for insect pest management employing EPFs, viz., (a) the classical biological control which is the intentional introduction of exotic strains for long term unmanaged control, (b) inoculative biocontrol which is the intentional release of endemic strains for long term unmanaged control of endemic pests, (c) inundative biological control which uses the fungi to limit pests when control is achieved exclusively by the mass release of the bioagent and (d) conservative biological control, which involves modification of the environment to enhance fungal infection. *Metarhizium anisopliae* and *Beauveria bassiana* are perhaps the most heavily researched upon EPFs probably because of their high host-specificity, non-persistence and non-toxicity to environment, unique mode of action and appreciable shelf life. They belong to class Hyphomycetes and are characterized by mycelial forms that bear asexual resting spores called “conidia”. The conidia serve as the infective propagule. Both of these are cosmopolitan in occurrence.

Both *M. anisopliae* and *B. bassiana* have a broad host range with individual isolates being more specific. *M. anisopliae* causes “green muscardine” disease while *B. bassiana* causes “white muscardine” in various insect pests belonging to orders lepidoptera, coleoptera and orthoptera. They have the ability to invade insects through the external cuticle. The physiology of EPF-pest interaction depends upon the following events, viz., attachment of infective unit to insect cuticle, germination on the insect cuticle, penetration of the cuticle, either directly by germ tube or by infection pegs from appressoria, multiplication in the yeast phase (hyphal bodies) in the insect haemocoel, production of toxic metabolites, death of the host, growth in the mycelial phase with invasion of virtually all host organs, penetration of the hyphae from the interior through the cuticle to the exterior of the host and finally production of infective unit to the exterior of the host (Figure 1.1).The advantages of EPFs over chemical pesticides are their significantly higher host specificity, the reduction of hazards and the inability of the insects to develop resistance. The constraints include the slow efficacy in comparison to the chemicides and on-field exposure to various biotic and abiotic stresses.

Significance of the EPF studies: The present thrust

The answer to the question as to why to pursue biological control is in simplest jargon "corporate commitment". It is now evident that the stage is set for the fungal biological disease control agents to play a greater part in commercial agriculture. The research and development sectors, the world over, commit funds to ensure that an efficacious, cost-effective and eco-friendly product will reach the market. The commitment is not limited to just the sale of products but also includes the follow-through to ensure that the end-users will be satisfied and successful when using their products. The search for and development of commercially viable EPFs entails several steps viz., isolation of the EPFs from the environment/ host insect, followed by experiments and studies to generate knowledge of the ecology, physiology and taxonomy of potential myco-insecticide. The knowledge of EPF ecology such as tolerance to environmental stress can contribute to a better understanding of the effect of optimum factors on the survival and distribution of EPFs in field. This in turn can enable prediction and application time and/ or promote habitats that encourage amplification of natural inoculum and the induction of epizootics. The study of virulence determinants like cuticle-hydrolyzing enzymes is the most imperative feature towards the success of EPFs. The high producers of the cuticle-degrading enzymes (the maximum extent of enzyme production by an isolate) are definitely the most attractive initial candidates towards isolate screening of EPFs.

Entomopathogens are cosmopolitan in nature. There are numerous isolates for each species in *M. anisopliae* and *B. bassiana*. Virulence and host specificity are the major criteria used towards the selection of a suitable isolate for commercial biological control. A particular species of an entomopathogen may have a wide host range but it might have differences in host-specificity and virulence traits among its isolates. Distinctive markers that characterize individual isolates are used to determine their host specificity, virulence efficacy, longevity and niche distribution in field. Molecular fingerprinting techniques like RAPD, RFLP and AFLP are commonly employed.

EPFs have significantly higher host specificity in comparison to the conventional bio - control agents like bacteria, protozoa, nematodes, predatory insects and viruses. It is theoretically impossible for the insects to develop resistance, as EPFs simultaneously use several modes of action, and as a “living pesticide” is subjected to adaptation too. This is in contrast to the single site of action of the much-hyped Bt-based bio - pesticides. Unlike the chemical pesticides, which are the ‘silent killers’ responsible for environmental persistence and toxicity, the EPF based biopesticides are safe to environment and mankind. The shelf life of EPF based mycopesticide is up to 8 months at room temperature but can retain viability for unto 8 years when stored at low temperatures. The use of myco - pesticides is slowly but gradually gaining momentum and its utilization for commercial purposes, inevitably leads to considerations of ways of improving its on - field performance. There can be many features that might benefit from genetic improvement. Some of the limitations of bio - insecticides, such as slow rate of action, restricted host range and limited persistence in field can be addressed by various strategies involving genetic manipulation. PEG, electroporation and biolistic methods have been employed to transform *M. anisopliae* using benomyl resistance as selectable marker (Bogo et al., 1996).

Three major aspect of the fungal biology were investigated in the present research viz., first, abiotic stress tolerance and extra-cellular enzyme production by *M. anisopliae* and *B. bassiana* isolates; second, establishing genetic relationships amongst the *M. anisopliae* and *B. bassiana* isolates and the third aspect studied was destruxin production, characterization and toxicity testing. The three aspects studied, together form the 11 Chapters of the thesis.

1.2 ASPECT 1: Abiotic stress tolerance and extracellular enzyme production by *Metarhizium anisopliae* and *Beauveria bassiana* isolates

UV-B and Temperature stress studies in EPF's

Upon field application, the entomopathogens are exposed to an array of abiotic stresses like temperature (Rangel et al., 2005a), UV radiations (Rangel et al., 2006a) humidity-osmolarity (Lazzarini et al., 2006), edaphic factors and nutrient source (Shah et al., 2005), which negatively affect the field use of entomopathogens as biocontrol agents. UV-photons, in particular those belonging to the UV-B type, form covalent bonds between adjacent thymine bases resulting in thymine dimers. Thymine dimers do not base pair normally, which causes distortion of the DNA helix, stalled replication, gaps and mis- incorporation. These can lead to mutations and ultimately disrupt the normal functioning of the organism (Diffy, 1991). Soil temperature is a major factor, which affects the success or failure in the establishment and production of fungal inoculum (Thomas and Jenkins, 1997). The entomopathogenic fungi not only have to be tolerant to the soil temperature but also have to survive through thermoregulatory defence response of the host insect (Inglis et al., 1996; Ouedraogo et al., 2003). It has been demonstrated that stress temperature alters the vegetative growth among isolates of entomopathogenic fungi (Ouedraogo et al., 2004). Dry heat exposure causes DNA damage through base loss leading to depurination and this may cause mutation (Nicholson et al., 2000). Wet heat *i.e.* heat in conjunction with high humidity results in protein denaturation and membrane disorganization. Rangel et al. (2006a) reported that conidial pigmentation is involved in protection against heat and UV radiation in several fungal species including *M.anisopliae*. Rangel et al. (2006b) reported that growth of *M.anisopliae* on non-preferred carbon sources like minimal media (MM) or MM plus a non-preferred carbon source greatly improved UV-B tolerance. Rangel et al. (2005b) studied variability in conidial thermo tolerance of *M.anisopliae* isolates and observed that most of the isolates tolerated 40^{o} C very well, with relative germination above 90% after 12 h of exposure to heat (40^{o} C or 45^{o} C for 2,4,8 and 12 h). Rangel et al. (2008) reported induction of elevated tolerance to UV-B radiation and heat in conidia of *M.anisopliae* produced on fungi exposed to sub lethal stress (other than heat or UV-B) during mycelial growth. Uma Devi et al. (2005) screened about 29 *B.bassiana* isolates against temperature stress for *in-vitro* germination and growth. Fernandes et al. (2007) studied variability in tolerance to UV-B radiation among *Beauveria* sp. isolates.

Nutrient sources

Nutrients are substances used in biosynthesis and energy release and therefore serve as cardinal impetus towards the viability, survival and sustainance of any organism (Safavi et al., 2007). For elite cultivation of microorganism, it is imperative to have an in-depth knowledge of their nutritional requirements. The macro-elements like carbon, oxygen, hydrogen, nitrogen, sulfur and phosphorus are integral components of carbohydrates, lipids, proteins and nucleic acids and these metabolically active groups are directly/ indirectly involved in host-pathogen interaction and self-defense and perpetuation mechanisms. Although saprophytic fungi are able to utilize a wide range of nutrient sources (Liu and Chen, 2002), simple and less expensive media are needed to permit their mass-production and commercialization (Shah et al., 2005). The CN ratio is said to significantly affect the number of conidia produced and conidial characteristics of *Colletotrichum truncatum* (Jackson and Bothast, 1990; Jackson and Schisler, 1992; Jackson and Slininger, 1993). Shah et al. (2005) studied the influence of nutrition on growth and virulence of *M. anisopliae*. Doust et al. (1983) studied the effect of growth substrates on conidial viability and virulence. An optimum nutrient source is the cardinal prerequisite for the healthy sustenance of any individual inclusive of the tiny but diverse world of microbes. The germination, growth and sporulation feature of a vast majority of EPFs is clearly affected by the supply of nutrients provided by the affected host insect (Humber et al., 2008). *Hirsutella thompsonii* which grows luxuriously on *in-vitro* lab culture media has sparse growth and sporulation on mycotized mites (Sampson et al., 1980). Hywel-Jones and Samuels (1998) emphasized on the power of nutrients to demonstrate the transition of emtomopathogenic mode (on scale insects) of three species of *Hypocrella* to plant parasitic mode, once the underlying insect host (on the plants) is destroyed. Shah et al. (2005) reported the significance of nutrition on the growth and virulence of *M.anisopliae* isolates. They reported maximum radial growth of two *M.anisopliae* isolates on CN ratio of 35:1 and 75:1 and least on osmotic stress medium. The conidial production too was highest in CN 35:1 media and least in osmotic stress medium. They found that conidia produced on nutrient poor/stress sources (1% Yeast and osmotic stress medium respectively) were relatively more virulent than conidia produced on CN 35:1 media. Engelkes et al. (1997) studied the effect of carbon, nitrogen and CN ratios on growth, sporulation and biocontrol efficacy of *Talaromyces flavus*. Zhang et al. (2005) reported influence of carbon-to-nitrogen ratio and carbon loading of production media on freeze-drying survival and biocontrol efficacy of *Cryptococcus nodaensis*. Lopez et al. (2003) studied the effect of CN source and ratio on the production of metabolite lovastatin by *Aspergillus terreus*.

Li and Holdom (1995) studied effects of nutrients on colony formation, growth and sporulation of *M.anisopliae* and observed that soy peptone was much better than simple nitrogen compounds for growth and sporulation. They also concluded that organic acids contribute to persistence of conidia in soil. Doust and Roberts (1983) found that the storage conditions were more critical to spore survival and virulence than the substrate upon which conidia were produced. Nutrition might affect the response of an organism towards stress. Rangel et al. (2004) studied variation in UV-B tolerance and germination speed of *M.anisopliae* conidia produced on insects and artificial substrate. Alves et al. (2002) demonstrated that Yeast-like structures produced on MacConkey agar are effective as inoculums for *B.bassiana* applications against arthropod pests, and possibly superior to conidia against some species. Obtaining well-defined Yeast-phase cultures of entomopathogenic fungi may be an important pre-requisite to study pathogenesis. The yield and biomass of entomopathogneic fungi, largely depends on the nutrient source. Bidochka et al. (1987) observed that the yield of blastopores of *B.bassiana* isolate was four-fold higher in peptone-glucose-yeast extract. They also observed that biomass production was highest in peptone-glucose-yeast extract. Nutrients also affect the release of extra-cellular enzymes by entomopathogneic fungi. Qazi and Khachatourians (2008) observed that addition of exogenous carbon and nitrogen sources to aphid eluvia modulates synthesis of proteases and chitinase by germinating conidia of *B.bassiana*. The natural nutritional environments of most organisms are spatially non-uniform and that alters their relative growth response. Ritz (1995) studied growth responses of some soil fungi to spatially heterogeneous nutrients by devising a system comprised of tessellations of agar tiles of contrasting nutrient status separated by air gaps. They observed that growth in low-nutrient tiles was greater when high nutrient tiles were included in the tessellation. They also observed that reproductive structures tended to be formed only in low nutrient tiles with *Trichoderma* and *Rhizoctonia* and only high nutrient tiles with *Alternaria*.

Extracellular enzyme production by EPF’s and their role in pathogenicity

Interaction of the entomopathogenic fungi and host depends on the occurrence of non-specific and specific events between the conidia and the insect cuticle (Chengshu et al., 2002). Amongst the series of specific events is involved the production of a range of cuticle-degrading enzymes corresponding to the different polymers of the insect cuticle. Several workers have reported a correlation between production of extra-cellular enzymes and pathogenicity. Gupta et al. (1992) studied cuticle-degrading enzymes from five strains of *B.bassiana* and concluded that enzyme expression differed considerably as a function of cuticle source. St.Leger et al. (1986c) found the mechanisms of interaction between cuticle degrading enzymes and insect cuticle. St. Leger et al. (1997) studied the lytic enzyme systems (proteases and carbohydrases) of *Verticillium lecanni*, *B. bassiana* and *M.anisopliae* and observed that the pathogens showed enzymatic adaptation to the polymers present in the integument of their particular hosts. St.Leger et al. (1991) elucidated the penetration events during infection of host cuticle by *M. anisopliae* and suggested early involvement of cuticle degrading enzymes in infection by the presence of chymoelastase protease (Pr1), esterase and N-acetylglucosaminidase in ungerminated spores. Fan et al. (2007) reported the construction and characterization of several *B.bassiana* hybrid chitinases where the chitinase Bb chitI was fused to chitin-binding domains derived from plant, bacterial or insect sources. Pedrini et al. (2007) reviewed the biochemistry of insect epicuticle degradation by entomopathogenic fungi and reported that over-expression of virulence enzymes is advantageous, since no concern to using genetically modified fungi has been provided and hence is an ecologically safe alternative to chemical pesticides. The cuticle degrading enzymes have been variously used for characterization of entomopathogenic fungi. Dong et al. (2007) characterized an *M.anisopliae* isolate for isoenzyme analysis. Mustafa and Kaur (2009) characterized fourteen *M.ansiopliae* isolates for cuticle hydrolyzing enzymes. Chengshu et al. (2002) detected and characterized Pr1 virulent gene deficiencies in *M.anisopliae*. Various studies emphasize on the enhancers and inhibitors of extracellular enzymes of entomopathogenic fungi. Hegedus and Khachatourians (1988) studied the production of lipase by *B.bassiana* and found that olive oil induces lipase production. They also reported that iron and copper inhibited the lipase activity, while magnesium increased the lipase activity. Silva et al. (2005) studied lipase production by *M.anisopliae* using different lipid sources like olive oil and sunflower oil and found that 32^{o}C was the best temperature for lipase production. Smith and Grula (1983) reported that chitinase production by entomopathogenic fungi is inducible by chitin oligomers, N-acetylglucosamine and glucosamine. St. Leger et al. (1986c) observed that chitosonase is co-induced with chitinase in *M.anisopliae*. Bidochka and Khachatourians (1988) suggest that the chitinase and protease activities are coordinately regulated. Gooday (1990) studied the physiology of microbial degradation of chitin and chitosan.

1.3 ASPECT 2: Establishing genetic relationships amongst the *Metarhizium anisopliae* and *Beauveria bassiana* isolates

DNA marker technologies and their significance in the study of entomopathogenic fungi

Entomopathogens are cosmopolitan in nature. There are numerous isolates for each species in *M. anisopliae* and *B. bassiana*. Virulence and host specificity are the major criteria used towards the selection of a suitable isolate for commercial biological control. A particular species of an entomopathogen may have a wide host range but it might have differences in host-specificity and virulence traits among its isolates. The breakthrough development of DNA-based markers has significantly revolutionized and impacted the microbial genetics. Distinctive markers that characterize individual isolates are used to determine host specificity, virulence efficacy, longevity and niche distribution of entomopathogenic fungi in field. With the use of DNA markers, it becomes possible to observe and exploit genetic variation in the entire genome. The popular genetic markers include allozymes, RAPD, RFLP, AFLP, IISR, SNP and EST markers. The DNA markers find use in investigations of genetic variability, parentage and phylogeny, species and strain identification and in construction of high resolution genetic linkage maps for identification of quantitative trait loci.

Type of DNA markers on the basis of gene function

On the basis of gene segment functionality, the DNA markers are classified into two types, viz., type I and type II. The former are associated with genes of known function while the latter are associated with anonymous genomic segments (O'Brien, 1991). RAPD markers which involves the use of random primers, amplifies bands from anonymous genomic regions via the polymerase chain reaction and so it falls under the category of type II markers. Most RFLP markers are type I markers because they are identified during analysis of known genes.

Random Amplified Polymorphic DNA (RAPD)

RAPD typing is based on the fact that short oligonucleotides i.e. the primers, whose sequences are not directed at any specific sequence in the genome, will hybridize at random site to initiate DNA polymerization when subjected to PCR (Welsh and McClelland, 1990). The primer pair is identical and 8-10 bp in length. RAPD polymorphisms can occur due to base substitutions at the primer binding sites or due to insertion/ deletions in the regions between the sites. The RAPD technology has been used to detect polymorphism in studies examining genetic diversity

(Grayson et al., 2000), pedigrees (Tinker et al., 1993), genetic map construction (Binelli et al., 1994), species identification and like. The technique involves use of arbitrary 'random' primers, and if genomic sequence of two or more template or test samples is different, then the PCR products display different banding patterns called 'polymorphism'. RAPD require no prior knowledge of the genome under investigation. It holds an edge in being simple and economical. Differences in band intensity as well as presence / absence of RAPD bands, forms the basis of diagnosis through phenetic and genetic analysis. The phenetic analysis considers all clear / reproducible bands, irrespective of whether they are diagnostic or not; whereas, in genetic analysis, an absent band corresponds to 'an unexpressed phenotypic locus'. The former involves scoring of bands as '1' for it being 'present' and '0' for it being 'absent' (Atienzar et al., 2006).

Analysis through RAPD is often criticized as non – reliable, but careful optimization does yield reproducible profiles (Hedrick et al., 1992; Fraga et al., 2005). The quality of genomic DNA plays a cardinal role in the RAPD banding patterns. RAPD assays performed with different DNA concentrations yields difference in the intensity of bands. Highly pure DNA free from macromolecules and possible inhibitory compounds produces clear and diagnostic RAPD profiles (Benter et al., 1995).

The technique has been successfully used to measure genetic diversity among fungal entomopathogens like *M. anisopliae* and *B. bassiana*. Urtz and Rice (1997) characterized *B. bassiana* isolates from the rice water weevil *Lissorhotrus oryzophilus* using RAPD – PCR technology. Fungaro et al. (1996) established diversity among soil and insect isolates of *M. anisopliae* var. *anisopliae* by RAPD. Tigano- Milani et al. (1995) established genetic variability among Brazilian isolates of *M. anisopliae* using RAPD – PCR. Tigano and Aljanabi (2000) showed that RAPD analysis provides a powerful tool for investigation of the genetic variability of *Nomuraea rileyi* isolates within and among population. Zengzhi (1998) detected variation between three species and isolates of *B. bassiana*. Bidochka et al. (1994) explained differentiation of species and strains of entomopathogenic fungi by RAPD technology. Dalzoto et al. (1993) studied recombination processes in *B. bassiana* using RAPD analysis. Huafeng et al. (1999) analyzed relation between DNA amplified fingerprints and source of *B. bassiana* isolates. Ozino et al. (1998) emphasized upon the suitability of RAPD – PCR to specifically characterize and identify entomopathogenic isolates of *B. brongniartii*, useful as potential bio - control agent against *M. melelontha*. Leal et al. (1994) characterized 20 isolates of *M. anisopliae* by RAPD – PCR and observed similarities between profiles of isolates from the same countries than from different countries. Fegan et al. (1993) examined 30 isolates of *M. anisopliae* and concluded that isolates exhibiting > 80 % similarity in RAPD profiles tended to be isolated from the same geographic area. They also observed that the *M. anisopliae* as assessed on morphological grounds represents a very diverse genetic group; and their results also suggested that RAPD markers may be useful for the tracking of specific bio – control strains in the field. Maurer et al. (1997) subjected 38 strains of *B. bassiana* from various geographical sites and variable insect hosts, to RAPD analysis, and observed that there exists a distinct similarity relationship between isolates obtained through same host. Kaur and

Padmaja (2008) characterized 27 isolates of *B. bassiana* isolates by RAPD – PCR.

Restriction Fragment Length Polymorphism (RFLP)

RFLP markers are perhaps the first shot in the genome revolution. If flanking sequences are known for a locus, the segment containing the RFLP region is amplified via PCR. If length polymorphism is caused by relatively large insertions-deletions, gel electrophoresis of the PCR products should reveal the size difference. However, if length polymorphism is caused by base substitution at a restriction site, PCR products must be digested with a restriction enzyme to reveal the RFLP. There are ‘universal’ primers commercially available, which can target DNA regions that are either relatively conserved or rapidly evolving, depending on the amount of variation observed and the taxonomic level under examination. The PCR products obtained can be digested with restriction enzymes and visualized by simple staining with ethidium bromide. Destefano et al. (2004) used RFLP of ITS – 5.8S rDNA regions to detect and identify *M. anisopliae* isolates after 48 hrs of its infection to sugarcane borer *Diatraea saccharalis*. Han et al. (2002) evaluated phylogenetic relationships among strains of *N. rileyi* as revealed by partial β – tubulin sequences. Mavridou et al. (2000) identified group – I introns at three different positions within 28s rDNA gene of *M. anisopliae* var. *anisopliae* using RFLP. Neuveglise and Brygoo (1994) used RFLP of 28s rDNA of the fungus *B. brongniartii* for the identification of group – I introns. Sugimoto et al. (2003) characterized 46 isolates of *Verticillium lecanii* using RFLP of the β – tubulin gene. Couteaudier and Viaud (1997), examined DNA variations among isolates belonging to different vegetative compatibility groups in population of *B. bassiana* using telomeric RFLP.

1.4 ASPECT 3: Studies on destruxin production, characterization and toxicity testing

After enzymatic hydrolysis of the cuticle, the EPF reaches the haemolymph of the insect where it grows as yeast like blastospores or hyphal bodies, rather than in mycelial form. Before proliferation in the haemocoel, the EPFs produce toxins to suppress the insect defense response. *B. bassiana* produces various toxic compounds including beauvericin, bassianolide and oosporin, and *M. anisopliae* produces numerous cyclic depsipeptides like destruxins and swansonine.

Destruxin production by *M. anisopliae*

The entomopathogenic fungus *Metarhizium anisopliae* is known to produce low molecular weight, bioactive secondary metabolite, destruxin, which is a cyclodepsipeptide. A typical structure of destruxin is illustrated in Figure 1.2. These are of special interest in context of the biological control of insect pests as these are the only mycotoxins detected in the insect body at advanced stages of infection in sufficient quantities to cause mycosis (Brousseau *et al.,* 1996; Kershaw *et al.,* 1999). In the group of destruxins biosynthesized by *M. anisopliae* isolates, the destruxin A, B and E are predominant. Destruxin E is the most toxic for invertebrates, particularly insects (Dumas *et al.,* 1994; Loutelier *et al.,* 1996; Pedras *et al.,* 2002; Strasser *et al.,* 2000). Biochemical and analytical studies showed that both *in vivo* and *in vitro* diffusion of destruxin is a very rapid process from endogenous mycelia, although their ratios being different (Cherton *et al.,* 1991; Amiri-Besheli *et al.,* 2000; Butt *et al.,* 1994).

Correlation between destruxin production and virulence

A positive correlation between virulence and destruxin production has been evidenced from several studies. Destruxins are produced by *M. anisopliae* during *in-vivo* colonization of host insect tissues (Pedras et al., 2002) and they are excreted into the growth medium during *in-vitro* cultivation of the fungus (Jegorov et al., 1998a). After injection destruxins show a range of insecticidal activities against insect pests. Al-Aidroos and Roberts (1978) reported a link between destruxin content and virulence against mosquito larvae. It is suggested that destruxins also inhibit Malphigian tubule fluid secretion (James et al., 1993) as well as ecdysteroid secretion by prothoracic glands (Sloman and Reynolds, 1993). Destruxin E acts on the midgut, Malpighian tubules and circulating haemocytes (Dumas et al., 1996). Muroi et al., (1994) reported the inhibitory effect of destruxin B on vacuolar type H^+ translocating ATPase. Destruxin prevent ecdysteroids by prothoracic glands of *Manduca sexta* (Sloman and Reynolds, 1993) and inhibit Malphigian tubule fluid secretion of *Schistocerca gregaria*. Bio-pharmacological studies reveal that the paralysis induced by destruxin in insects results from a calcium-dependent effect on muscular cells (Kodaira, 1961; Samuels *et al.,* 1988a, b, c). Studies show that there is an interaction between the mycotoxin and the plasma membrane and that the surface of the cells becomes modified. Electron microscopic studies reveal development of dilated vesicles at the level of brush borders. Several studies have reported immunomodulatory effects of destruxins (Huxam *et al.,* 1989; Vilcinskas *et al.,* 1997) and reveal that inhibition of cellular immune response in the haemolymph may be the key factor for destruxin induced mycosis. It is established that destruxin induce calcium influx and phosphorylation of intracellular proteins within lepidopteron cell lines (Dumas *et al.,* 1996). All in all, it can be concluded that destruxins alter and hamper one or more basic aspects of insect biology and thus are positively correlated with virulence.

Antifeedant properties of destruxin

. Because of their peptidic nature and insecticidal properties, the destruxins are attributed the status of a new generation of pesticides. Several studies report the injection and/ or forced feeding effects of different destruxins on insect pests. Destruxins are stomach poisons and must be necessarily ingested to exert their insecticidal effects but few reports suggest their topical and/ or contact application for insect mortality. Reported bioactivities of destruxins to insect pests utilize different mode of action such as contact and antifeedant (leaf dip method as in studies of Hu et al., 2009). Amiri-Besheli et al. (1999) reported the antifeedant properties of destruxin and their use for the control of crucifer pests. Hu et al. (2009) studied the antifeedant effect of destruxin A and B on *Bemisia tabaci* by leaf dip method.

The concept of LD_{50}, LC_{50} and LT_{50} for the expression of destruxin toxicity

The toxin - based bioassays report the insect mortality in terms of LD_{50}, LC_{50} and LT_{50} (Robertson and Preisler, 1992). The median lethal dose LD50 is defined as the statistically derived exposure dosage of a pesticide expected to cause mortality in 50 % of an insect population. Similarly LC_{50} is the concentration of the toxin required to kill 50 % of the insect pest population and LT_{50} is the time required by a particular dose/ concentration of toxin to kill 50 % of the insect pests out of a given population. A typical dose – response relationship is analyzed with models like LOGISTIC, PROBIT, CATMOD and GENMOD (Finney, 1964). All these methods predict the probability of a positive response (insect death) as a function of the pesticide/ toxin dosage applied. The response variable may be binary or measured ordinally rather than continuously. For binary response, PROBIT analysis, based on 'maximum likelihood' estimation methods, are best suited (Finney, 1962; Finney, 1952). The binary responses are measured as '1' if insect death occurs and '0' if it does not.

Virulence of natural and insect passaged isolates of entomopathogenic fungi

The life cycle of an entomopathogenic fungus in the natural environment is characterised by multi-step mode of pathogenesis (as discussed under Section 1.1 and illustrated in Figure 1.1). On the contrary, laboratory culture of entomopathogenic fungi requires their *in-vitro* maintenance on rich nutrient media like Sabouraud Dextrose Agar, Potato Dextrose Agar and Sabouraud Maltose Agar, in which the conidiation and mycelial aspects of the EPF life cycle predominate alternatively. In the *in-vitro* process the fungus does produce its characteristic lytic enzymes (Chapter 4 of the thesis deals with *in-vitro* extracellular enzyme production) and toxins (Chapter 8 of the thesis deals with *in-vitro* production of mycotoxin, destruxin) but the extent of production may 'considerably vary' and reports suggest that 'the *in-vivo* traits gradually decline in the *in-vitro* conditions'. In other words, the *in-vitro* life cycle of the entomopathogenic fungi may be 'similar' to its *in-vivo* counterpart but 'exact simulation of the *in-vivo* life cycle is not possible in the *in-vitro* life cycle of entomopathogenic fungi'. This decline in intensity of virulence of the entomopathogenic fungi in the *in-vitro* conditions (in comparison to the *in-vivo* mode) is termed as 'strain/ isolate attenuation' and it is supposed to be a gradual process which manifests over generations of sub-culturing on artificial culture media (Schaerffenberg, 1964; Aizawa, 1971, Boucias and Pendland, 2000). Variation in isolate virulence has been frequently associated with hyphomycetous fungi like *M. anisopliae* (Latch et al., 1976; Doust and Roberts, 1982), *Nomuraea rileyi* (Ignoffo et al., 1976) and *B. bassiana* (Latch, 1976). The attenuated generation may or may not have visible morphological symptoms. As an example, during routine sub-culturing of the *M. anisopliae* and *B. bassiana* isolates, it was occasionally noticed that certain isolates produced 'sectors' (Figure 1.3) on the culture media, which upon analysis showed that they produced almost no or very sparse conidiation *i.e.* they were basically sterile and lytic enzyme activity by such colony was insignificant. Another example of attenuated generation that was observed during the routine culturing of fungi was with the isolate UM8 and UM9 which although produced extra-cellular enzymes, did not produce appreciable sporulation and growth. Thus the concept of strain/ isolate attenuation was the motivation for the Chapter 10 of the thesis (Virulence of insect-passaged conidia vs. native conidia).

There have been reported instances in which the fungus, re - isolated from the insect cadaver, after successful completion of the intended bioassays, showed an increase in virulence response. Al- Aidroos and Roberts (1978) were able to enhance virulence of *M. anisopliae* by passage through mosquito larvae, the same host which they used for bioassays. Alternatively virulence can also be enhanced by passing the fungus through an alternate host (Hartmann and Wasti, 1974). Doust and Roberts (1982) passaged *M. anisopliae* isolates through *Culex pipiens* larvae and brown plant hoppers to enhance virulence. Hall (1980) enhanced virulence and growth rate of

Verticillium lecanii by passing it through insect hosts. Shah et al. (2005) demonstrated that the conidia of two *M. anisopliae* isolates (V245 and V275) recovered from *Tenebrio molitor* and *Galleria mellonella* were more virulent and also germinated faster than those recovered from synthetic culture media. They also reported that spore bound Pr1 was significantly higher in conidia produced on artificial substrate. Lane et al. (1991) studied the influence of cultural conditions on virulence of *B. bassiana* to green leaf hopper. Kawakami (1960) reported an increase in virulence of *M. anisopliae* through successive insect passage. A probable reason for enhancement of fungal virulence through insect passage might be that the insects and insect - derived components (insect homogenate/ insect cuticle) provide a complex nutritional component which lacks in routine mycological culture media (Shah et al., 2005). Al- Sayed et al. (1993) reported an increase in protease and chitinase activity with increasing concentration of *Trichoplusia ni* and *Helicoverpa zea* cuticle.

The following objectives were set for the present study in an attempt to obtain information on the lines stated above.

1.5 OBJECTIVES

1. Screening the most robust isolate(s) on the basis of abiotic stress tolerance. Effect of temperature, UV radiation and nutrition on the germination, growth and sporulation was studied for *M. anisopliae* and *B. bassiana* isolates.
2. Studies on the production of extra-cellular lytic enzymes by *M. anisopliae* and *B. bassiana* isolates.
3. Establishing genetic relationships amongst the 14 *M. anisopliae* and 17 *B. bassiana* isolates (RAPD and RFLP).
4. Production and characterization of destruxin produced by *M. anisopliae* ARSEF-2735.
5. Toxicity studies of destruxin produced by *M. anisopliae* ARSEF-2735.
6. Studies on the characteristics and virulence of native and insect-passaged conidia.

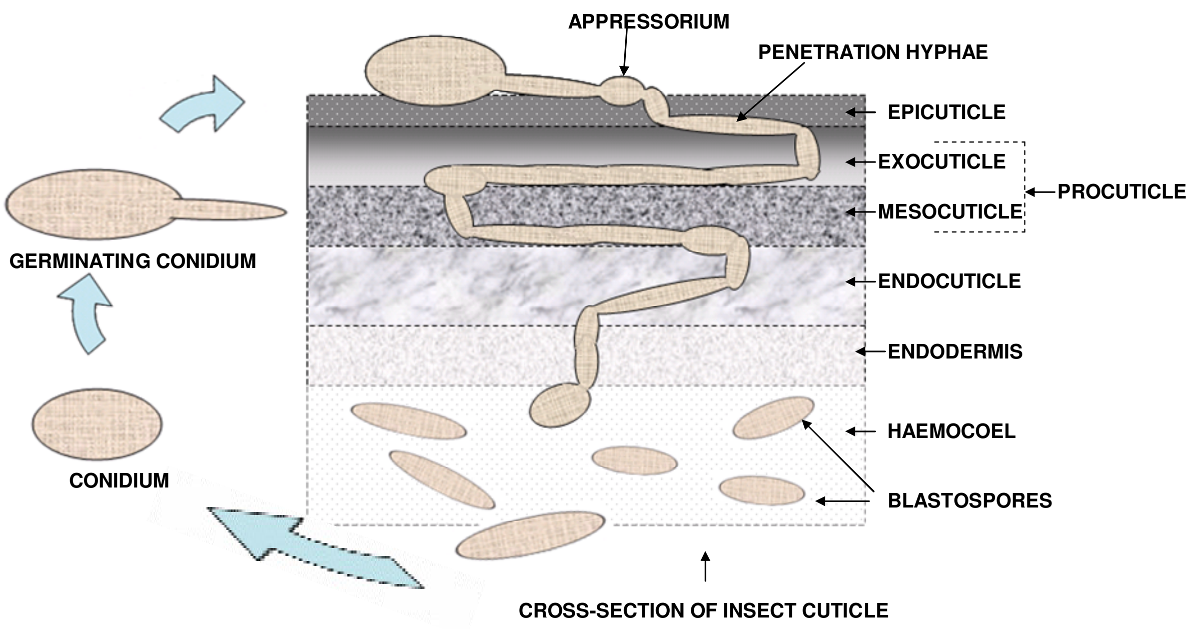
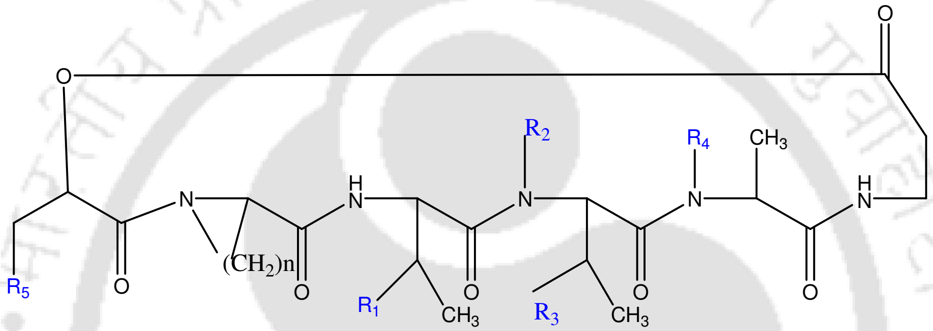


Fig. 1.1: A schematic representation of interaction between entomopathogenic fungi and insect cuticle.



General Formula: cyclo-[-D-HA-L-Pro^1/ Pip^1-L-Ile^2/ Val^2-L-MeVal^3-L-MeAla^4-β-Ala^5-]

n: n=3 for Pro and n=4 for Pip
HA: Hydroxy acids
Pro^1: Proline residue; the members have no subscript (A-E) and these members have isoleucine at 3rd place.
Pip^1: Pipecoloic acid residue; the members are designated with subscript 1 (A_1-E_1).
Ile^2: Isoleucine.
Val^2: Valine residue; the members are designated with subscript 2 (A_2-E_2).
Ala: Alanine.

Fig. 1.2: A typical structure of dextruxin.

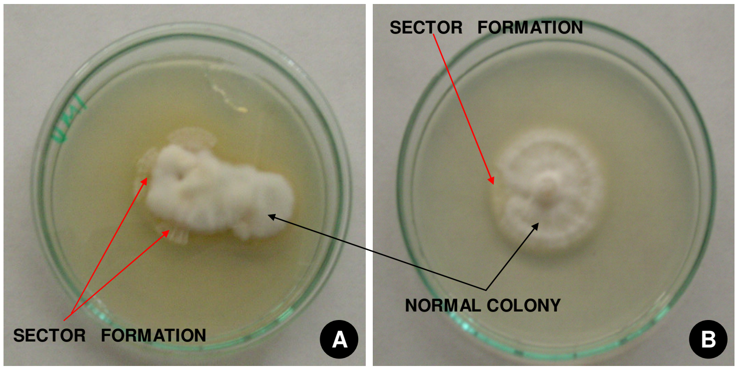


Fig. 1.3A & B: Sector formation in *B. bassiana* isolates.

2.1 Introduction

Commercial biological control involving entomopathogenic fungi in the present global scenario is a hi-tech venture both in terms of safety and sustenance. But the credibility of hi-fidelity management of insect pests by fungus is besotted upon attaining, maintaining and novel sustaining of such fungal strains, in the turmoil multitude of abiotic stresses (Tang and Hou, 2001). The continuance of viability and virulence of fungal inoculum (conidia) after field application is the pre-requisite threshold for their efficacy (Doust and Roberts, 1983). Various isolates of *B. bassiana* and *M. anisopliae* have been the most entrusted entomopathogens that have been heavily researched upon and find appreciable on-field commercial usage for insect pest management (Easwaramoorthy, 2003). Upon field application, the entomopathogens are exposed to an array of abiotic stresses like temperature (Rangel et al., 2005a), UV radiations (Rangel et al., 2006a), humidity-osmolarity (Lazzarini et al., 2006), edaphic factors and nutrient source (Shah et al., 2005) that negatively affect the field use of entomopathogens as biocontrol agents. Continued abiotic stress can either have absolute deleterious effect or force natural selection in them (Rangel et al., 2006b).

The solar radiation, which includes visible light, ultraviolet radiations, infrared rays and radio waves have been the dominant source in which all organisms evolved and adapted. In biological context, the UV radiations acclaim a special mention in terms of their impact on life (Bjorn, 2006). Ultraviolet (UV) light is electromagnetic radiation with a wavelength shorter than that of visible light, but longer than soft X-rays. When considering the effect of UV radiations on organisms and the environment, the range of UV wavelength is often subdivided into UV-A or long wave or black light (400-315 nm), UV-B or medium wave (315-280 nm), and UV-C or short wave or germicidal (‹ 280 nm). UV-photons, in particular those belonging to the UV-B type, form covalent bonds between adjacent thymine bases resulting in thymine dimers. Thymine dimers do not base pair normally, which causes distortion of the DNA helix, stalled replication, gaps and misincorporation. These can lead to mutations and ultimately disrupt the normal functioning of the organism (Diffy, 1991). Soil temperature is a major factor, which affects the success or failure in the establishment and production of fungal inoculums (Thomas and Jenkins, 1997). The entomopathogenic fungi not only have to be tolerant to the soil temperature but also have to survive through thermoregulatory defense response of the host insect (Inglis et al., 1996; Ouedraogo et al., 2003). It has been demonstrated that stress temperature alters the vegetative growth among isolates of entomopathogenic fungi (Ouedraogo et al., 2004). Dry heat exposure causes DNA damage through base loss leading to depurination and this may cause mutation (Nicholson et al., 2000). Wet heat i.e. heat in conjunction with high humidity results in protein denaturation and membrane disorganization. It has been reported that *M. anisopliae* has temperature tolerance upper limit as 37 - 40 ºC (Thomas and Jenkins, 1997). *B. bassiana* on the other hand can survive up to a maximum temperature of 37 ºC (Fargues et al., 1997). In fungi the temperature range for germination and mycelial growth has been reported to be similar.

The entomopathogenic fungi are natural and cosmopolitan in occurrence. The purpose of this study has been to identify those naturally occurring entomopathogenic fungi, which are naturally resilient to abiotic stresses. With such an objective, *in-vitro* laboratory studies were undertaken to investigate the stress-tolerant attributes among fourteen *M. anisopliae* and seventeen *B. bassiana* isolates. The abiotic stresses, which were studied, were temperature stress and UV-B radiation stress. A comparative analysis based on germination, growth and sporulation parameters was done in case of temperature stress. In UV-B radiation stress studies, only germination assay was undertaken.

2.2 Materials and Methods

Fungal cultures

The different isolates of the fungus *M. anisopliae* and *B. bassiana* were either procured from ARSEF (USDA-ARS Plant Protection Unit) or isolated locally in India. The fourteen isolates of the fungus *M. anisopliae* were designated as UM1-UM13 and AR1 and the seventeen *B. bassiana* isolates were designated as UB1-UB16 and AB1 (Figure 2.1). The isolates with their accession no., their geographical origin and the host insect from which they were isolated are detailed in Table 2.1 and Table 2.2. The isolates were routinely sub cultured on SDA (Sabouraud dextrose Agar) slants at 28 °C in incubators and maintained at 4 ^{o}C.

Colony growth and sporulation (Temperature stress)

Seven day old cultures on SDA slants were used for preparing spore suspension in 0.02 % Tween 80 solution at 1x10^6 spores/ ml. A 200 µl of 10^6 spores were plated on SDA medium and were incubated for 3 days at 28 °C. At the end of 3 days, 5 mm agar disc with mycelia was retrieved with the help of a cork borer and placed in middle of fresh SDA plates (5 replicates / isolate were maintained) and incubated at 28 °C, 30 °C, 34 °C, 37 °C and 40 °C respectively. Radial growth was measured from 3rd day onwards till 8th day. Radial growth rate (mmd-^{1}) was calculated from the linear portions of the curves plotted from these values. The plates at 28 °C served as control. At the end of 8th day, 5 mm agar discs were randomly taken with the help of a cork borer. The discs were placed in 10 ml of 0.02 % (v/v) Tween 80 solution and vortexed to suspend the spores. Spore concentration was determined using a Neubauer Haemocytometer.

Germination (Temperature stress)

Agar slide technique was used for studying the rate of germination. Petri plates were lined with blotting paper discs and 2 glass slides were placed in each of the plates and autoclaved. A 1ml SDA medium was evenly spread on each of the glass slides using micropipette. Conidial suspension was prepared from seven day old cultures with concentrations maintained at 10^6 conidia/ ml. Approximately 100 µl of 10^6 spores/ ml of fungal isolates were spread on the SDA coated slides. The slides were placed back in the Petri plates and the blotting paper discs were moistened with sterilized water. The Petri plates were kept for incubation at 28 °C, 30 °C, 34 °C, 37 °C and 40 °C (2 replicates/

isolate/ treatment were maintained). The slides were observed under compound microscope (40X) for germination, every 2 hrs, starting from 4th hr after inoculation. A conidium was considered to be germinated when a distinct germ tube projected from it, and was at least twice the diameter of the conidium (Milner et al., 1991). Approximately 300 conidia were scored per replicate for each of the treatments and the rate of germination determined.

Germination (UV-B radiation stress)

A stock solution of 10^6 spores/ ml was prepared in a viol and vortexed and divided into 1 ml each in 5 polystyrene tubes. The control-experiment tubes were wrapped in aluminum foil (0 hr exposure as the aluminum foil prevented UV-B penetration) and placed together with the test treatment tubes on UV-Trans illuminator, which served as the source of UV-B radiations (320 nm) and gave the desired exposure. Treatments (exposure duration) were 1, 2, 3 and 4 hrs exposure to UV-B radiations. Petri plates were lined with blotting paper discs and 2 glass slides were placed in each of the plates and autoclaved. A 1 ml SDA medium was evenly spread on each of the glass slides. Approximately 100 µl of 10^6 spores/ ml, were UV-B treated at different exposure durations, and spread on the SDA coated slides. The slides were placed back in the Petri plates and the blotting paper discs were moistened with sterilized water. The Petri plates were kept for incubation at 28 °C in dark (2 replicates/ isolate/ treatment were maintained). Observations were taken from 4th hr onwards till 24th hr, and repeated at every 4-hr interval. The slides were observed under compound microscope (40X) for germination. A conidium was considered germinated when a distinct germ tube projected from it. On an average about 300 conidia were scored/ replicate/ treatments and the rate of germination determined.

Statistical analysis

Variances in germination and growth counts among the different treatments and the sample time (days/ hrs) were analyzed using procedures for two factor analysis of variance (ANOVA). The data of percentage germination were arc sine percentage square root transformed before analysis.

2.3 Results

Colony growth and sporulation (Temperature stress)

The fourteen *M. anisopliae* isolates showed radial mycelial growth at all the test temperatures except at 40 °C. All the *M. anisopliae* isolates showed similar tolerance ($P > 0.05$) towards temperature stress. But the response of each of the isolates at different temperature showed significant variation ($P < 0.05$). In *M. anisopliae* isolates the radial growth was more at 28 °C but decelerated sharply with increase in temperature at 34 °C (Table 2.3). At 37 °C, the *M. anisopliae* isolates showed rosette like limited growth which reverted to normal growth pattern on reversion to lower temperature (28 °C). No growth was observed in plates at 40 °C and these plates when reverted to lower favorable temperature did not show any resumption of normal growth pattern. All the *M. anisopliae* isolates showed a 100 fold increase in spore yield at both 28 °C (control) and 30 °C but remained practically constant at 34 °C and 37 °C, *i.e.* 10^6 (the same value as the starting concentration used for spread plating). Isolates UM1, UM2, UM6, UM7 and AR1 showed appreciable growth and sporulation potential at almost all the stress temperatures studied.

The response of different *B. bassiana* isolates towards temperature stress was significant ($P < 0.05$). All the seventeen *B. bassiana* isolates showed mycelial growth at 28 °C and 30 °C only and the growth was more at 28 °C than at 30 °C (Table 2.4). Isolates UB2, UB6, UB8, UB9, UB15 and UB16 showed maximum relative radial growth (in comparison to other isolates) at both 28 °C and 30 °C. In case of other *B. bassiana* isolates, the mycelial growth at 30 °C decreased to an extent with increase in temperature. The *B. bassiana* isolates showed a 100 - fold increase (over the initial concentration of 10^6 spores/ isolate with which the experiment was started) in spore yield at both 28 °C (control) and 30 °C (Table 2.4). Like mycelial growth, the sporulation in *B. bassiana* isolates was more at 28 °C than at 30 °C. Isolate UB1, UB6 and UB16 showed maximum sporulation (in comparison to other isolates) at 28 °C and at 30 °C isolates UB2, UB6, UB8, UB9, UB 15 and UB16 showed highest sporulation compared to other isolates.

Germination (Temperature stress)

The effect of temperature on the *M. anisopliae* isolates was significant ($P< 0.05$), meaning, that the different isolates exhibited different germination potentiality with varying temperature. Isolates UM2, UM3 and AR1 were the best germinating isolates at all the temperatures studied and had more than 50 % of their conidia germinated even at 37 °C (Table 2.5). It was observed that the germination rate declined with increase in temperature. Isolates UM6, UM9, UM10, UM12 and UM13 were poor isolates as they did not show any initiation of germination at 8th hr of incubation. None of the isolates could germinate at 40 °C and when transferred to lower temperatures could not resume normal growth.

The *B. bassiana* isolates showed good germination at 28 °C and 30 °C (Table 2.6), the germination rate being faster at the former temperature. Isolates UB1 to UB6 and AB1 showed 100 % germination at 28 °C and 30 °C at 16th hr of incubation. Isolates AB1, UB3 and UB5 showed similar germination response at both 28 °C and 30 °C. At 12th hr of incubation at 34 °C, good pegs (size of pegs and their corresponding spores being almost equal) were seen in almost all the spores of UB5, UB6 and AB1. At 16th hr of incubation at 34 °C, very good pegs (size of pegs almost twice the size of corresponding spore) were seen in almost all the spores scored, of UB5 and AB1, but these did not germinate further, probably due to the desiccation of the nutrient source on which they were spread. None of the isolates could germinate at 37 °C and 40 °C. It was seen that the *B. bassiana* isolates were equally tolerant ($P > 0.05$) to temperature stress in context of the germination rate.

Germination (UV-B radiation stress)

The UV-B dependent germination in *M. anisopliae* (Table 2.7) isolates was significant ($P < 0.05$). The germination potential of each of the isolates showed significant variation ($P < 0.05$) in context to the UV-B exposure duration *i.e.* different germination rate at 0 (control), 1, 2, 3 and 4 hrs of UV-B exposure. Isolates UM2, UM3 and AR1 were the most robust candidates in response to UV-B radiation stress. Isolates UM6, UM9, UM10, UM12 and UM13 were poor isolates as they did not show any initiation of germination at 8th hr of incubation.

The *B. bassiana* isolates showed varying tolerance towards UV-B radiation stress and their response was significantly ($P < 0.05$) affected by the dose of UV-B exposure duration. The *B. bassiana* isolates exhibited appreciable germination at all the four UV-B exposure durations (Table 2.8) and did not vary much from the control setup (the aluminum foil wrapped tubes). Isolates UB1 to UB7, UB16 and AB1 showed more than 80 % effective germination at 1 hr of UV-B exposure at 16th hr of incubation. Isolate AB1 was the most robust candidate in response to UV-B radiation stress and was almost unaffected to changes in UV-B radiation dose. It was generally observed that the germination potential decreased with increase in UV-B radiation dose. Isolate UB5 was almost unaffected at one and two hrs of UV-B exposure but showed decline in germination potential at higher exposure durations. Isolate UB7 and UB12-UB15 did not initiate any germination at 8th hr of incubation and so these were considered as poor isolates. Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

2.4 Discussion

Abiotic stresses, notably extremes in temperature, photon irradiance, water stress and variable concentration of organic-inorganic solutes, frequently limit growth and pathogenic potential of entomopathogenic fungi (Inglis et al., 2001). In addition, more than one abiotic stress can occur at one time. Furthermore, one abiotic stress can decrease an organism's ability to resist a secondary stress or vice-versa (Rangel et al., 2006b). Great variations in germination, growth and sporulation parameters was observed as isolate response to UV-B radiation and temperature stress, as anticipated from previous reports (Braga et al., 2001; Rangel et al., 2005a). Certain isolates were appreciably robust in response to these abiotic stresses. Probably the physiology of tolerant isolates is more suited towards abiotic stresses and is thus indicative of their complex genetic base. The tolerant isolates exhibited variation in the phenotypic states depending on the stress stimuli and this capacity is supposed to be adaptive. Phenotypic plasticity exhibited by the entomopathogens was either a reversible or an irreversible plastic response, depending upon the reversion or persistence of the stress stimuli (Kassen and Rainey, 2004; Rangel et al., 2005b). In the present study, it was observed that all *M. anisopliae* isolates, showed a constant rosette like limited radial growth of 4.5 mm at 37 °C on all the days of observations, and this could revert to normal growth pattern on reversion to lower temperature (Figure 2.2). On the other hand, *B. bassiana* isolates which could not show any biological activity at 34 °C and above exemplifies an irreversible change at either persistence and/ or reversion of temperature stress stimulus.

Abiotic stress tolerance is a polygenically determined attribute. Resilience to abiotic stress by inherent polygenic mechanisms is disadvantageous in terms of a rather complex genetic manipulation strategy for strain improvement. Consequently *in vitro* assays of abiotic stress inducers, for strain selection (in lieu of strain improvement) offers immediate solutions for identifying robust isolates and considering them for further commercial usage (St. Leger and Screen, 2001). The generalization, and its converse, that an increment in abiotic stress dose (above the condition optima) decrements the biological activities (*viz.* germination, growth and sporulation) holds true in *in - vitro* assays on *M. anisopliae* and *B. bassiana* isolates. Hedgecock et al., 1995 have reported that temperature is a key factor for conidial viability and longevity of *M. anisopliae*. James et al., 1998 reported that *B. bassiana* could grow most rapidly at a continuous temperature of 25 - 32 °C, but at temperatures higher than 32 ± 1 °C, germination was delayed and/ or decreased in some isolates. Results of the present study have shown that the *M. anisopliae* isolates hold an edge over the *B. bassiana* isolates for temperature tolerance. The *B. bassiana* isolates germinated and grew at 28 °C and 30 °C; and this is almost consistent with James et al., 1998. All the *M. anisopliae* isolates exhibited conidial germination, mycelial growth and sporulation up to a maximum temperature of 37 °C which was 3 °C more than the tolerance limit of *B. bassiana* isolates; which is in consistence with the observations of Thomas and Jenkins, 1997.

Studies on UV-B tolerance suggest that with increasing exposure time, the rate of conidial germination declines (Rangel et al., 2006a). Fargues et al., 1996 observed that conidia of *M. flavoviride* were generally more resistant to artificial UV-B irradiance, followed by conidia of *B. bassiana*, *M. anisopliae* and *Paecilomyces fumosoroseous.* In our study, with increase in UV-B exposure duration a corresponding decrease in conidial germination was observed in case of *B. bassiana* isolates but this decrease was not significant. On the other hand most of the *M. anisopliae* isolates were susceptible to the increasing dose of UV-B irradiance. It is essential that the entomopathogenic fungi must on priority survive on-field application and also retain activity in the environment. One of the major impending challenges that the entomopathogens face is survival against fluctuating physical environment. Consequently the artificial *in vitro* based assays for screening and selection, should adopt an ecological approach that takes into account the features of the environment in which it is intended to be used (Deacon, 1991). The northeastern part of India (where the work was carried out) encounters great fluctuations in day-night temperatures and solar radiations. The summer months witness diurnal temperature range of 35 – 40 °C, which drops by an amplitude of 5 °C at night. While during the winter months, the atmospheric temperature varies between 20 – 25 °C in the daytime and 10 - 15 °C at night. Also during daytime, the sunlight has UV-B component acting in conjunction with the solar temperature. From this study, it can be concluded that *B. bassiana* isolates are flexible in the sense of application time, as they were not significantly affected by change in UV-B dose. On the other hand, the *M. anisopliae* isolates could tolerate temperatures from 28 °C up to 37 °C and this supports their candidature for application during winter and summer seasons. But as far as their application time is concerned, most isolates can be applied after sunset, thus allowing suitable time for their acclimatization. On coming in contact with the insect cuticle, the most desirable trait for an entomopathogen is its rate of germination which facilitates its growing into the insect haemocoel. As a conclusion of this study, isolate AR1 (*M. anisopliae*) which showed around 88 % germination rate for up to 37 °C and could tolerate up to 4 hrs of UV-B radiation exposure, seems to be a promising candidate for field use.

2.5 Conclusions

The *M. anisopliae* isolates hold an edge over the *B. bassiana* isolates for temperature tolerance. The *B. bassiana* isolates germinated and showed growth and sporulation at 28 °C and 30 °C. All the *M. anisopliae* isolates exhibited conidial germination, mycelial growth and sporulation up to a maximum temperature of 37 °C which was 3 °C more than the tolerance limit of *B. bassiana* isolates. Isolates AR1 and UM2 were judged to be the better isolates.

Table 2.1: Source of *M. anisopliae* isolates

Isolates	**Code no. / Accession no. ARSEF/ Local**	**Host insect**	**Geographical location**
UM1	ARSEF 1745	*Nilaparvata lugens*	India
UM2	ARSEF 2735	*Spodoptera* sp.	Philippines
UM3	ARSEF 2153	*Nephotettix virescens*	Indonesia
UM4	ARSEF 2424	Lepidoptera larva	Indonesia
UM5	ARSEF 3210	Coleoptera	India
UM6	ARSEF 2596	*Pyrausta machaeralis*	India
UM7	ARSEF 1080	*Helicoverpa zea*	USA
UM8	ARSEF 1724	*Nilaparvata lugens*	India
UM9	ARSEF 1727	*Nilaparvata lugens*	India
UM10	ARSEF 3295	*Anticarsia gemmatalis*	Mexico
UM11	ARSEF 1729	*Nilaparvata lugens*	India
UM12	ARSEF 1744	*Nilaparvata lugens*	India
UM13	ARSEF 1823	*Nilaparvata lugens*	India
AR1	Local	Unknown	India

Table 2.2: Source of *B. bassiana* isolates

Isolates	**Code no. / Accession no. ARSEF/ Local**	**Host insect**	**Geographical location**
UB1	ARSEF 1788	*Helicoverpa virescens*	Spain
UB2	ARSEF 2041	*Cnaphalocrocis medinalis*	Philippines
UB3	ARSEF 5278	*Bemicia tabaci*	USA
UB4	ARSEF 2417	*Emmalocera depressella*	India
UB5	ARSEF 2597	*Hyblaea puer*	India
UB6	ARSEF 6646	*Spodoptera litura*	India
UB7	ARSEF 4027	*Coccinella septumpunctata*	Denmark
UB8	ARSEF 1166	*Helicoverpa armigera*	Spain
UB9	ARSEF 2033	*Coccinella* sp.	USA
UB10	ARSEF 2034	*Coccinella* sp.	USA
UB11	ARSEF 4018	Coccinella *septempunctata*	Denmark
UB12	ARSEF 1886	*Chilo infuscatellus*	India
UB13	ARSEF 2412	*Xyloryctes jamaicensis*	India
UB14	ARSEF 8250	*Basilepta fulvicornis*	India
UB15	ARSEF 6650	*Spodoptera litura*	India
UB16	ARSEF 2660	Adult Coleoptera	India
AB1	Local	Unknown	India

Table 2.3: Temperature dependent growth and sporulation of *M. anisopliae* isolates

Isolates	**28 ^{0}C**		**30 ^{0}C**		**34 ^{0}C**		**37 ^{0}C**	
	Growth rate (mmd^{-1})	Sporulation (x10^6)	Growth rate (mmd^{-1})	Sporulation (x10^6)	Growth rate (mmd^{-1})	Sporulation (x10^6)	Growth rate (mmd^{-1})	Sporulation (x10^6)
UM1	2.04^{bc}	15.5^{de}	2.02^{b}	14.5^{cd}	1.92^{b}	1.30^{cd}	1.50^{bcd}	1.05^{bcdef}
UM2	1.97^{cd}	19.5^{cd}	1.98^{b}	18.5^{bc}	1.70^{cd}	1.65^{bc}	1.73^{b}	1.45^{bcd}
UM3	1.73^{e}	15.5^{de}	1.68^{c}	14.0^{cd}	1.59^{d}	1.25^{cd}	1.59^{bcd}	1.15^{bcde}
UM4	1.16^{h}	25.0^{c}	1.00^{f}	23.0^{b}	0.95^{f}	1.95^{b}	1.11^{ef}	1.55^{b}
UM5	1.68^{ef}	23.5^{c}	1.61^{d}	22.5^{b}	1.57^{d}	2.05^{b}	1.00^{f}	1.50^{bc}
UM6	3.11^{a}	35.0^{b}	3.00^{a}	33.5^{a}	2.80^{a}	0.50^{ef}	2.10^{a}	0.40^{f}
UM7	2.10^{b}	22.0^{c}	2.00^{b}	20.0^{bc}	2.00^{b}	2.05^{b}	1.67^{bc}	1.40^{bcd}
UM8	0.61^{i}	11.5^{e}	0.57^{g}	11.0^{d}	0.54^{g}	0.95^{def}	0.54^{g}	0.80^{cdef}
UM9	1.52^{g}	09.5^{e}	1.51^{de}	09.0^{d}	1.27^{e}	0.90^{def}	1.00^{f}	0.75^{def}
UM10	1.90^{d}	48.0^{a}	1.95^{b}	38.5^{a}	1.85^{bc}	1.20^{cd}	1.40^{cde}	0.95^{bcdef}
UM11	1.55^{g}	14.0^{de}	1.55^{de}	12.0^{d}	1.50^{d}	1.10^{cde}	1.00^{f}	1.00^{bcdef}
UM12	1.59^{fg}	13.0^{e}	1.56^{de}	11.5^{d}	1.53^{d}	1.00^{def}	1.18^{ef}	0.55^{ef}
UM13	1.49^{g}	12.0^{e}	1.49^{e}	10.5^{d}	1.47^{d}	0.45^{f}	1.30^{def}	0.40^{f}
AR1	1.52^{g}	38.0^{b}	1.51^{de}	35.5^{a}	1.50^{d}	2.90^{a}	1.29^{def}	2.35^{a}

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

Table 2.4: Temperature dependent growth and sporulation of *B. bassiana* isolates

Isolates	**28 ^{0}C**		**30 ^{0}C**	
	Growth rate (mmd^{-1})	Sporulation (x10^{7})	Growth rate (mmd^{-1})	Sporulation (x10^{7})
UB1	1.00^{d}	3.20^{ab}	1.00^{f}	3.30^{a}
UB2	1.53^{ab}	2.95^{bc}	1.51^{ab}	2.95^{a}
UB3	1.06^{d}	2.10^{de}	1.26^{cd}	2.20^{bc}
UB4	1.14^{cd}	2.55^{cd}	1.07^{ef}	2.50^{b}
UB5	1.40^{b}	2.95^{bc}	1.37^{bc}	3.00^{a}
UB6	1.59^{a}	3.45^{a}	1.59^{a}	3.40^{a}
UB7	0.75^{e}	1.80^{ef}	0.75^{f}	1.80^{cd}
UB8	1.50^{ab}	0.90^{h}	1.50^{ab}	0.90^{f}
UB9	1.54^{ab}	1.50^{fg}	1.50^{ab}	1.50^{de}
UB10	1.18^{cd}	2.05^{e}	1.14^{def}	2.00^{c}
UB11	1.40^{b}	1.15^{gh}	1.34^{bc}	1.15^{ef}
UB12	1.14^{cd}	0.95^{h}	1.14^{def}	0.95^{f}
UB13	1.00^{d}	1.00^{h}	1.04^{ef}	1.00^{f}
UB14	1.00^{d}	1.15^{gh}	1.00^{f}	1.15^{ef}
UB15	1.61^{a}	2.20^{de}	1.50^{ab}	2.20b^{c}
UB16	1.49^{ab}	3.05^{ab}	1.49^{ab}	3.05^{a}
AB1	1.23^{c}	2.10^{de}	1.21^{cde}	2.05^{c}

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

Table 2.5: Temperature dependent percentage germination of *M. anisopliae* isolates

Isolates	**28 ^{0}C**		**30 ^{0}C**		**34 ^{0}C**		**37 ^{0}C**	
	8th h	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**
UM1	42.09^{f}	100^{a}	39.55^{e}	100^{a}	23.20^{d}	61.20^{e}	18.64^{d}	57.80^{c}
UM2	97.00^{a}	100^{a}	94.60^{a}	100^{a}	75.78^{a}	97.60^{ab}	0^{e}	31.80^{d}
UM3	87.51^{c}	100^{a}	89.31^{b}	100^{a}	70.54^{b}	89.80^{c}	0^{e}	0^{h}
UM4	65.14^{d}	100^{a}	64.74^{c}	100^{a}	51.11^{c}	99.70^{a}	42.20^{b}	66.94^{b}
UM5	23.40^{g}	100^{a}	23.40^{f}	100^{a}	21.40^{d}	95.20^{ab}	0^{e}	32.10^{d}
UM6	0^{i}	21.80^{g}	0^{h}	21.80^{g}	0^{f}	21.00^{h}	0^{e}	6.20^{g}
UM7	14.00^{h}	100^{a}	14.00^{g}	100^{a}	14.00^{e}	94.00^{bc}	0^{e}	5.40^{g}
UM8	22.00^{g}	33.30^{e}	22.00^{f}	33.30^{e}	22.00^{d}	32.70^{g}	0^{e}	4.60^{g}
UM9	0^{i}	52.80^{d}	0^{h}	52.80^{d}	0^{f}	50.60^{f}	0^{e}	9.00^{f}
UM10	0^{i}	67.27^{c}	0^{h}	67.27^{c}	0^{f}	64.27^{e}	0^{e}	13.00^{e}
UM11	50.80^{e}	87.10^{b}	50.80^{d}	87.10^{b}	50.80^{c}	84.40^{d}	35.20^{c}	32.00^{d}
UM12	0^{i}	26.60^{f}	0^{h}	26.60^{f}	0^{f}	26.00^{h}	0^{e}	9.60^{f}
UM13	0^{i}	22.35^{g}	0^{h}	22.35^{g}	0^{f}	22.00^{h}	0^{e}	15.12^{e}
AR1	92.40^{b}	100^{a}	93.00^{a}	100^{a}	69.80^{b}	100^{a}	57.60^{a}	87.60^{a}

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

Table 2. 6: Temperature dependent percentage germination of *B. bassiana* isolates

Isolates	**28 ^{0}C**		**30 ^{0}C**	
	8th h	**16th h**	**8th h**	**16th h**
UB1	31.93^{d}	100^{a}	25.24^{g}	100^{a}
UB2	37.55^{c}	100^{a}	32.30^{d}	100^{a}
UB3	38.14^{c}	100^{a}	37.17^{c}	100^{a}
UB4	25.75^{f}	100^{a}	30.72^{de}	100^{a}
UB5	42.51^{b}	100^{a}	41.37^{b}	100^{a}
UB6	29.50^{e}	100^{a}	27.06^{f}	100^{a}
UB7	0^{i}	91.60b	0^{j}	91.60^{b}
UB8	2.20^{hi}	53.50^{e}	1.80^{ij}	53.50^{e}
UB9	2.60^{h}	66.80^{d}	2.60^{i}	66.80^{d}
UB10	6.80^{g}	35.34^{h}	7.80^{h}	35.34^{h}
UB11	7.60^{g}	27.58^{i}	8.80^{h}	27.58^{i}
UB12	0^{i}	47.94^{f}	0^{j}	47.94^{f}
UB13	0^{i}	45.10^{fg}	0^{j}	45.10^{fg}
UB14	0^{i}	42.70^{g}	0^{j}	42.70^{g}
UB15	0^{i}	46.00^{f}	0^{j}	46.00^{f}
UB16	30.10^{e}	81.60^{c}	30.10^{e}	81.60^{c}
AB1	60.22^{a}	100^{a}	58.80^{a}	100^{a}

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

Table 2.7: UV-B dependent percentage germination of *M. anisopliae* isolates

Isolates	**Control**		**1 h exposure**		**2 h exposure**		**3 h exposure**		**4 h exposure**	
	8th h	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**
UM1	43.09^{e}	100^{a}	42.22^{e}	99.90^{a}	40.20^{e}	96.70^{a}	30.00^{f}	82.40^{b}	18.80^{f}	65.20^{c}
UM2	96.36^{a}	100^{a}	95.80^{a}	100^{a}	92.80^{a}	100^{a}	89.80^{a}	100^{a}	89.40^{a}	100^{a}
UM3	88.69^{b}	100^{a}	88.80^{b}	100^{a}	87.40^{b}	100^{a}	76.60^{c}	100^{a}	73.60^{c}	100^{a}
UM4	63.57^{c}	100^{a}	63.39^{c}	100^{a}	61.99^{c}	100^{a}	50.60^{d}	83.60^{b}	43.60^{d}	76.60^{b}
UM5	23.00^{f}	88.60^{b}	22.40^{f}	84.60^{b}	19.50^{f}	82.80^{b}	7.40^{g}	67.40^{c}	0^{g}	56.00^{d}
UM6	0^{i}	21.80^{h}	0^{i}	21.20^{h}	0^{i}	20.40^{i}	0^{i}	4.20^{h}	0^{g}	0^{i}
UM7	13.40^{h}	100^{a}	12.40^{h}	70.20^{c}	10.20^{h}	66.80^{d}	2.80^{h}	38.60^{d}	0^{g}	25.60^{e}
UM8	19.40^{g}	32.10^{f}	17.80^{g}	31.90^{f}	14.20^{g}	31.50^{g}	4.20^{h}	25.60^{f}	0^{g}	15.00^{g}
UM9	2.60^{i}	52.40^{e}	2.60^{i}	50.60^{e}	2.00^{i}	47.20^{f}	0.60^{i}	29.60^{e}	0^{g}	22.00^{f}
UM10	0^{i}	66.72^{d}	0^{i}	65.70^{d}	0^{i}	63.60^{e}	0^{i}	25.00^{f}	0^{g}	16.20^{g}
UM11	51.00^{d}	85.20^{c}	50.20^{d}	84.20^{b}	46.40^{d}	79.60^{c}	36.00^{e}	69.00^{c}	25.40^{e}	55.80^{d}
UM12	0^{i}	26.00^{g}	0^{i}	24.50^{g}	0^{i}	23.30^{h}	0^{i}	10.60^{g}	0^{g}	5.60^{h}
UM13	0^{i}	21.00^{h}	0^{i}	20.60^{h}	0^{i}	19.00^{i}	0^{i}	9.40^{g}	0^{g}	0.80^{i}
AR1	95.37^{a}	100^{a}	94.30^{a}	100^{a}	91.30^{a}	100^{a}	85.80^{b}	100^{a}	84.20^{b}	100^{a}

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

Table2. 8: UV-B dependent percentage germination of *B. bassiana* isolates

Isolates	**Control**		**1 h exposure**		**2 h exposure**		**3 h exposure**		**4 h exposure**	
	8th h	16th h	8th h	16th h	8th h	16th h	8th h	16th h	8th h	16th h
UB1	30.63^{e}	100^{a}	31.40^{d}	100^{a}	29.09^{d}	100^{a}	27.16^{d}	100^{a}	21.53^{d}	99.20^{a}
UB2	35.49^{d}	100^{a}	0^{j}	100^{a}	0^{g}	100^{a}	0^{h}	100^{a}	0^{i}	99.80^{a}
UB3	39.41^{c}	100^{a}	36.24^{c}	100^{a}	31.51^{c}	100^{a}	29.19^{c}	100^{a}	28.00^{c}	98.80^{a}
UB4	26.87^{f}	100^{a}	27.27^{f}	100^{a}	21.49^{e}	100^{a}	16.40^{f}	97.60^{a}	14.60^{f}	96.80^{a}
UB5	44.96^{b}	100^{a}	44.40^{b}	100^{a}	40.72^{b}	100^{a}	32.99^{b}	100^{a}	29.62^{b}	100^{a}
UB6	23.50^{g}	100^{a}	23.72^{g}	100^{a}	22.24^{e}	100^{a}	19.78^{e}	98.80^{a}	16.58^{e}	98.40^{a}
UB7	0^{j}	90.80^{b}	0^{j}	88.80^{b}	0^{g}	88.20^{b}	0^{h}	86.80^{b}	0^{i}	86.40^{b}
UB8	2.20^{ij}	53.30^{e}	2.20^{i}	53.30^{e}	2.20^{g}	52.30^{e}	2.20^{h}	51.40^{e}	2.20^{h}	51.00^{e}
UB9	2.60^{i}	64.80^{d}	2.60^{i}	64.60^{d}	2.60^{g}	62.80^{d}	2.60^{h}	61.00^{d}	2.60^{h}	60.60^{d}
UB10	6.80^{h}	34.94^{h}	6.80^{h}	34.94^{g}	6.80^{f}	34.34^{g}	6.80^{g}	33.54^{g}	6.80^{g}	32.74^{g}
UB11	7.60^{h}	27.60^{i}	7.60^{h}	26.80^{h}	7.60^{f}	26.10^{h}	7.60^{g}	26.10^{h}	7.60^{g}	25.50^{h}
UB12	0^{j}	46.54^{f}	0^{j}	45.94^{f}	0^{g}	45.04^{f}	0^{h}	44.34^{f}	0^{i}	43.94^{f}
UB13	0^{j}	44.10^{fg}	0^{j}	44.30^{f}	0^{g}	43.74^{f}	0^{h}	43.34^{f}	0^{i}	43.14^{f}
UB14	0^{j}	42.30^{g}	0^{j}	42.70^{f}	0^{g}	42.10^{f}	0^{h}	42.10^{f}	0^{i}	41.80^{f}
UB15	0^{j}	45.00^{fg}	0^{j}	44.80^{f}	0^{g}	44.20^{f}	0^{h}	43.80^{f}	0^{i}	43.80^{f}
UB16	30.10^{e}	79.40^{c}	30.10^{e}	78.60^{c}	30.10^{cd}	78.40^{c}	30.10^{c}	77.40^{c}	30.10^{b}	77.20^{c}
AB1	47.42^{a}	98.00^{a}	46.62^{a}	97.40^{a}	47.42^{a}	96.30^{a}	46.41^{a}	95.70^{a}	48.08^{a}	95.00^{a}

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul’s multiple range test.

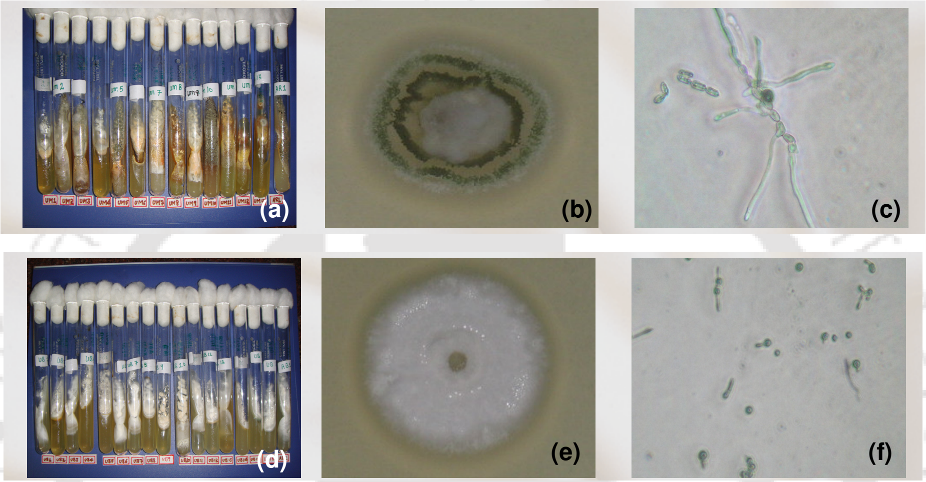


Fig.2.1. (a). The *M. anisopliae* cultures (UM1-13, AR1) (b).Green colony of *M. anisopliae* (c).The oval-shaped germinating spores of *M. anisopliae* (10X magnification) (d).The *B. bassiana* cultures (UB1-16, AB1) (e).White colony of *B. bassiana* (f).The round germinating spores of *B. bassiana* (10X magnification).

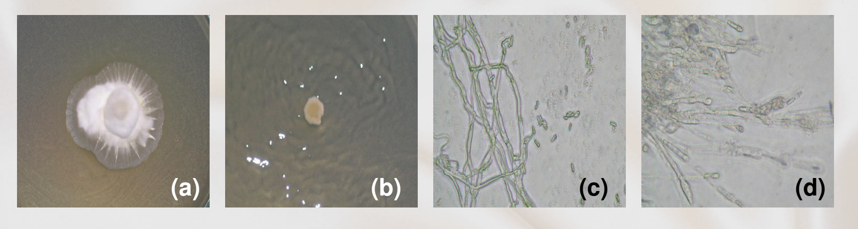


Figure 2.2. (a).Normal colony formation at 28°C (b).Restricted colony growth at 37°C (c).Normal mycelial growth showing conidia formation at 28°C (d).Globose mycelium with restricted vegetative activity at 37°C.

3.1 Introduction

The last century has been witness to both the keynote augmentation of the chemical pesticides as well as its backlashes, so much so, that the development of entomopathogenic fungi as environmentally friendly alternative is fast gaining momentum (Inglis et al., 2001). Several microorganisms are currently under consideration as control agents of insects, with particular emphasis on *Metarhizium anisopliae* and *Beauveria bassiana*, probably because of their high host-specificity, non-persistence and non-toxicity to environment, unique mode of action and appreciable shelf life. Mycelial forms that bear asexual resting spores called ‘conidia’ that serves as the infective propagule characterizes them. Good indicators of virulence include factors like germination rate, growth and sporulation (Chandler et al., 1993; Altre et al., 1999). It has been reported that nutrient source is an integral determinant of growth and virulence of entomopathogenic fungi (Shah et al., 2005). Nutrients are substances used in biosynthesis and energy release and therefore serve as cardinal impetus towards the viability, survival and sustainance of any organism (Safavi et al., 2007). For elite cultivation of microorganism, it is imperative to have an in-depth knowledge of their nutritional requirements. The macro-elements like carbon, oxygen, hydrogen, nitrogen, sulfur and phosphorus are integral components of carbohydrates, lipids, proteins and nucleic acids and these metabolically active groups are directly/ indirectly involved in host-pathogen interaction and self-defense and perpetuation mechanisms. Carbon is required as the skeletal element of all organic molecules, and molecules serving as carbon sources normally also contribute both oxygen and hydrogen. Gao et al. (2007) studied the effect of different nutrient sources on the growth and sporulation of several biocontrol agents. The mycelial growth and spore yield on artificial media depends upon the fungal isolates and the components used in the culture media. Although saprophytic fungi are able to utilize a wide range of nutrient sources (Liu and Chen, 2002), simple and less expensive media are needed to permit their mass-production and commercialization (Shah et al., 2005). For commendable flourishment, the microorganism must be able to incorporate appreciable quantities of nitrogen, phosphorus and sulfur, which are the essential ingredients of growth factors viz., amino acids, purines and pyrimidines and vitamins. Although these elements may be acquired from the same sources that supply carbon, the microbes have the capacity of employing inorganic sources as well (Li and Holdom, 1995). The CN ratio is said to significantly affect the number of conidia produced and conidial characteristics of *Colletotrichum truncatum* (Jackson and Bothast, 1990; Jackson and Schisler, 1992; Jackson and Slininger 1993). Shah et al. (2005) studied the influence of nutrition on growth and virulence of *M. anisopliae*. Li and Holdom (1995) characterized two *M. anisopliae* isolates on basis of various carbohydrate and nitrogen supplements. Doust and Roberts (1983) studied the effect of growth substrates on conidial viability and virulence. Engelkes et al. (1997) reported the effect of CN ratio on growth, sporulation and biocontrol efficacy of *Taloromyces flavus*. An understanding of the growth characteristic with respect to the growth substrates shall also be handy in tolerance-selection studies.

Laboratory culture medium be it in solid (Shah and Butt, 2005) or liquid form (Adour et al., 2002), are preparations used to grow, transport and store microorganisms. To be effective, the medium must contain all the nutrients, the microorganisms require for growth. This study on variable nutrient source had been conducted for fourteen *M. anisopliae* and seventeen *B. bassiana* isolates so as to evaluate the fungi for their conidial germination, mycelial growth and sporulating potential on different media containing carbon and nitrogen sources and ratios. The culture media which supports better germination, growth and sporulation promises to be effective for low cost development of biopesticides. The cardinal objective of this work has been the development of cost effective methods for fungal propagation so as to yield effective inoculum levels towards mass production and formulation studies.

3.2 Materials and Methods

Fungal cultures

The different isolates of the fungus *M. anisopliae* and *B. bassiana* were either procured from ARSEF (USDA-ARS Plant Protection Unit) or isolated locally in India (Table 2.1 & 2.2). The isolates were routinely sub cultured on SDA (Sabouraud dextrose Agar) slants at 28 °C in incubators and maintained at 4 ^{o}C.

Culture media

Culture media representing disparate carbon and nitrogen sources and ratios were used in this study. They included 1) Sabouraud Dextrose Agar (SDA) with intermediate CN ratio (35 : 1) consisting of glucose (4 %) and peptone (1 %) 2) low

CN (10 : 1) medium consisting of glucose (0.6 %) and peptone (1 %) 3) high CN source (75 : 1) consisting of glucose (9.1 %) and peptone (1 %) 4) nutrient poor media, peptone (2 %) with CN ratio (8 : 1) 5) Yeast extract (1 %) with CN ratio (3.6 : 1), 6) Potato Dextrose Agar (PDA) with CN ratio (10 : 1) and finally 7) 'osmotic stress' medium (OSM) consisting of glucose (8 %), peptone (2 %), KCl (5.5 %) and an exceptionally higher level of agar (5.5 %) for solidification. The rest of the media were solidified in agar (2 %). The pH of all the media was maintained at 7.0 and was sterilized at 121 ^{0}C at 15 psi for 15 min and 15 ml poured into 9 cm diameter Petri plates.

Assay of germination

Agar slide technique was used for studying the rate of germination. Petri plates were lined with blotting paper discs and 2 glass slides were placed in each of the plates and autoclaved. 1 ml medium was evenly spread on each of the glass slides using micropipette. Conidial suspension was prepared from seven to ten day old cultures with concentrations maintained at 10^6 conidia/ ml. About 100 µl of 10^6 spores of fungal isolates were spread on the different media coated slides. The slides were placed back in the Petri plates and the blotting paper discs were moistened with sterilized water. The Petri plates were kept for incubation at 28 °C. The slides were observed under compound microscope (40X) for germination, every 2 h starting after 3 h. A conidium was considered germinated when a distinct germ tube projected from it, and was at least twice the diameter of the conidium (Milner *et al.,* 1991). Approximately 300 conidia were scored per replicate for each of the treatments.

Assay of colony growth and sporulation

Seven day old cultures on SDA slants were used for preparing spore suspension in 0.02 % Tween 80 solution at 1 x 10^6 spores/ ml. 200 µl of 10^6 spores were spread plated on SDA medium and were incubated for 3 d at 28 °C. At the end of 3 d, 5 mm agar disc with mycelia was retrieved with the help of a cork borer and placed in middle of fresh 9 cm Petri plates and incubated at 28 °C. This was done for all the nutrient source media and five replicates were maintained for each media and for each isolate. Radial growth was measured every 2nd day till 8th day. Radial growth rate (mmd^{-1}) was calculated from the linear portions of the curves plotted from these values. At end of 8th day, 5 mm agar discs were randomly taken with the help of a cork borer. The discs were placed in 10 ml of 0.02 % (v/v) Tween 80 solution and vortexed to suspend the spores. Spore yield was determined using a Haemocytometer.

Statistical analysis

Statistical analysis of all the data for fungal growth, sporulation and germination were subjected to one-way analysis of variance (ANOVA) and the means were separated by Student-Newman-Keuls multiple range test of comparisons of means at P= 0.05.

3.3 Results

The effect of different carbon and nitrogen sources on the conidial germination, growth and sporulation of *M. anisopliae* and *B. bassiana* isolates showed variations among the isolates studied. The results are presented in Tables 3.1 and 3.2 (*M. anisopliae*) and 3.3 and 3.4 (*B. bassiana*).

Effect of CN sources and ratio on germination potential of *M. anisopliae* and *B. bassiana* isolates

Germination potential of *M. anisopliae* isolates on Sabouraud Dextrose Agar (CN= 35: 1) showed isolate UM2 with 75 % germination potential at 8th hr of incubation amongst the 14 isolates of *M. anisopliae* studied. This was followed by isolate UM3, UM4 and AR1 with germination potential of ≥ 65 %. All of these isolates showed 100 % germination potential at 16th hr of incubation. Amongst the 17 isolates studied for *B. bassiana*, isolate AB1 was identified to have up to 62 % germination potential at 8th hr of incubation. This was followed by isolate UB2 and UB5 with germination potential of about 40 %. In case of low CN media (CN= 10: 1), *M. anisopliae* isolate UM2 exhibited germination potential of more than 70 % at 8th hr incubation. This was followed by germination rate of > 60 % by isolate UM4 and > 50 % by isolate AR1. Among the *B. bassiana* isolates, isolate AB1 exhibited germination potential of more than 50 % at 8th hr of incubation. This was followed by germination rate of > 45 % by isolate UB2 and ≥ 40 % by isolate UB5. For High CN source (CN= 75 : 1), isolates UM2 and UM4 with germination rate of > 60 % at 8th hr incubation, was followed by isolates UM3 and UM11 with germination rate of > 50 %, and isolate UM1, UM5 and AR1 with > 40 % germination potential among the *M. anisopliae* isolates. All these isolates exhibited an appreciable germination in the range of 75 – 100 % at 16th hr of incubation. Among the *B. bassiana* isolates, isolate AB1 with germination rate of ≥ 56 % at 8th hr incubation, was followed by isolate UB5 and UB3 with germination rate of about 40 %. For the media containing Yeast extract (1 %) (CN= 3.6: 1), isolate UM4 showed maximum germination rate of 65 % at 8th hr of incubation among the *M. anisopliae* isolates. Isolates UM3 and UM11 exhibited > 50 % germination potential and this was followed by isolate UM1, UM5 and AR1 with around 40 % germination at 8th hr of incubation. Among the *B. bassiana* isolates, isolate AB1 showed maximum germination rate of 67 % at 8th hr of incubation. Isolates UB2 and UB16 exhibited > 40 % germination potential at 8th hr of incubation, and had all their conidia germinated at 16th hr observation. In the medium containing Peptone (2 %) (CN= 8 : 1), it was observed that isolate UM2 and AR1 had germination rate > 60 %, isolates UM3, UM4 and UM5 with around 50 % germination rate and isolate UM1 with 40 % germination potential among the *M. anisopliae* isolates at 8th hr of incubation. Among the *B. bassiana* isolates at 8th hr of incubation, it was observed that isolate UB16 and AB1 had germination rate > 45 %, followed by isolates UB5 with 40 % germination potential. Among the *M. anisopliae* isolates at 8th hr of incubation, it was observed that isolate UM2 exhibited > 70 % germination potential, followed by isolate UM4 and AR1 with > 50 % germination rate and isolate UM3 with germination potential of > 45 % for Potato Dextrose Agar (CN= 10 : 1). For isolate UM9, PDA was a stress media. Among the *B. bassiana* isolates at 8th hr of incubation, it was observed that isolate AB1 exhibited > 45 % germination potential, followed by isolate UB16 and UB5 with > 40 % germination rate and isolate UB3 with germination potential of > 35 %; all these isolates showed complete germination at 16th hr of incubation. For Osmotic Stress media, among the *M. anisopliae* isolates at 8th hr of incubation, isolate UM1 and UM2 demonstrated > 60 % germination potential, followed by isolate UM3 and UM4 with around 50 % and isolate UM5 and AR1 with > 40 % germination potential. Among the *B. bassiana* isolates at 8th hr of incubation, isolate AB1 demonstrated > 50 % germination potential, followed by isolate UB3 with around 40 % and isolate UB5 with > 38 % germination.

Effect of CN sources and ratio on growth rate and spore yield of *M. anisopliae* and *B. bassiana* isolates

The study on colony growth rate on SDA (CN= 35 : 1), showed isolates UM2, UM3 UM4 and AR1 exhibiting an appreciable growth rate of ≥ 1.5 mmd^{-1} and spore yield in the varying range of 1-6 x 10^7 spores/ml at 8th day of incubation. Isolate UM6 though showed profuse growth rate of 2.6 mmd^{-1} which was considered to be highest on SDA media for the isolates studied, had less than 50 % of its conidia germinated at 16th hr of incubation. The colony growth rate of the good germinating *B. bassiana* isolates like UB2, UB5 and AB1 also exhibited an appreciable colony growth rate of ≥ 1.0 mmd^{-1}

and spore yield in the varying range ≥ 2 x 10^7 spores/ ml at 8th day of incubation. Isolate UB15 though showed profuse growth rate (1.67 mmd^{-1}) which was considered to be highest on SDA media among isolates studied, had none of its conidia germinated at 16th hr of incubation and so it was not considered to be good candidate. The growth rate in the low CN source (CN= 10 : 1) showed isolates UM2, UM4 and AR1 in the range 1.5 - 2.0 mmd^{-1} but highly varying spore yield (1-6 x 10^7 spores/ ml). Isolate UM10 and AR1 showed similar sporulation potential of 5.5 x 10^7 spores/ ml. Here again isolate UM6 showed maximum growth rate (2.58 mmd^{-1}) but had poor germination potential (< 50 %) even at 16th hr of incubation. The growth rate of isolate AB1, UB2 and UB5 were in the range 1.1 - 1.7 mmd^{-1} and spore yield of 3-4 x 10^7 spores/ ml. Isolate UB15 showed maximum growth rate of 3.0 mmd^{-1} but it did not initiate any germination at 8th hr of incubation. Isolate UB6 showed best spore yield of 4.40 x 10^7 spores/ ml but had poor germination rate of only 26 % at 8th hr of incubation. The isolates UM1, UM2, UM4 and AR1 exhibited growth rate in the range 1 - 2 mmd^{-1} and variable sporulation potential (1 - 4.5 x 10^7 spores/ ml) in high CN source (CN= 75 : 1). Isolate UM8 and UM9, for which SDA and low CN source was a stress media, showed some amount of growth on high CN nutrient source. Isolate UM6 and UM7 had an exceptionally high growth rate (> 2.0 mmd^{-1}) and spore yield (≥ 2.0 x 10^7 spores/ ml) on 8th day of incubation, but had poor germination potential. Isolate UB12 had maximum growth rate of 1.85 mmd^{-1} but showed poor germination and spore yield. In the media containing Yeast extract (1 %) (CN= 3.6 : 1), the growth rate of all the isolates studied was quite appreciable in the range 1.5 - 2.5

mmd^{-1} with the only exception of isolates UM8 and UM9 for which this media was nutrient poor. The spore yield was maximum for isolate UM10 (5.60 x 10^{7} spores/ ml), followed by isolates UM1-7 and AR1 in the range of 1.25 - 3.75 x 10^{7} spores/ ml. The growth rate of all the *B. bassiana* isolates studied was quite variable in the range 0.7 - 3.0 mmd^{-1}, with maximum growth rate shown by isolate UB14. Isolate UB15 sporulated best on Yeast extract (1 %) and this was followed by isolate UB9. All the *M. anisopliae* isolates studied showed an appreciable growth rate in the range 1.0 - 2.5 mmd^{-1}, with maximum growth potential shown by isolate UM6 in Peptone (2 %) (CN= 8 : 1). The best sporulating isolate was UM10 (5.8 x 10^{7} spores/ ml) followed by isolates UM1 (4.4 x 10^{7} spores/ ml) and AR1 (3.6 x 10^{7} spores/ ml). All the *B. bassiana* isolates studied, showed an appreciable growth rate in the range 1.5 - 3.0 mmd^{-1}, with maximum growth potential shown by isolate UB15. The best sporulating isolate was UB2 (6.02 x 10^{7} spores/ ml) followed by isolates UB15 (5.65 x 10^{7} spores/ ml) and UB8 (5.0 x 10^{7} spores/ ml). For Potato Dextrose Agar (CN= 10 : 1), all *M. anisopliae* isolates showed good growth rate in the range 0.8 - 2.0 mmd^{-1}, the best being for isolate UM6 (1.90 mmd^{-1}), followed by isolate UM3, UM4, UM5, UM10 and AR1 ($\geq$ 1.4 mmd^{-1}). Isolates UM10 and AR1 exhibited maximum spore yield (> 6 x 10^{7} spores/ ml) and isolate UM3 and UM7 with $\geq$ 4 x 10^{7} spores/ ml. All *B. bassiana* isolates showed good growth rate in the range 1.0 - 1.8 mmd^{-1}, the best being for isolate AB1 (1.76 mmd^{-1}) and UB4 (1.74 mmd^{-1}), followed by isolate UB5 and UB6 ($\geq$ 1.5 mmd^{-1}). Isolate UB2 exhibited maximum spore yield (5.56 x 10^{7} spores/ ml), followed by isolate UB6, UB8 and UB16 with $\geq$ 4 x

10^7 spores/ ml. Amongst *M. anisopliae* isolates, maximum growth potential was demonstrated by isolate UM10 (2.8 mmd^{-1}), followed by isolate UM4 (2.5 mmd^{-1}) and UM5 (2.15 mmd^{-1}) in Osmotic Stress media. Isolate AR1 exhibited maximum spore yield (4.4 x 10^7 spores/ ml) followed by isolate UM10 (3.15 x 10^7 spores/ ml) and for rest of the isolates OSM was a stress media. Amongst *B. bassiana* isolates maximum growth potential was demonstrated by isolate UB15 (2.04 mmd^{-1}), followed by isolate UB4 (1.35 mmd^{-1}) and UB6 (1.19 mmd^{-1}). Isolate UB6 exhibited maximum spore yield (4.20 x 10^7 spores/ ml) followed by isolate UB15 (3.60 x 10^7 spores/ ml). In general OSM was a stress media for *B. bassiana* isolates too like *M. anisopliae*.

3.4 Discussion

The isolates showed varied germination growth and sporulation response on different nutrient sources (Figure 3.1 and 3.2). Such is the utility of nutrients in the survival ability of microorganisms, that, finally it must be emphasized that they require it in a balanced mix. If an essential element is below threshold in supply, then microbial growth will be limited regardless of the concentrations of other nutrients. The different *M. anisopliae* isolates showed significant variation ($P < 0.05$) with respect to the differing preference for the carbon sources. One of the most remarkable characteristics of microorganisms is their extraordinary flexibility with respect to carbon sources. Laboratory experiments indicate that most naturally occurring organic molecules can be utilized by most microbes but with varying success rate of germination, growth and sporulation. Gao et al. (2007) concluded that the requirement for carbon and CN ratio varied among fungal species and isolates and that the growth characteristics might be strain dependent. In this study we observed that each of the isolate had specific preference for a particular nutrient source. Isolates UM8 and UM9 were the most susceptible isolates and it was observed that the original lyophilized cultures of these were difficult to grow on almost all the nutrient media studied and so these isolates were screened out of further studies. It has been reported that nutrient source have significant effects on the spore yield of fungus (Jackson and Bothast, 1990). Jackson and Schisler, 1992, reported that the nutritional environment alters the composition and attributes of fungal spores. Schisler et al. (1991) reported the influence of nutrition on conidial germination. We observed that SDA, low CN source, high CN source, peptone (2 %), PDA and OSM supported maximum germination for isolate UM2, while Yeast extract (1 %) did the job for isolate UM4. It was seen that isolate UM6 had maximum growth rate on almost all the six media investigated, but had poor germination potential on all of them, even after 16 hr of incubation, and hence it was considered to be a poor isolate. Shah et al. (2005) observed that an increase in radial growth did not result in simultaneous increase in conidial yield. Our study too showed that the best sporulation supporting media was not the same as the media, which induced best colony growth in most *M. anisopliae* isolates, for most nutrient sources. We observed that SDA that produced maximum sporulation in isolate UM2, UM3 and UM4, did not produce maximum radial growth in them. Although there is a tendency for more sporulation on carbon rich source, the threshold varies with the species, strains and the nature of the CN source (Engelkes et al., 1997). Wyss et al. (2001) evaluated agar and grain media for mass production of conidia and found varying spore yield on different substrates. In this study, it was observed that OSM was a stress medium for spore harvestation in most isolates. Several authors have reported that an intermediate CN source achieves best yield in terms of growth. Shah et al., 2005, achieved maximum yields in 35 : 1 CN medium (similar to SDA). Similarly Jackson and Schisler (1992) observed maximum spore yield in 30 : 1 CN medium and not at higher or lower CN ratios. In contrast, we found that SDA (intermediate CN source) which is the routine culture media for entomopathogenic fungi did not always support ample growth and sporulation; and high CN source and low CN source were not always marginal to the intermediate source.

In *B. bassiana* isolates, different nutrient media had a significant impact on conidial germination. It was observed that at 8th hr of incubation, all the CN sources showed capability of initiating germination in all the *B. bassiana* isolates except isolates UB7 and UB12 - 15 and hence these were considered poor isolates. Isolate AB1 was the most robust isolate in terms of germination potential on all the nutrient stress media used. It germinated best on Yeast extract (1 %) (up to 67 % at 8th hr of incubation). Isolate UB2, UB3, UB5 and UB16 also depicted commendable germination potential on different nutrient sources. A general trend observed was that, Yeast extract (1 %) induced a higher germination rate in most of the *B. bassiana* isolates. The different *B. bassiana* isolates showed significant difference ($P < 0.05$) in context of radial growth with respect to the nutrient source. The colony growth also varied significantly ($P < 0.05$) with respect to the observation time. Isolate UB15, UB16 and AB1 were better isolates in terms of specific growth rate and spore yield on most of the nutrient sources studied. Yeast extract (1 %) supported better mycelial growth than any other media. OSM was a stress medium for most isolates as it did not induce appreciable colony growth in these isolates when compared to other nutrient source. Like *M. anisopliae* isolates, in *B. bassiana* isolates too, the best sporulation supporting media was not the same as the media which induced best colony growth in them.

Upon field application, the major impending challenges that the entomopathogens face is survival against fluctuating physical environment (Tang and Hou, 2001). The germination and acclimatization of the fungal conidia, before its subsequent growth and sporulation, is the pre-requisite threshold for their efficacy, and we considered germination potential as the major criteria for isolate selection. It may be stated that some of the nutrient sources used in this study were the stress media which might mimic the on-field abiotic stress such as osmotic stress and/or nutrient poor soil. The knowledge of entomopathogenic fungal ecology such as tolerance to environmental stress can contribute to a better understanding of the effect of optimum factors on the survival and distribution of these fungi in filed. This in turn can enable prediction or promote habitats that encourage amplification of natural inoculum and the induction of epizootics. The growth characteristic of vast majority of entomopathogenic fungi is clearly affected by the supply of nutrients (Humber, 2008).

Conclusion

Our findings show that isolates UM2 (*M. anisopliae*) and AB1(*B. bassiana*) was the fastest germinating isolate on almost all nutrient sources studied and possessed commendable growth rate and sporulation potential on them. High germination ability on nutrient poor sources as well as osmotic stress sources highlights the endurance of the infective propagule of the isolates UM2 and AB1.

Table 3.1: Germination percentage of *M. anisopliae* isolates on various media at 8th and 16th h of incubation

Isolates	**CN ratio – 35 : 1** (Glucose (4 %) and Peptone (1 %))		**CN ratio – 10 : 1** (Glucose (0.6 %) and Peptone (1 %))		**CN ratio – 75 : 1** (Glucose (9.1 %) and Peptone (1 %))		**CN ratio – 8 : 1** Peptone (2 %)		**CN ratio – 3.6 : 1** Yeast extract (1 %)		**CN ratio – 10 : 1** (Potato Dextrose agar)		**OSM** (Glucose (8 %), Peptone (2 %), KCl (5.5 %))	
	8th h	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**
UM1	40.15^{d*}	100.00^{a}	40.71^{e}	100.00^{a}	40.97^{e}	100.00^{a}	40.84^{d}	100.00^{a}	44.42^{d}	100.00^{a}	27.39^{g}	90.00^{b}	60.42^{b}	100.00^{a}
UM2	75.00^{a}	100.00^{a}	72.00^{a}	100.00^{a}	61.57^{a}	100.00^{a}	68.22^{a}	100.00^{a}	51.20^{c}	100.00^{a}	71.17^{a}	100.00^{a}	68.87^{a}	100.00^{a}
UM3	68.98^{b}	100.00^{a}	49.42^{d}	100.00^{a}	55.60^{b}	87.98^{b}	48.00^{c}	100.00^{a}	50.93^{c}	100.00^{a}	44.60^{d}	100.00^{a}	50.40^{c}	100.00^{a}
UM4	67.92^{b}	99.50^{a}	64.52^{b}	100.00^{a}	62.43^{a}	76.00^{d}	49.00^{c}	100.00^{a}	64.95^{a}	83.80^{c}	51.31^{c}	81.20^{c}	47.45^{d}	78.80^{b}
UM5	29.82^{e}	100.00^{a}	36.80^{f}	100.00^{a}	23.20^{f}	100.00^{a}	49.20^{c}	86.20^{b}	41.20^{e}	100.00^{a}	41.80^{e}	100.00^{a}	43.40^{f}	100.00^{a}
UM6	9.60^{j}	47.50^{e}	0^{j}	27.60^{f}	0^{i}	21.80^{i}	22.20^{g}	65.00^{d}	23.40^{h}	53.20^{f}	20.70^{h}	44.30^{g}	0^{g}	17.30^{c}
UM7	12.50^{i}	79.70^{b}	15.20^{g}	82.28^{c}	14.00^{h}	100.00^{a}	18.10^{i}	100.00^{a}	17.11^{i}	100.00^{a}	0^{j}	67.60^{d}	0^{g}	0^{e}
UM8	0^{k}	0^{g}	0^{j}	0^{i}	22.00^{g}	33.80^{g}	0^{j}	0a	0^{k}	0^{h}	35.07^{f}	55.20^{e}	0^{g}	0^{e}
UM9	0^{k}	0^{g}	0^{j}	0^{i}	0^{i}	53.20^{f}	0^{j}	32.20^{h}	0^{k}	0^{h}	0^{j}	0^{j}	0^{g}	0^{e}
UM10	25.40^{f}	59.50^{c}	15.60^{g}	42.10^{d}	0^{i}	68.4740^{e}	34.20^{e}	83.57^{c}	28.20^{g}	76.92^{d}	0^{j}	25.10^{i}	0^{g}	0^{e}
UM11	17.10^{h}	100.00^{a}	0^{j}	13.52^{h}	50.80^{c}	86.20^{c}	20.20^{h}	50.60^{f}	54.00^{b}	90.20^{b}	0^{j}	33.40^{h}	0^{g}	0^{e}
UM12	0^{k}	43.44^{f}	11.00^{i}	39.20^{e}	0^{i}	26.40^{h}	30.20^{f}	62.80^{e}	29.00^{g}	67.40^{e}	14.70^{i}	50.80^{f}	0^{g}	14.60^{d}
UM13	23.40^{g}	50.50^{d}	13.60^{h}	23.40^{g}	0^{i}	21.55^{i}	0^{j}	38.42^{g}	7.04^{j}	41.40^{g}	0^{j}	24.20^{i}	0^{g}	0e
AR1	64.40^{c}	100.00^{a}	50.80^{c}	96.80^{b}	44.20^{d}	100.00^{a}	60.60^{b}	100.00^{a}	37.60^{f}	100.00^{a}	55.56^{b}	100.00^{a}	44.80^{e}	100.00^{a}

*Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

Table 3.2: Growth rate and spore yield of *M. anisopliae* isolates on various media

Isolates	**CN ratio – 35 : 1** (Glucose (4 %) and Peptone (1 %))		**CN ratio – 10 : 1** (Glucose (0.6 %) and Peptone (1 %))		**CN ratio – 75 : 1** (Glucose (9.1 %) and Peptone (1 %))		**CN ratio – 8 : 1** Peptone (2 %)		**CN ratio – 3.6 : 1** Yeast extract (1 %)		**CN ratio – 10 : 1** (Potato Dextrose agar)		**OSM** (Glucose (8 %), Peptone (2 %), KCl (5.5 %))	
	Growth rate (mmd^{-1})	**Spore yield (x 10^{7})**	**Growth rate** (mmd^{-1})	**Spore yield (x 10^{7})**	**Growth rate** (mmd^{-1})	**Spore yield (x 10^{7})**	**Growth rate** (mmd^{-1})	**Spore yield (x 10^{7})**	**Growth rate** (mmd^{-1})	**Spore yield (x 10^{7})**	**Growth rate** (mmd^{-1})	**Spore yield (x 10^{7})**	**Growth rate** (mmd^{-1})	**Spore yield (x 10^{7})**
UM1	2.04^{b*}	1.75^{f}	2.05^{b}	2.10^{de}	2.04^{bc}	1.75^{g}	1.30^{h}	4.40^{b}	1.66^{f}	3.75^{b}	1.02^{e}	1.50^{gh}	1.54^{d}	1.50^{d}
UM2	1.99^{b}	3.30^{d}	2.02^{b}	1.00^{i}	1.97^{cd}	1.00^{hi}	1.60^{ef}	1.90^{g}	1.62^{f}	2.00^{e}	0.88^{f}	2.50^{d}	1.54^{d}	1.20^{e}
UM3	1.68^{c}	1.00^{h}	1.92^{c}	1.60^{g}	1.72^{e}	1.65^{g}	2.12^{c}	2.00^{g}	2.06^{c}	2.00e	1.42^{b}	3.95^{c}	1.13^{e}	1.00^{ef}
UM4	1.48^{d}	2.55^{e}	1.50^{d}	3.00^{c}	1.16^{h}	3.30^{d}	2.17^{c}	1.60^{h}	2.50^{b}	1.65^{f}	1.47^{b}	2.60^{d}	2.50^{b}	2.65^{c}
UM5	1.37^{e}	3.60^{c}	1.98^{bc}	3.25^{b}	1.67^{ef}	2.40^{e}	2.18^{c}	2.00^{g}	2.00^{cd}	1.25^{g}	1.39^{b}	4.60^{b}	2.15^{c}	2.50^{c}
UM6	2.52^{a}	1.10^{g}	2.58^{a}	1.30^{h}	3.10^{a}	3.60^{c}	2.60^{a}	2.25^{f}	2.86^{a}	2.50^{d}	1.90^{a}	1.85^{ef}	1.00^{ef}	1.05^{ef}
UM7	1.39^{e}	1.15^{g}	1.04^{f}	0.85^{i}	2.10^{b}	2.00^{f}	1.66^{e}	1.15^{i}	1.62^{f}	1.30^{g}	1.04^{e}	4.15^{c}	0.50^{h}	0.60^{g}
UM8	0^{h}	0^{i}	0^{g}	0^{j}	0.60^{i}	1.00^{hi}	0^{j}	0^{j}	0^{g}	0^{j}	1.28^{c}	1.05^{i}	0^{i}	0^{h}
UM9	0^{h}	0^{i}	0^{g}	0^{j}	1.52^{g}	0.90^{i}	1.48^{g}	1.55^{h}	0^{g}	0^{j}	0^{g}	0^{j}	0^{i}	0^{h}
UM10	1.07^{f}	5.10^{b}	1.50^{d}	5.50^{a}	1.90^{d}	5.20^{a}	1.88^{d}	5.80^{a}	1.76^{e}	5.60^{a}	1.42^{b}	6.10^{a}	2.80^{a}	3.15^{b}
UM11	0.95^{g}	1.35^{g}	0.99^{f}	1.70^{fg}	1.55^{fg}	1.15^{hi}	1.14^{i}	3.00^{d}	1.99^{cd}	0.55^{i}	1.13^{d}	2.10^{e}	0.80^{g}	0.85^{fg}
UM12	1.04^{f}	1.20^{g}	1.45^{d}	1.90^{ef}	1.59^{fg}	1.30^{h}	1.49^{g}	2.50^{e}	2.01^{cd}	0.75^{h}	1.08^{de}	1.70^{fg}	0.75^{g}	0.85^{fg}
UM13	0.99^{f}	1.80^{f}	1.34^{e}	2.20^{d}	1.49^{g}	1.55^{g}	1.52^{fg}	2.35^{ef}	1.95^{d}	0.55^{i}	1.24^{c}	1.35^{h}	0.89^{fg}	1.00^{ef}
AR1	1.50^{d}	5.40^{a}	1.94^{c}	5.50^{a}	1.52^{g}	4.70^{b}	2.41^{b}	3.60^{c}	2.50^{b}	3.10^{c}	1.42^{b}	6.15^{a}	1.07^{e}	4.40^{a}

*Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

Table 3.3: Germination percentage of *B. bassiana* isolates on various media at 8th and 16th h of incubation

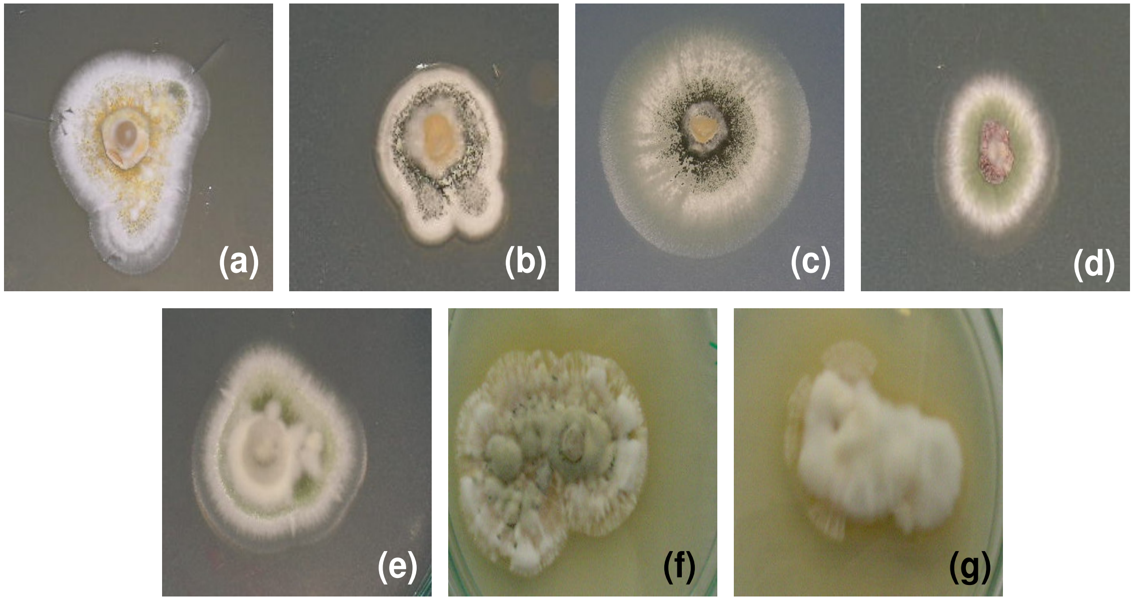
Isolates	**CN ratio – 35:1** (Glucose (4%) and Peptone (1%))		**CN ratio – 10:1** (Glucose (0.6%) and Peptone (1%))		**CN ratio – 75:1** (Glucose (9.1%) and Peptone (1%))		**CN ratio – 8:1** Peptone (2%)		**CN ratio – 3.6:1** Yeast extract (1%)		**CN ratio – 10:1** (Potato Dextrose agar)		**OSM** (Glucose (8%), Peptone (2%), KCl (5.5%))	
	8th h	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**	**8th h**	**16th h**
UB1	31.00^{d}	100^{a}	31.60^{e}	100^{a}	25.50^{e}	100^{a}	36.20^{d}	100^{a}	30.00^{g}	100^{a}	27.60^{e}	100^{a}	20.50^{f}	100^{a}
UB2	38.60^{b}	100^{a}	45.70^{b}	100^{a}	35.00^{d}	100^{a}	45.80^{b}	100^{a}	35.60^{e}	100^{a}	32.10^{d}	100^{a}	30.00^{d}	100^{a}
UB3	36.64^{c}	100^{a}	36.00^{d}	100^{a}	36.90^{c}	100^{a}	36.40^{d}	100^{a}	36.10^{d}	100^{a}	36.00^{c}	100^{a}	40.60^{b}	100^{a}
UB4	26.97^{e}	100^{a}	26.50^{f}	100^{a}	25.60^{e}	100^{a}	24.40^{f}	100^{a}	31.00^{f}	100^{a}	28.00^{e}	100^{a}	23.01^{e}	100^{a}
UB5	40.00^{b}	100^{a}	39.80^{c}	100^{a}	41.40^{b}	100^{a}	38.00^{d}	100^{a}	40.80^{c}	100^{a}	40.10^{b}	100^{a}	37.50^{c}	100^{a}
UB6	30.20^{d}	100^{a}	26.10^{f}	100^{a}	24.40^{e}	100^{a}	32.70^{e}	100^{a}	27.62^{h}	100^{a}	25.40^{f}	100^{a}	20.45^{f}	100^{a}
UB7	0^{g}	92.70^{b}	0^{i}	45.40^{c}	0^{i}	35.00^{f}	14.40^{g}	60.20^{c}	12.10^{k}	73.00^{c}	10.60^{j}	73.60^{b}	0^{j}	49.50^{c}
UB8	1.40^{g}	54.20^{e}	2.20^{h}	24.60^{g}	1.00^{hi}	20.40^{i}	11.50^{hi}	36.80^{f}	13.00^{j}	41.60^{j}	11.50^{i}	38.80^{h}	0^{j}	27.60^{g}
UB9	2.20^{g}	67.80^{d}	2.20^{h}	25.80^{g}	1.60^{h}	27.50^{g}	5.00^{k}	36.80^{f}	12.00^{k}	51.60^{i}	6.00^{k}	26.50^{i}	1.80^{i}	19.40^{j}
UB10	6.60^{f}	35.80^{i}	2.60^{h}	29.00^{f}	3.50^{g}	25.20^{h}	9.40^{ij}	55.20^{d}	7.60^{m}	69.00^{e}	6.10^{k}	52.00^{g}	4.00^{h}	27.60^{g}
UB11	7.00^{f}	28.20^{j}	6.00^{g}	43.00^{d}	5.80^{f}	45.60^{c}	7.70^{j}	47.20^{e}	10.70^{l}	83.00^{b}	6.00^{k}	23.30^{j}	0^{j}	20.50^{i}
UB12	0^{g}	49.00^{f}	0^{i}	51.20^{b}	0^{i}	42.00^{d}	0^{l}	59.80^{c}	0^{n}	66.40^{f}	0^{l}	61.20^{e}	0^{j}	51.70^{b}
UB13	0^{g}	46.20^{g}	0^{i}	45.90^{c}	0^{i}	42.50^{d}	13.60^{gh}	52.96^{d}	12.00^{k}	60.60^{g}	15.10^{g}	66.60^{d}	0^{j}	40.00^{e}
UB14	0^{g}	44.00^{h}	0^{i}	41.80^{d}	0^{i}	37.90^{e}	0^{l}	46.20^{e}	0^{n}	54.60^{h}	0^{l}	53.42^{f}	0^{j}	45.00^{d}
UB15	0^{g}	46.40^{g}	0^{i}	31.60^{e}	0^{i}	26.90^{g}	10.80^{i}	69.40^{b}	15.10^{i}	70.60^{d}	12.10^{h}	68.50^{c}	0^{j}	21.50^{h}
UB16	30.40^{d}	83.40^{c}	39.20^{c}	100^{a}	25.60^{e}	73.40^{b}	41.20^{c}	100^{a}	45.90^{b}	100^{a}	40.60^{b}	100^{a}	5.10^{g}	35.40^{f}
AB1	61.80^{a}	100^{a}	51.30^{a}	100^{a}	56.00^{a}	100^{a}	67.40^{a}	100^{a}	54.40^{a}	100^{a}	47.40^{a}	100^{a}	50.20^{a}	100^{a}

*Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test

Table 3.4: Growth rate and spore yield of *B. bassiana* isolates on various media

Isolates	**CN ratio – 35:1** (Glucose (4%) and Peptone (1%))		**CN ratio – 10:1** (Glucose (0.6%) and Peptone (1%))		**CN ratio – 75:1** (Glucose (9.1%) and Peptone (1%))		**CN ratio – 8:1** Peptone (2%)		**CN ratio – 3.6:1** Yeast extract (1%)		**CN ratio – 10:1** (Potato Dextrose agar)		**OSM** (Glucose (8%), Peptone (2%), KCl (5.5%)	
	Growth rate (mm/d)	**Spore yield (x 10^7)**	**Growth rate (mm/d)**	**Spore yield (x 10^7)**	**Growth rate (mm/d)**	**Spore yield (x10^7)**	**Growth rate (mm/d)**	**Spore yield (x10^7)**	**Growth rate (mm/d)**	**Spore yield (x 10^7)**	**Growth rate (mm/d)**	**Spore yield (x 10^7)**	**Growth rate (mm/d)**	**Spore yield (x 10^7)**
UB1	1.00^{e}	3.60^{b}	1.03^{h}	3.75^{b}	0.84^{g}	3.05^{cd}	0.70^{k}	4.40^{bcd}	1.23^{l}	4.08^{d}	1.15^{h}	3.04^{c}	1.00^{f}	2.75^{c}
UB2	1.52^{abc}	3.00^{c}	1.53^{e}	3.00^{c}	0.99^{ef}	3.50^{c}	1.10^{i}	3.75^{d}	1.85^{g}	6.02^{a}	1.03^{i}	5.56^{a}	1.02^{ef}	3.00^{c}
UB3	1.13^{de}	2.25^{ef}	1.33^{f}	2.95^{c}	1.19^{d}	2.50^{de}	1.94^{d}	3.60^{d}	1.95^{e}	4.12^{d}	1.43^{de}	2.02^{de}	1.06^{e}	2.80^{c}
UB4	1.22^{d}	2.60^{d}	1.02^{h}	2.10^{de}	0.83^{g}	4.20^{b}	1.67^{g}	4.85^{bc}	1.92^{ef}	5.06^{c}	1.74^{a}	2.54^{d}	1.35^{b}	3.15^{c}
UB5	1.40^{c}	3.10^{c}	1.78^{d}	4.10^{ab}	1.09^{e}	3.40^{c}	1.95^{d}	1.30^{f}	2.03^{d}	3.06^{f}	1.57^{b}	3.52^{c}	1.14^{d}	3.05^{c}
UB6	1.59^{ab}	4.05^{a}	1.52^{e}	4.40^{a}	0.93^{f}	5.50^{a}	1.62^{g}	1.80^{f}	2.01^{d}	2.02^{g}	1.52^{c}	4.58^{b}	1.19^{c}	4.20^{a}
UB7	0.75^{f}	1.90^{f}	1.45^{e}	1.80^{e}	1.18^{d}	1.10^{h}	1.24^{h}	2.00^{f}	1.38^{k}	3.00^{f}	0.95^{j}	2.35^{d}	0.63^{i}	1.30^{e}
UB8	1.49^{bc}	0.90^{i}	1.20^{g}	1.05^{f}	1.22^{d}	4.30^{b}	1.04^{ij}	4.50^{bcd}	2.09^{c}	5.00^{c}	0.62^{k}	4.35^{b}	0.94^{g}	3.55^{b}
UB9	1.57^{abc}	1.55^{g}	2.01^{c}	1.75^{e}	1.35^{c}	2.40^{def}	1.83^{f}	5.05^{b}	2.03^{d}	4.50^{d}	1.03^{i}	2.10^{de}	0.65^{i}	2.05^{d}
UB10	1.18^{de}	2.15^{ef}	2.02^{c}	2.10^{de}	1.50^{b}	2.10^{efg}	2.00^{cd}	4.10^{cd}	2.25^{b}	4.10^{d}	1.33^{g}	3.15^{c}	0.75^{h}	2.10^{d}
UB11	1.40^{c}	1.30^{gh}	1.56^{e}	1.70^{e}	1.42^{bc}	2.25^{def}	1.85^{ef}	4.05^{cd}	2.29^{b}	4.10^{d}	1.42^{de}	1.85^{de}	0.53^{j}	0.90^{e}
UB12	1.09^{de}	1.00^{hi}	2.23^{b}	1.05^{f}	1.85^{a}	1.40^{gh}	1.18^{h}	2.75^{e}	1.72^{h}	5.05^{c}	1.40^{ef}	1.10^{f}	1.00^{f}	1.80^{d}
UB13	1.00^{e}	0.95^{hi}	1.00^{h}	1.10^{f}	1.00^{ef}	1.60^{fgh}	1.00^{j}	1.90^{f}	1.45^{j}	1.50^{h}	1.00^{i}	1.50^{ef}	0.75^{h}	1.05^{e}
UB14	1.03^{e}	1.25^{ghi}	1.48^{e}	1.55^{ef}	1.27^{d}	1.05^{h}	3.00^{a}	4.35^{bcd}	1.88^{fg}	4.60^{cd}	1.37^{f}	2.05^{de}	0.38^{k}	1.05^{e}
UB15	1.67^{a}	2.50^{de}	3.01^{a}	2.55^{cd}	1.48^{b}	4.70^{b}	2.05^{c}	5.70^{a}	2.98^{a}	5.65^{b}	1.47^{d}	3.35^{c}	2.04^{a}	3.60^{b}
UB16	1.48^{bc}	3.05^{c}	1.09^{h}	3.95^{ab}	1.50^{b}	4.35^{b}	1.92^{de}	1.95^{f}	1.62^{i}	2.35^{g}	1.02^{i}	4.35^{b}	1.04^{ef}	2.00^{d}
AB1	1.13^{de}	2.20^{ef}	1.11^{h}	3.00^{c}	1.00^{ef}	2.50^{de}	2.13^{b}	3.80^{d}	2.27^{b}	3.56^{e}	1.76^{a}	2.10^{de}	1.01^{ef}	3.05^{c}

*Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test



a. SDA b. PDA c. 1%Yeast extract d. 2% Peptone e. Low CN source
f. High CN source g. OSM

Fig. 3.1. Nutrient dependent change in colony morphology in a *M. anisopliae* isolate.

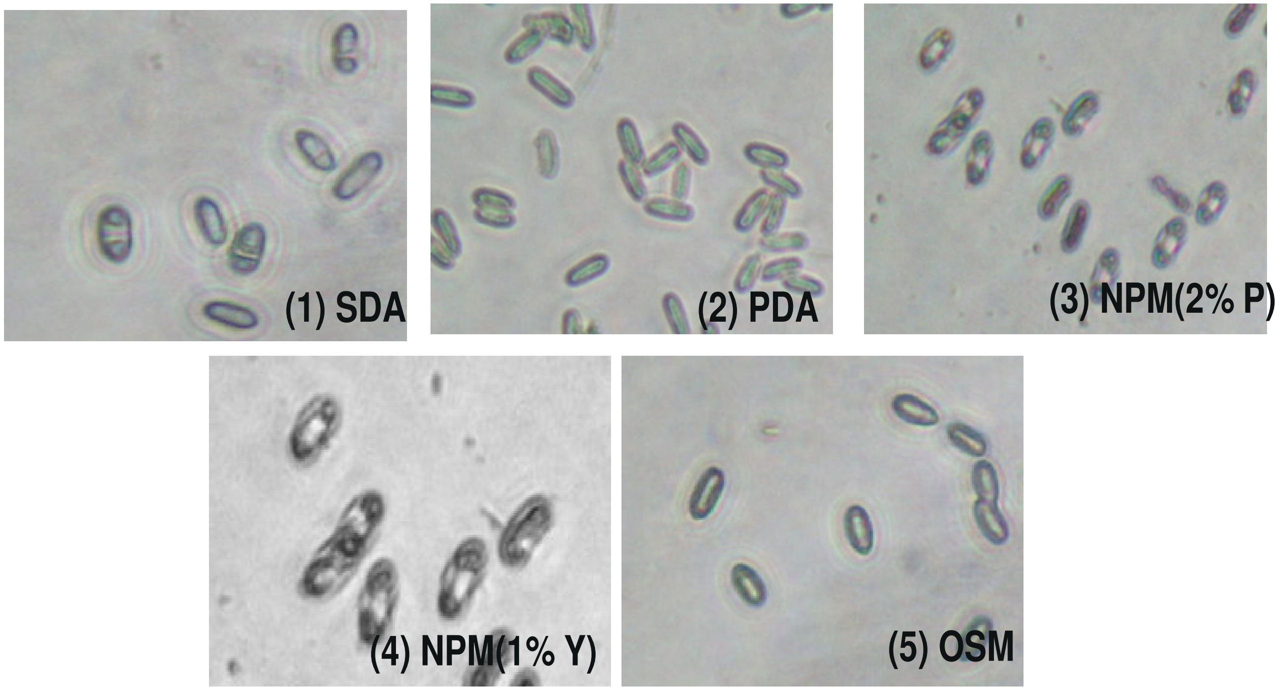


Fig. 3.2. Nutrient dependent change in conidial morphology in a *M. anisopliae* isolate.

4.1 Introduction

Biocontrol of pests works to create a system where the insects and pathogens that cause diseases are kept at manageable levels, utilizing the natural processes within an ecosystem. Entomopathogenic fungi in common with other insect natural enemies can be employed for biocontrol strategies (Shah and Pell, 2003). They are unique when compared to other microbes causing diseases in insects, because they cause infection by growing through the insect cuticle and are not required to be ingested, thus showing great potential for control of even sucking pests. They work by contact mode of action and have a broad host range. Entomopathogenic fungi like *Metarhizium anisopliae* and *Beauveria bassiana* have shown good potential for the management of various insect pests (Inglis et al., 2001). Interaction of the entomopathogenic fungi and host depends on the occurrence of non-specific and specific events between the conidia and the insect cuticle (Chengshu et al., 2002). Amongst the series of specific events is involved the production of a range of cuticle-degrading enzymes corresponding to the different polymers of the insect cuticle (Charnley and St. Leger, 1991). Many extra-cellular cuticle-hydrolyzing enzymes have been purified from entomopathogenic fungi and their induction in cultures containing cuticle as substrates has been studied (St. Leger et al., 1986a; St. Leger et al., 1986b). The biochemistry of insect epicuticle degradation by entomopathogenic fungi has been thoroughly reviewed (Pedrini et al., 2007). Several workers have reported a correlation between production of extracellular enzymes and pathogenicity (Bidochka and Khachatourians, 1994).

In-vitro production of extracellular enzymes by entomopathogens has been the subject of many studies (Smith et al., 1981). Available studies on extracellular enzyme production have examined not many isolates of entomopathogenic fungi. Gupta et al., (1992) examined five isolates of *B. bassiana* for cuticle degrading enzymes and Niranjana (2004) reported studies with two isolates of *B. bassiana*. The results of enzyme production are difficult to compare because of differences in isolates and culturing methods (Hegedus and Khachatourians, 1988). To the best of our knowledge, our study has been more inclusive and plate assays with twelve isolates for five different enzymes was evaluated and this data was used for screening the best isolate amongst those tested.

The entomopathogenic fungi are natural and cosmopolitan in their occurrence. In nature, certain isolates hold an edge in terms of rate of kill over certain other isolates and enzyme secretion is one of the key components in designating them as virulent/ non-virulent. The purpose of this study has been to identify those naturally occurring entomopathogenic fungi, which are the high secretors and thus possess high potential of enzymatic-pathogenesis. With such an objective, *in-vitro* laboratory studies were undertaken to investigate the potential of enzymatic degradation by twelve *M. anisopliae* isolates and the enzymes studied are chitinase, proteinase, caseinase lipase and amylase. An enzyme assay was also conducted for chitinase and proteinase activity for all the fourteen isolates. Morphological characteristics were also taken into account to ascertain their pathogenic potential.

4.2 Materials and Methods

Fungal cultures

The different isolates of the fungus *M. anisopliae* and *B. bassiana* were either procured from ARSEF (USDA - ARS Plant Protection Unit) or isolated locally in India (Table 2.1 & 2.2). The isolates were routinely sub cultured on SDA (Sabouraud Dextrose Agar) slants at 28 °C and maintained at 4 °C until further use.

Studies on growth characteristics and colony morphology on Sabouraud dextrose agar (SDA) medium

For germination assay agar slide technique was used. One ml medium (SDA) was evenly spread on each of the glass slides using micropipette. Conidial suspension was prepared from seven day old cultures with concentration maintained at 10^6 conidia per ml. Around 100 µL of conidial suspension was spread on the media coated slides and incubated at 28 °C. The slides were observed under compound microscope (40X) for germination, every 2 hrs, starting after 3 hrs. A conidium was considered germinated when a distinct germ tube projected from it, and was at least twice the diameter of the conidium (Milner et al., 1991). Approximately 300 conidia were scored per replicate for each of the isolates.

For assay of colony growth and sporulation, the fresh SDA Petri plates were inoculated with a plug (diameter 5 mm) of agar containing pre - grown mycelia on the same medium for 3 days and the plates were incubated at 28 °C (five replicates were maintained for each isolate). Radial growth was measured every 2nd day till 8th day. Radial growth rate (mmd^{-1}) was calculated from the linear portions of the curves plotted from these values. At end of 8th day, 5 mm agar discs were randomly taken with the help of a cork borer. The discs were placed in 10 ml of 0.02 % (v/ v) Tween 80 solution and vortexed to suspend the spores and spore yield determined using a Haemocytometer. The Petri plates (8 days old) prior to spore count were studied for colony morphology characteristics like fluffy/powdery growth and sector formation.

***In-vitro* enzyme plate assays**

For disc preparation and inoculation on different enzymatic substrates, the methodology followed for studying the colony growth characteristics was repeated with a difference that the discs were placed on different test substrates for *in-vitro* enzyme assays. The control Petri plates contained only minimal media solidified with agar (2 %). Enzyme activities were calculated as an index of the total diameter of the colony + halo divided by the diameter of the colony (St. Leger et al., 1997) at the end of 8 days. Enzymatic index value > 1.0 indicates enzyme activity.

Five enzymes viz. chitinase, proteinase, caseinase, lipase and amylase were studied. For chitinase plate assay, colloidal chitin (2 %) was used. Colloidal chitin was prepared by a modification of the method suggested by Shimahara and Takiguchi (1988). At the end of incubation period, a thin layer of 0.002 % Calcofluor white (Gohel et al., 2005; Anil et al., 2007) was spread on the culture plates and left for incubation for few more hrs after which they were observed under UV transilluminator. Presence of a zone of clearance indicated chitinase activity. For proteinase plate assay, gelatin extract (1 %) in minimal media (0.003 % NaCl, 0.03 % $MgSO_4$ and 0.015 % K_3PO_4) and pH 7.0 was used. At the end of the incubation period, the culture plates were flooded with a solution of 15 % Mercuric chloride in 2 M/ L HCL. A distinct transparent zone of clearance could be seen around the colony while the rest of the plate appeared translucent white in color. Caseinase plate assay contained milk agar (24 gm/ L) as a substrate (pH 7.2 at 25 °C). At the end of the incubation period, a clear transparent halo could be seen around the colony while the rest of the plate appeared opaque white in color. For lipase plate assay, Tween 20 (1 %) along with peptone (1 %), 500 mg NaCl, 10 mg $CaCl_2$ and agar (2 %) was used (pH 6.0). At the end of the incubation period, formation of lipolytic enzymes by a colony was seen as either a visible precipitate due to the formation of crystals of the calcium salt of the lauric acid liberated by the enzyme, or as a clearing of such a precipitate around a colony due to complete degradation of the salt of the fatty acid. Amylase plate assay contained starch agar (30 gm/ L) with pH 7.4 at 25 °C (Niranjana, 2004). At the end of the incubation period, the culture plates were flooded with Lugol's Iodine solution and a yellow colored halo could be seen around the colony in an otherwise blue medium indicating amylolytic activity.

Enzyme assays for chitinase and protease

Chitinase assay

Chitinase assay was done by the method of Yanai et al. (1992). 500 µL of culture supernatant was incubated with 300 µL of 10 % (w/ v) colloidal chitin and 200 µL of 0.2

mol/ L acetate buffer (pH 4) at 37 °C for 2 hrs. The reaction product N-acetyl glucosamine was determined by using para-dimethyl-Amino benzaldehyde reagent (Reissig et al., 1955). Absorbance at 585 nm was taken against water as blank. Sampling was done every two days till tenth day of culture incubation. One unit of chitinase activity was defined by the amount of enzyme that produced 1 µM of N - acetyl glucosamine per min under the above conditions.

Proteinase assay

Proteolytic activity was assayed by a modified method of Kunitz (1947). A 100 µL enzyme sample was incubated with 400 µL of 0.5 % (w/ v) gelatin in 50 mM Tris-HCl buffer, pH 10.0, at 50 ^{0}C for 20 min. The enzyme reaction was terminated by addition of 500 µl 10 % (w/ v) trichloroacetic acid and kept at room temperature for 10 min. The reaction mixture was centrifuged at 10,000 g for 10 min at 4 ^{0}C and the absorbance measured against water as blank at 280 nm. One unit of proteases was defined as the amount of enzyme releasing the equivalent of 1 µM of tyrosine per min under the defined assay conditions.

Statistical analysis

Statistical analysis of all the data for fungal growth, sporulation and germination were subjected to one-way analysis of variance (ANOVA) and the means were separated by Student-Newman-Keuls multiple range test of comparisons of means at $P = 0.05$.

4.3 Results

Studies on growth characteristics and colony morphology on SDA medium

Amongst the twelve isolates studied, isolate UM2 was identified to have up to 75 % germination potential at 8th hr of incubation. This was followed by isolates UM3, UM4 and AR1 with germination potential of ≥ 65 %. All of these isolates showed 100 % germination potential at 16th hr of incubation (Table 4.1). The colony growth rate of these good germinating isolates (viz. UM2, UM3 UM4 and AR1) also exhibited an appreciable colony growth rate of ≥ 1.5 mm/ day and spore yield in the varying range of 1-6 x 10^7 spores per ml at 8th day of incubation. Isolate UM6 though showed profuse growth rate (of 2.6 mmd^{-1}), which was highest on SDA media when compared with other isolates studied, had less than 50 % of its conidia germinated at 16th hr of incubation. Isolate UM1, UM6, UM7 and UM11 - 13 formed fluffy colonies, while isolate UM4, UM5 and UM10 formed powdery colony. Isolate UM1, UM5 and AR1 showed sector formation.

At 8th hr of incubation, isolate AB1 showed up to 62 % germination and this was followed by isolate UB1 (61 %), isolates UB2 and UB5 which had > 35 % of its conidia germinated (Table 4.2). Isolate UB7, UB12 - UB15 did not show any initiation of germination at 8th hr of incubation. Isolate UB1 - UB6 and AB1 had all of their conidia germinated by 16th hr of incubation. Isolate UB2, UB6, UB9, UB15 and UB16 showed specific growth rate in the range of 1.5 - 1.6. Isolates UB1 and UB6 were the best sporulating isolates when compared to the rest of the isolates. Colony morphology was also studied and the isolates UB2, UB5 and UB11 showed sector formation when grown on SDA plates. Isolate UB1, UB2 and UB6 formed powdery colony whereas isolates UB7, UB9 - 14 and UB16 formed fluffy colony. Isolates UB3 - 5, UB8 and AB1 showed both fluffy and powdery traits.

***In-vitro* enzyme activity**

The isolates exhibited chitinase activity in the enzymatic index value range of 1.04 - 1.53 (Table 4.3). Isolate UM2 with enzymatic index value of 1.53 was the highest secretor of chitinase in *in-vitro* plate assays, and this was closely followed by isolate UM1 and AR1. The highest index value of 2.67 for proteinase activity was observed for UM5 and this was preceded by an index of 2.00 for UM1 and UM2. High caseinase production was observed in isolate UM10 (2.07) followed by isolates UM2 (1.92) and UM3 (1.94) respectively. The other isolates produced caseinase in the index range of 1.45 - 1.81. The isolates exhibited lipase activity in the range 1.83 - 2.96. The highest secretor of extra-cellular lipase was isolate UM10. The isolates exhibited amylase activities with enzymatic index value in the range 1.05 - 1.58, the highest producer being isolate UM8. Isolates UM5, UM6 and AR1 showed a constant enzymatic index value of 1.14.

It was observed that the *in-vitro* chitinase activity was exhibited in the range 1.05 - 1.35, the highest producer being isolate UB1 (1.34) followed by isolate AB1 (1.33). Isolates UB6, UB7, UB8, UB10 and UB11 exhibited similar activity with enzymatic index value of 1.1. The highest protease production was seen in UB1 with enzymatic index value of 2.11 followed by isolate UB6 (1.94). No protease activity was observed in isolates UB7, UB8, UB9, UB10 and UB11. Isolates UB3 and AB1 showed similar activity with an index of 1.56 (Table 4.4). The isolates exhibited caseinase index values in the range 1.50 - 3.11 (Table 4.4), the highest producer being UB12 with enzymatic index value of 3.11, followed by UB1 and UB6 with enzymatic index value of 2.63 and 2.35 respectively. Similar to isolate UB3, the isolate UB7 had an enzymatic index value of 1.71, but contrary to the former, the latter did not show any initiation of mycelial growth from the disc, it just produced a zone of clearance, suggestive of enzymatic activity. The isolates exhibited lipase activity with index values ranging from 2.06 - 3.89 (Table 4.4), the most potent secretor being isolate UB1. Six of the isolates, viz., UB1, UB3, UB6, UB7, UB12 and AB1 showed presence of two distinct zones of clearance around the fungal colony. Of the two zones, the first zone, immediately after the fungal colony was a rainbow-colored film with rosette like structures embedded in scattered fashion throughout the depth of the media. These structures when observed under 10X and 40X magnification of compound microscope, showed profuse leafy growth, which when teased with needle (taking care not to shear the filaments), showed the organism growing in a very organized manner. The second zone, which was present beyond the first zone, was a distinct halo with no special structures present. Both the haloes were considered as a whole for calculating the enzymatic index value. Isolate UB16 with enzymatic index value of 2.36 was the highest amylase producer (Table 4.4). Isolate UB1 did not show any amylase activity at all. Rest of the *B. bassiana* isolates showed amylase index value in the range of 1.14 - 1.2.

Enzyme assays

The *M. anisopliae* isolates showed maximum chitinase activity on 4th day of culture incubation with the only exception of isolate UM3 which had maximum activity on day 2 (Table 4.5). The activity declined gradually from day 6 - 10, with exception of isolate UM4 and UM5 (similar activity on day 4 - 6) and isolate UM6 and UM7 (similar activity on day 2 - 8). Isolate UM2 exhibited maximum chitinase activity on all the days of culture incubation and it was closely followed by isolate AR1.

Amongst the isolates assayed for proteinase, maximum activity was observed from day 6 to 8 of culture incubation. Isolate UM5 had maximum activity (0.96 U/ ml) on 6th day of culturing, and this was closely followed by similar activity by isolates UM1 and UM2 (1.52 U/ ml) (Table 4.6). Isolate UM13 and AR1 showed maximum proteinase activity of 0.59 and 0.67 U/ ml respectively on 8th day of culture conditions.

The *B. bassiana* isolates showed maximum chitinase activity on fourth day of culture incubation (Table 4.7). Isolates UB1 and UB2 exhibited maximum chitinase activity when compared with other isolates on fourth day of incubation followed by isolates UB1 and AB1 on sixth day. On day two, the chitinase activity was high for isolates UB2 and AB1. The chitinase activity decreased with the increase in days of incubation and the isolates showed less chitinase activity on days eight and ten.

Studies on proteinase activity demonstrated highest activity on eighth day for all the isolates studied (Table 4.8). On day two UB1 showed highest enzyme production

(0.56U/ ml) followed by the isolate UB6 (0.22U/ ml). Isolate UB1 showed maximum activity on day six and eight followed by isolate UB6 on eighth day.

4.4 Discussion

To be successful, the entomopathogenic fungi ought to breach the outer integument of their hosts by non-specific and specific events between the conidiospores and the insect cuticle (Jeffs et al., 1999). Most of the members amongst the 14 isolates that were tested in the present study, produced an appreciable spectrum of protein and polysaccharide-hydrolyzing enzymes, which could be useful in the degradation of the complex living and non-living organic substrates and implies greatest genetic and biochemical versatility. This versatility reflects their ability to exploit resources as and when it becomes available to them (Smith et al., 1981). *In-vivo*, the sequence of enzyme secretion corresponds to the sequence of the polymers present in the cuticle (St. Leger et al., 1986c). In plate screening assays, enzyme production is typically indicated by large clearing zones (halos) around the growing colony or by the formation of colored product. The most significant inference from the current study is the surprising degree of variability of enzyme secretion by the *M. anisopliae* isolates. Isolate variability has been observed in the production of cuticle-hydrolyzing enzymes in *M. anisopliae* isolates (St. Leger et al., 1986b; Gupta et al., 1991). The involvement of proteinases and chitinases has been proven and it has been suggested that lipases might also be participating in the infection process (Clarkson and Charnley, 1996). The isolate variability was not limited to the quantitative levels of enzyme produced, but extended to the pattern of enzyme expression. Studies on colony morphology showed that sector formation was a negative attribute as it either did not form any spores or produced sterile spores. Isolate UM1 which showed good production of chitinase and proteinase exhibited sector formation on SDA as well as on the enzyme substrates. The spores produced on SDA medium when checked for viability showed less germination which when further analyzed was observed to be sterile. Powdery colony is preferred over fluffy as it produces more spores and aids in wind dispersal. Isolate UM2 was observed having both fluffy and powdery traits and when the spores were checked for its germination potential on SDA, it was observed to show maximum germination rate in comparison to other isolates and also showed appreciable growth rate and sporulation. Current understanding of the initial events of entomo-pathogenesis by fungi reflects that the enzyme system of the entomopathogenic fungi is unique and is of great interest as potential criteria for mycoinsecticide improvement. Genetic optimization of entomopathogenic fungi by addition and expression of insecticidal genes in *M. anisopliae* has been successfully used to engineer a fungal isolate over-expressing a toxic protease, resulting in a considerable shortening in the time of kill (St. Leger *et al.,* 1996c, Hu and St. Leger, 2002). Fang et al. *(*2005) reported that cloning of *B. bassiana* chitinase gene led to increment in its virulence potential. Several workers have reported a positive correlation between production of extracellular enzymes and virulence potential (Bidochka and Khachatourians, 1994). Specific enzymes or several of them in an enzyme cascade may serve as key virulence determinants. Since enzymes were differentially expressed in test media, it is also possible that the enzymes may be involved in host range determination (Gupta et al., 1992). Isolate UM2 showed high *in-vitro* enzyme production and when characterized for enzyme assays also showed maximum chitinase production on day 4, which declined gradually. Maximum proteinase production was observed on day 6. Appreciable germination, growth rate and sporulation characteristics were also shown by isolate UM2. Information generated through such data, may be used to rationally screen and select a potent isolate from a population and there after for its genetic optimization which might be host-specific/ broad host range or rhizosphere competent or has upgraded ability in the speed of kill (Fang et al., 2004). Amongst *B. bassiana* isolates, UB1 and AB1 showed good levels of extracellular enzyme secretion in *in-vitro* plate studies. These isolates showed similar response in chitinase and protease assays. Morphological and growth studies showed that these isolates had similar germination and growth rates, but isolate UB1 formed powdery colony while colony of isolate AB1 exhibited both powdery and fluffy traits. These isolates when further analyzed for spore yield showed that isolate UB1 had better sporulation in comparison to isolate AB1.

4.5 Conclusions

Isolates UM2, UB1 and AB1 are the best producers of extra-cellular lytic enzymes. Thus, these isolates can be rationally advocated towards production of improved myco-pesticides.

Table 4.1: Studies on germination, growth, sporulation and colony morphology of *M. anisopliae* on SDA medium

Isolates	**Germination (%)**		**Specific growth (mmd^{-1})**	**Sporulation ($x\ 10^{7}$)**	**Colony morphology**	
	8^{th} h	**16^{th} h**			**Sector formation**	**F/ P**
UM1	40.15^{d}	100.00^{a}	2.04^{b}	1.75^{f}	Yes	F
UM2	75.00^{a}	100.00^{a}	1.99^{b}	3.30^{d}	No	F+P
UM3	68.98^{b}	100.00^{a}	1.68^{c}	1.00^{h}	No	F+P
UM4	67.92^{b}	99.50^{a}	1.48^{d}	2.55^{e}	No	P
UM5	29.82^{e}	100.00^{a}	1.37^{e}	3.60^{c}	Yes	P
UM6	9.60^{j}	47.50^{e}	2.52^{a}	1.10^{g}	No	F
UM7	12.50^{i}	79.70^{b}	1.39^{e}	1.15^{g}	No	F
UM10	25.40^{f}	59.50^{c}	1.07^{f}	5.10^{b}	No	P
UM11	17.10^{h}	100.00^{a}	0.95^{g}	1.35^{g}	No	F
UM12	0^{k}	43.44^{f}	1.04^{f}	1.20^{g}	No	F
UM13	23.40^{g}	50.50^{d}	0.99^{f}	1.80^{f}	No	F
AR1	64.40^{c}	100.00^{a}	1.50^{d}	5.40^{a}	Yes	F+P

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.
F = fluffy colony, P = powdery colony

Table 4.2: Studies on germination, growth, sporulation and colony morphology of *B. bassiana* on SDA medium

Isolates	**Germination**		**Specific growth (mmd^{-1})**	**Sporulation (x 10^{7})**	**Colony morphology**	
	8^{th} h	**16^{th} h**			**Sector formation**	**F/ P**
UB1	61.00^{a}	100^{a}	1.00^{e}	3.60^{b}	No	P
UB2	38.60^{b}	100^{a}	1.52^{abc}	3.00^{c}	Yes	P
UB3	36.64^{c}	100^{a}	1.13^{de}	2.25^{f}	Yes	F+P
UB4	26.97^{e}	100^{a}	1.22^{d}	2.60^{d}	No	F+P
UB5	40.00^{b}	100^{a}	1.40^{c}	3.10^{c}	Yes	F+P
UB6	30.20^{d}	100^{a}	1.59^{ab}	4.05^{a}	No	P
UB7	0^{g}	92.70^{b}	0.75^{f}	1.90^{f}	No	F
UB8	1.40^{g}	54.20^{e}	1.49^{bc}	$.90^{i}$	No	F+P
UB9	2.20^{g}	67.80^{d}	1.57^{abc}	1.55^{g}	No	F
UB10	6.60^{f}	35.80^{i}	1.18^{de}	2.15^{ef}	No	F
UB11	7.00^{f}	28.20^{j}	1.40^{c}	1.30^{gh}	Yes	F
UB12	0^{g}	49.00^{f}	1.09^{de}	1.00^{hi}	No	F
UB13	0^{g}	46.20^{g}	1.00^{e}	$.95^{ehi}$	No	F
UB14	0^{g}	44.00^{h}	1.02^{e}	1.25^{ghi}	No	F
UB15	0^{g}	46.40^{g}	1.66^{a}	2.50^{de}	No	F+P
UB16	30.40^{d}	83.40^{c}	1.48^{bc}	3.05^{c}	No	F
AB1	61.80^{a}	100^{a}	1.13^{de}	2.20^{ef}	No	F+P

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.
F = fluffy colony, P = powdery colony

Table 4.3: Enzymatic Index Values of extra-cellular enzymes produced by *M. anisopliae* isolates

Isolates	**Enzymes**				
	Chitinase	**Protease**	**Caseinase**	**Lipase**	**Amylase**
UM1	1.40^{b}	2.00^{b}	1.66^{d}	1.83^{g}	1.13^{f}
UM2	1.53^{a}	2.00^{b}	1.92^{b}	1.72^{h}	1.21^{e}
UM3	1.23^{d}	1.67^{d}	1.94^{b}	1.88^{f}	1.10^{f}
UM4	1.11^{e}	1.42^{e}	0^{h}	1.87^{f}	1.05^{g}
UM5	1.10^{e}	2.67^{a}	1.81^{c}	1.91^{e}	1.14^{f}
UM6	1.04^{f}	1.11^{g}	0^{h}	0^{j}	1.14^{f}
UM7	1.23^{d}	0^{h}	1.45^{g}	1.24^{i}	1.58^{a}
UM10	1.10^{e}	0^{h}	2.07^{a}	2.97^{a}	1.27^{d}
UM11	1.22^{d}	0^{h}	1.77^{c}	2.17^{c}	1.38^{b}
UM12	1.09^{e}	0^{h}	1.68^{d}	2.19^{c}	1.33^{c}
UM13	1.10^{e}	1.18^{f}	1.53^{f}	2.33^{b}	1.34^{c}
AR1	1.36^{c}	1.84^{c}	1.63^{e}	2.11^{d}	1.14^{f}

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.
Enzymatic Index Value = (total diameter of the colony + halo) / diameter of the colony.

Table 4.4: Enzymatic Index Values of extra-cellular enzymes produced by *B. bassiana* isolates

Isolates	Enzymes				
	Chitinase	**Protease**	**Caseinase**	**Lipase**	**Amylase**
UB1	1.34^{a}	2.12^{a}	2.63^{b}	3.89^{a}	0^{k}
UB2	1.18^{de}	1.44^{e}	1.73^{g}	2.63^{d}	1.72^{d}
UB3	1.15^{ef}	1.56^{d}	1.71^{h}	2.62^{d}	1.41^{f}
UB4	1.18^{de}	1.46^{e}	1.57^{i}	2.78^{c}	1.20^{i}
UB5	1.12^{fg}	1.76^{c}	2.04^{d}	2.73^{c}	1.32^{gh}
UB6	1.11^{g}	1.94^{b}	2.35^{c}	2.17^{g}	1.37^{fg}
UB7	1.10^{g}	0^{f}	$1.71^{h\ \#}$	2.91^{b}	2.12^{b}
UB8	1.10^{g}	0^{f}	0^{k}	0^{i}	1.37^{fg}
UB9	1.14^{fg}	0^{f}	1.98^{e}	2.19^{g}	1.53^{e}
UB10	1.10^{g}	0^{f}	1.50^{j}	0^{i}	1.52^{e}
UB11	1.10^{g}	0^{f}	0k	2.24^{g}	1.57^{e}
UB12	1.22^{c}	0^{f}	3.11^{a}	2.38^{f}	1.80^{c}
UB13	1.28^{b}	0^{f}	0^{k}	0^{i}	0^{k}
UB14	1.20^{cd}	0^{f}	0^{k}	0^{i}	1.14^{j}
UB15	1.05^{h}	0^{f}	0^{k}	0^{i}	1.39^{f}
UB16	1.23^{c}	0^{f}	0^{k}	2.06^{h}	2.36^{a}
AB1	1.33^{a}	1.56^{d}	1.80^{f}	2.50^{e}	1.27^{h}

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.
Enzymatic Index Value = (total diameter of the colony + halo) / diameter of the colony.
No growth from disc observed but halo (due to enzyme activity) was present.

Table 4.5: Chitinase activity in *M. anisopliae* isolates

Isolates	**Enzyme Activity (U/ ml) as on day**				
	2	**4**	**6**	**8**	10
UM1	3.27^{eE}	6.47^{cA}	5.74^{cB}	4.29^{eC}	3.71^{deD}
UM2	6.49^{aC}	7.63^{aA}	7.04^{aB}	6.37^{aC}	5.21^{aD}
UM3	5.11^{bA}	3.79^{fB}	2.99^{fC}	2.49^{gD}	1.98^{gE}
UM4	4.30^{cC}	5.92^{dA}	5.61^{cA}	4.95^{cB}	2.93^{fD}
UM5	3.93^{cdB}	5.32^{eA}	5.01^{dA}	4.21^{eB}	3.54^{eC}
UM6	3.93^{cdA}	4.12^{fA}	3.89^{eA}	3.56^{fB}	2.98^{fC}
UM7	4.99^{bAB}	5.65^{deA}	5.64^{cA}	5.39^{bA}	4.52^{cB}
UM10	3.66^{deE}	5.78^{deA}	5.40^{cdB}	5.03^{cC}	4.47^{cD}
UM11	3.61^{deC}	5.34^{eA}	3.93^{eB}	2.72^{gD}	2.18^{gE}
UM12	3.63^{deC}	5.53^{deA}	4.85^{dB}	4.58^{dB}	3.16^{fD}
UM13	4.19^{cD}	5.82^{deA}	5.34^{cdB}	4.70^{cdC}	3.86^{dD}
AR1	5.20^{bD}	7.03^{bA}	6.49^{bB}	6.21^{aC}	4.97^{bE}

Values followed by the same lower/ upper case alphabets in the same column/ row are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

Table 4.6: Proteinase activity in *M. anisopliae* isolates

Isolates	**Enzyme Activity (U/ ml) as on day**				
	2	**4**	**6**	**8**	**10**
UM1	0.91^{bC}	0.96^{bB}	1.52^{bA}	1.00^{bBC}	0.92^{bC}
UM2	0.94^{bB}	0.96^{bB}	1.52^{bA}	1.03^{bB}	0.90^{bB}
UM3	0.31^{dB}	0.42^{eA}	0.40^{fA}	0.42^{dA}	0.31^{eB}
UM4	0.23^{eD}	0.73^{cB}	0.95^{cA}	0.97^{bA}	0.28^{fC}
UM5	1.10^{aC}	1.03^{aC}	1.92^{aA}	1.95^{aA}	1.67^{aB}
UM6	0.27^{deE}	0.51^{dB}	0.56^{dA}	0.47^{dC}	0.36^{dD}
UM13	0.32^{dD}	0.34^{fC}	0.39^{fB}	0.59^{cA}	0.34^{dC}
AR1	0.38^{cE}	0.48^{dC}	0.47^{eD}	0.67^{cA}	0.52^{cB}

Values followed by the same lower/ upper case alphabets in the same column/ row are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test

Table 4.7: Chitinase activity in *B. bassiana* isolates

Isolates	**Enzyme activity (U/ ml) as on day**				
	2	**4**	**6**	**8**	**10**
UB1	18.44^{bD}	45.53^{bA}	33.25^{aB}	20.92^{aC}	10.26^{bE}
UB2	19.20^{aC}	46.49^{aA}	28.59^{cB}	17.87^{bD}	8.54^{cE}
UB3	6.55^{fD}	22.93^{fA}	17.10^{fB}	11.28^{eC}	5.30^{gE}
UB4	4.34^{jB}	6.74^{lA}	6.61^{jkA}	4.15^{kB}	1.58^{kC}
UB5	5.25^{hiC}	8.08^{kA}	5.89^{kB}	5.25^{jC}	2.02^{kD}
UB6	4.88^{iE}	15.42^{jA}	13.57^{hB}	9.45^{gC}	7.95^{dD}
UB7	3.73^{kC}	6.96^{lA}	6.57^{jkA}	4.96^{jB}	1.85^{kD}
UB8	5.98^{gC}	8.25^{kA}	7.40^{jB}	7.04^{iB}	5.56^{gC}
UB9	6.70^{fD}	19.06^{gA}	12.65^{iB}	9.00^{hC}	4.07^{iE}
UB10	5.42^{ghiE}	16.24^{iA}	13.51^{hB}	10.57^{fC}	8.00^{dD}
UB11	5.79^{ghE}	16.75^{hiA}	12.50^{iB}	10.25^{fC}	7.94^{dD}
UB12	10.35^{dD}	34.10^{dA}	20.59^{eB}	11.33^{eC}	7.36^{eE}
UB13	15.33^{cC}	33.76^{dA}	22.66^{dB}	12.32^{dD}	6.62^{fE}
UB14	5.21^{hiE}	17.24^{hA}	14.70^{gB}	13.98^{cC}	11.35^{aD}
UB15	7.61^{eD}	19.15^{gA}	17.47^{fB}	11.07^{eC}	4.66^{hE}
UB16	7.51^{eD}	23.68^{eA}	17.32^{fB}	12.54^{dC}	3.38^{jE}
AB1	18.07^{bD}	42.81^{cA}	32.01^{bB}	20.60^{aC}	10.97^{aE}

Values followed by the same lower/ upper case alphabets in the same column/ row are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

Table 4.8: Proteinase activity in *B. bassiana* isolates

Isolates	**Enzyme activity (U/ ml) as on day**				
	2	**4**	**6**	**8**	**10**
UB1	0.56^{aD}	0.58^{aC}	0.75^{aA}	0.75^{aA}	0.66^{aB}
UB2	0.02^{eC}	0.11^{fAB}	0.12^{fA}	0.12^{fA}	0.11^{eB}
UB3	0.16^{dE}	0.17^{eD}	0.19^{eC}	0.23^{eA}	0.20^{dB}
UB4	0.01^{fD}	0.06^{gC}	0.11^{gB}	0.12^{fA}	0.11^{eB}
UB5	0.20^{cD}	0.24^{cB}	0.26^{cA}	0.27^{cA}	0.22^{cC}
UB6	0.22^{bC}	0.29^{bA}	0.29^{bA}	0.30^{bA}	0.25^{bB}
AB1	0.16^{dD}	0.19^{dC}	0.21^{dB}	0.24^{dA}	0.21^{cdB}

Values followed by the same lower/ upper case alphabets in the same column/ row are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

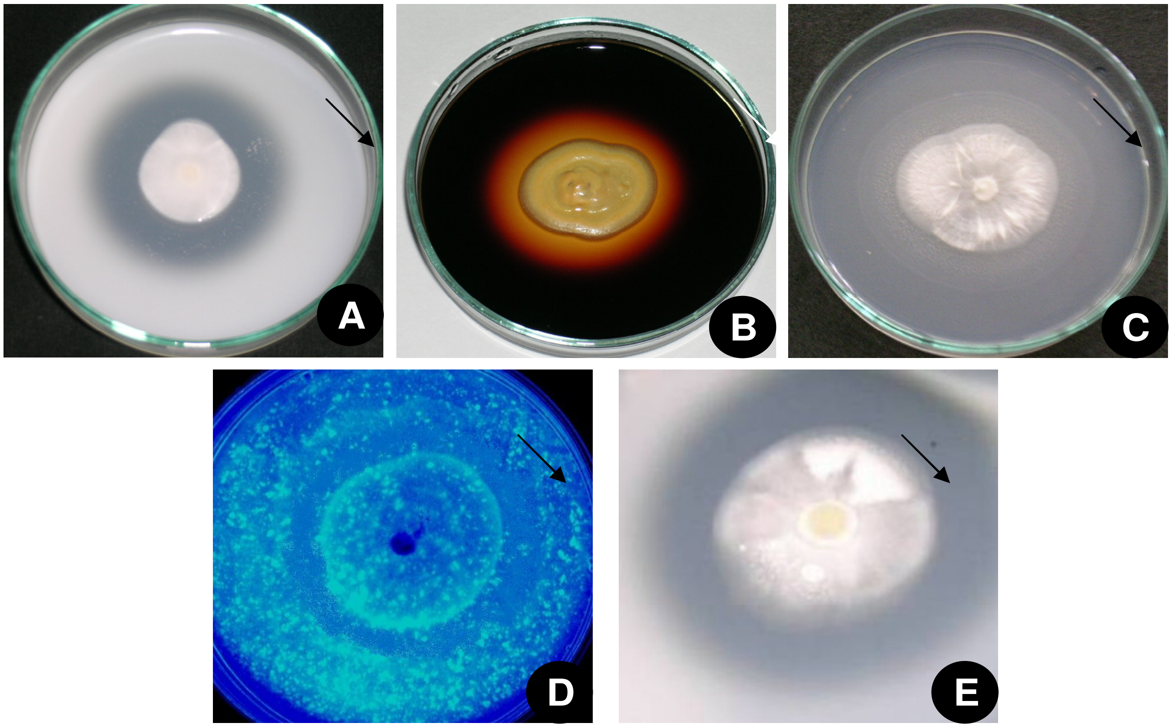


Fig. 4.1: Plate assay of extra - cellular enzymes for *B. bassaina* isolates. (A). Protease activity. (B). amylase activity. (C). Lipase activity (D). chitinase activity. (E). Caseinase activity. The zones of clearance (shown by arrow) produced by enzyme activity by the fungus.

5.1 Introduction

Since the first inception of specific synthesis of DNA *in – vitro* via a Polymerase–catalyzed Chain Reaction (Welsh and McCleland, 1990), the molecular analytical potentials of Random Amplified Polymorphic DNA (RAPD) are advocated till date for biodiversity evaluation (Karp et al., 1997). The technique addresses questions of genetic diversity at species, population and / or within population levels, and provides better understanding on the degree and distribution of genetic variation and helps in development of more efficient strategies for evaluation and conservation of biodiversity. It is one of the simplest and most economic shot for the differentiation of fungal isolates (Kaur and Padmaja, 2008; Tigano and Aljanabi, 2000; Huafeng et al., 1999; Ozino et al., 1998; Zengzhi, 1998; Maurer et al., 1997; Urtz and Rice, 1997; Leal et al., 1994; Fegan et al., 1993). The technique requires no prior knowledge of the genome under investigation and involves use of arbitrarily chosen 'random' primers of 10- 20 mers. If genomic sequence of two or more templates or test samples is different, then the PCR products display different banding patterns called 'polymorphism'. RAPD require no prior knowledge of the genome under investigation. Differences in band intensity as well as presence / absence of RAPD bands, forms the basis of diagnosis through phenetic and genetic analysis. The phenetic analysis considers all clear / reproducible bands, irrespective of whether they are diagnostic or not; whereas, in genetic analysis, an absent band corresponds to 'an unexpressed phenotypic locus'. The former involves scoring of bands as '1' for it being 'present' and '0' for it being 'absent' (Atienzar et al., 2006).

Isolate variation in terms of pathogenicity determinants and their genetic manipulations for isolate improvements are the key considerations when it comes to studies on commercial development of Entomopathogenic fungi (EPFs) based mycopesticide. This often requires handling of a large number of samples as in molecular typing studies like RAPDs, RFLPs and AFLPs. A large number of methods have been published for the extraction of total genomic DNA from filamentous fungi (Moller et al., 1992; Pfeifer and Khachatourians, 1993; Fredricks et al., 2005; Sanchez et al., 2008), some of which are microbe specific (Glee et al., 1987; Karakousis et al., 2006; Tussel et al., 2007; Francesconi et al., 2008). Cell disruption is a necessary early step in the extraction of DNA for which both chemical and physical alternatives are employed. Filamentous fungi are known to possess strong cell walls that are often resistant to traditional DNA extraction methodologies (Foster, 1992; Van Burik et al., 1997). The fungal pigments, polysaccharides and nucleases also contribute to difficulties in DNA isolation. Moreover, the previously described methods are often time consuming and take hours to days for processing when the sample size is large. Taubert et al. (2000) made a comparative study on the disintegration of filamentous fungi and identified two basic approaches for cells disruption, viz., lysis by shear mechanical forces in liquid or solid media or inducing cell wall permeability by use of enzymes (Andrews and Asenjo, 1987; Asenjo and Andrews, 1990), chemicals or physical treatments. Chemical methods typically rely on the use of detergents and chaotropes while tools for physical disruption of cells include desiccation through air-drying and lyophilization or use of mortar pestles, grinder mills, sonicators and hand held homogenizers. A variety of minipreparation methodologies have been elaborated for fungal genomic DNA isolation (Raeder and Broda, 1985; Yoon et al., 1991; Cenis, 1992). Many of these methods are not cost and time-effective where processing many samples simultaneously is required. Ample efforts have been expended to optimize the procedures for DNA extraction from various fungi, however little has been documented as to be the most apt and efficient homogenization methods.

The main objective of this chapter is to evaluate two separate populations of *B. bassiana* (17 isolates) and *M. anisopliae* (14 isolates). The isolates belong to different geographical regions and are isolated from different hosts. We have tried to correlate the nativity and / or host with the RAPD signature profiles of the isolates.

The other objective of this chapter was the incorporation of a methodology addendum which includes evaluating various cell lysis methodology means for rapid and efficient DNA extraction. The point to be noted here is that this was not the intended objective of this chapter but an overall routine observation over the routine research tenure of this thesis. Over the years, modifications for DNA extraction have evolved mainly for two reasons, viz., to simplify the existing protocols to allow processing of large number of samples, and secondly to better the quality DNA. The main idea of this procedure is to extract pure DNA from a large sample size of filamentous fungi with complex cell walls.

5.2 Materials and Methods

Fungal cultures

The different isolates of the fungus *M. anisopliae* and *B. bassiana* were either procured from ARSEF (USDA-ARS Plant Protection Unit) or isolated locally in India. The fourteen isolates of the fungus *M. anisopliae* were designated as UM1-UM13 and AR1 and the seventeen *B. bassiana* isolates were designated as UB1-UB16 and AB1. The isolates with their accession no., their geographical origin and the host insect from which they were isolated are detailed in Table 2.1 and Table 2.2. The isolates were routinely sub cultured on SDA (Sabouraud dextrose Agar) slants at 28 °C in incubators and maintained at 4 ^{o}C.

DNA extraction

For DNA extraction the isolates (seven day old culture @ approximately 10^6 spores concentration) were grown on YPD broth (yeast: peptone: dextrose in 1: 1: 4 ratio), for 72 h at 28 ^{0}C in an orbital shaker with 180 rpm. At the end of the incubation period, the mycelium was harvested and washed several times with sterile distilled water before grinding them to a fine powder in liquid nitrogen. DNA was extracted following a modified method of Lee and Taylor, (1990). Mycelia (500mg) were ground in 5 ml of lysis buffer (50 mM Tris HCl, 50 mM EDTA, 3 % SDS and 1 % β – mercaptoetanol). The mixture was incubated at 65 ^{0}C for 30 min and thereafter centrifuged to collect the supernatant, to which an equal volume of phenol: chloroform (1: 1 v/v) was added and then mixed by gentle inversion and centrifuged to collect the aqueous layer. To the aqueous phase, an equal volume of chloroform: isoamyl alcohol was added, mixed and then centrifuged. To the recovered aqueous layer, 1/ 10th volume of 3M sodium acetate and an equal volume of chilled isopropanol was added. After incubation at – 20^0 C for 30 min, the DNA pellet was recovered by centrifugation. The DNA pellet was washed with 70 % ethanol and dried in vacuum, before suspending it in 50- 100 µl of TE buffer (20 mM Tris HCl, 1mM EDTA) and stored at – 20 ^{0} C until further use.

As an addendum to the mycelium/ cell lysis part, seven different procedures of cell lysis were evaluated for their effectiveness in extracting genomic DNA from 31 isolates of the two filamentous fungi. The cultures were routinely sub-cultured on Sabouraud's Dextrose Agar (SDA) slants. For DNA extraction, the isolates were grown in YPD (yeast, peptone and dextrose in the ratio 1:1:4) for 72 hours at 28^0C. The mycelium was harvested and washed thoroughly with double distilled water. 100mg of the mycelium was taken as the starting material for each of the methods evaluated for efficiency of cell lysis towards DNA extraction. Homogenization was performed by mortar-pestles hand grinding (of freshly harvested mycelium, lyophilized samples, and liquid-nitrogen-dried samples and of lyophilized samples with addition of glass-wool), enzymatic lysis, water-bath sonication and probe-tip sonication. The underlying concept for the said techniques is detailed in Table 5.1. Purified DNA was analyzed spectrophotometrically at 260 and 280 nm to determine the yield and purity. DNA quality was confirmed by the successful PCR amplification of the conserved region of the fungal β-tubulin gene by analyzing the products on 0.8% agarose gels.

PCR amplification of DNA

A total of 80 different random primers from Operon technologies, USA (belonging to OPA 1-20, OPB 1-20, OPC 1-20 and OPF 1-20 series) were initially screened for amplification and polymorphism. The 25 µl reaction mixture consisted of 2.5 µl of PCR buffer (with 1.5mM $MgCl_2$), 1.25 µl 100 mM dNTP mixture, 0.5 µl primer (10 mM), 0.125 µl Taq DNA polymerase, 2 µl of DNA (25 -30 ng) and sterile water to make up for the final volume of 25 µl. The PCR reactions were carried out using a Thermal Cycler (Eppendorf) and the selected temperature profile included initial denaturation for 3 min at 94 °C followed by 35 cycles of denaturation for 40 sec at 94 °C, annealing for 40 sec at 65°C, extension for 1 min at 72 °C, and a final extension of 5 min at 72 °C. A 100bp ladder was used as molecular marker. The gels were stained in ethidium bromide and observed under UV. The gels were either photographed in Biorad Gel documentation system or Kodak Gel imaging system or with Sony digital camera over UV – Trans - illuminator using proper protection (the three different modes of imaging were used when one or the other was out of order).

Cluster analysis

The presence and absence of bands was characterized as a binary matrix of '1' and '0' respectively for their being present and absent. Based on the matrix data, simple matching coefficients between all possible pair combinations of isolates was calculated (Sokal and Michener, 1958). The similarity values were subjected to the Sequential

Agglomerative Hierarchical Nested (SAHN) clustering using the Unweighted Pair Group method (UPGMA), using NTSYS PC (version 2.2) to generate dendrograms.

5.3 Results and Discussion

Effect of cell lysis/ cell disruption methodology on DNA Yield and purity

Table 5.1 enlists the comparison of the different sample disruption procedures used for DNA extraction. Mortar and pestles hand grinding of the of freshly harvested mycelium, lyophilized samples, liquid-nitrogen-dried samples and of lyophilized samples with addition of glass-wool, resulted in loss of sample recovery, was more laborious when handling large number of samples and called for much higher amounts of starting material to get appreciable yield. Incubation of the sample with lysing enzyme from *Trichoderma harzianum* gave an appreciable yield of high quality DNA but then this incurred an additional cost of the enzyme and more so when the sample size is large. Water bath sonication which was carried in microfuge tube called for higher homogenization time and also demanded more amount of lytic solution per unit amount of the starting material, thus limiting the efficiency. Probe-tip sonication method offered efficiency in terms of rapidity but it calls for control in terms of amplitude and pulsar. The processing was carried out in microfuge tubes kept on ice bath so as to minimize extensive heating. Optimal results were obtained when sonication was performed at an amplitude of 25-30 (in no case should it increase beyond 35 so as to prevent shearing) and a pulsar of 10 sec on and 5 sec off cycle of 2 minutes per sample. The yield was almost the same irrespective of weather the 100 mg of the starting material was lyophilized, liquid-nitrogen dried or fresh centrifuged pellet (Fig. 1). The final DNA solution in molecular biology grade water (Sigma) was viscous and clear. All DNA isolated had OD_{260}/OD_{280} ratios between 1.5 and 1.9. An average DNA concentration of 1.5-2.0 µg/µl could be recorded for all the isolates (data not shown). The average yield of DNA for the *M. anisopliae* isolates was in the range 70-120 µg per 100 mg of the tissue taken as the starting material, while for *B. bassiana* isolates it was 60-100 µg per 100 mg of the tissue. The purity of the DNA preparations was further confirmed by successful PCR amplification of the conserved region of the fungal β-tubulin gene (Figure 6.1 and 6.2).

The major impending challenges for low quality and low quantity of DNA extraction is often due to cell walls or capsule that are not readily susceptible to lysis. The resistance to disruption of microorganisms has been previously referred (Wimpenny et al., 1967; Chisti and Moo-Young, 1986; Van Gaver and Huyghebaert, 1990; Foster, 1992). DNA isolation from fungus requires cell disintegration by physical, chemical or enzymatic means and such cell disruption techniques have been studied for many years. Some of these methods are suitable only for small sample sizes and some even adversely affect the DNA like producing low yield and/ or the quality is compromised or require large amount of starting material or are time consuming. The rapid methods are often expensive, involving use of enzymes like lyticase and proteinase K (Sanchez at al., 2008) or use of specialized high-throughput columns and homogenizers (Specht et al., 1982; Saunders et al., 1984; Branovic et al., 2004). Our results demonstrate that different DNA extraction methods may produce dramatically different yields of fungal DNA. The modified fungal cell wall lysis by controlled probe-tip sonication method used in this study, effectively released DNA from both *Metarhizium anisopliae* and *Beauveria bassiana* isoltes. The experiment provides another effective fungal DNA isolation procedure to complement several existing methods for use with other fungi.

Polymorphism

During the initial screening it was observed that most of the primers could amplify at least two members of each of the populations. But after careful selection we identified that out of the eighty random primers, five (OPA-18, OPB-12, OPB-19, OPC-16, OPF-12) of them produced polymorphic profile in *M. anisopliae* isolates (Table 5.2), while seven of them (OPA-13, OPA-19, OPB-07, OPC-12, OPC-20, OPF-09, OPF-17) showed reproducible polymorphism in *B. bassiana* isolates (Table 5.3).

Cluster analysis

M. anisopliae

The cluster analysis of *M. anisopliae* is shown in Fig. 5.14. It shows three main clusters bifurcating at 0.54 similarities. The similarity coefficient of *M. anisopliae* isolates as revealed by SAHN/UPGMA analysis is detailed in Table 5.4 and the summary of the relationship between the experimentally observed clustering and geographic origin is depicted in Table 5.5. Cluster 1 has 3 clads. The first clad has isolate UM1 and UM2 with, the second clad has isolates UM3 and UM6. Isolate UM4 and UM7 with 100% similarity form the 3rd clad. The isolates in 1st clad belong to different geographic regions and hence cannot be affirmed for origin similarity. The association of fungal isolates with host insect from which it was isolated bears positive relationship as 4 of the isolates were isolated from Hemiptera. Cluster 2 comprises of 3 clads and it was observed that the isolates bear similarity in origin (as 4 out of 5 isolates had Indian origin); and the members of the cluster also depicted host similarity. The 3rd cluster comprised of isolates UM9 only. Cluster 2 and cluster 3 analyzed together show origin and host similarity. To conclude the cluster pattern, it can be said that the *M. anisopliae* isolates belonging to same habitat were grouped in similar clusters. The cluster pattern also depicted the grouping of isolates with either common host insect from which it was isolated or from the same order of the insect from which it was isolated.

B. bassiana

The *B. bassiana* isolates formed 3 clusters separating at similarity 0.07 (Fig.5.15). The similarity coefficient of *B. bassiana* isolates as revealed by SAHN/UPGMA analysis is detailed in Table 5.6 and the summary of the relationship between the experimentally observed clustering and geographic origin is depicted in Table 5.7. Cluster 1 has 7 clads of 12 isolates showing certain limited degree of similarity in origin (as 7 out of 12 were from India); as well as some extent of similarity in their source (as half the population was isolated from Lepidoptera while half of them were isolated from coleopteran host. Cluster 2 had 4 isolates in 3 clads and did not bear any similarity with respect to their host, but had only partial relationship with respect to the country of origin. Cluster 3 with its only isolate (UB5) when accounted together with cluster2 bears similarity of habitat.

PCR provides selective amplification of a specific DNA segment from an organism. Fungaro et al. (1996) studied diversity among soil and insect isolates of *M. anisopliae var. anisopliae* by RAPD. The isolates were grouped by cluster analysis using Dice similarity index. The results showed that isolates of *M. anisopliae var. anisopliae* were extremely diverse but those isolated from *D. flavopicta* presented only a moderate degree of variation. Cobb and Clarkson, (1993) also studied DNA polymorphism in *M. anisopliae* and *M. flavoviride*. DNA fragments differed in size and number of fragments between isolates indicating considerable polymorphism. Bidochka et al. (1994) also studied PCR based technology for assessing the genomic variability between 24 isolates of *M. flavoviride* and *B. bassiana*. Castrillo and Brooks (1998) studied the two natural genetic markers, isozymes and RAPD to detect variation of *B. bassiana* isolates. RAPD markers provide better resolution of the differences between the isolates. Riverera et al. (1997) and Valdderrama et al. (2000) found no correlation between the clusters obtained by RAPD analysis of the insect host and the pathogenicity of *Hypothenemus hampei.* The existence of isolates with similar DNA fingerprints, like UM1 and UM2 (from India and Philippines respectively) implies that similar changes in the DNA sequences might have occurred independently in the said isolates at two different locations. Padmavathi et al. (2003) observed absolute similarity in *M. anisopliae* isolate ARSEF 1166 and ARSEF 1512 although they belonged to different geographic habitat. Isolate UB1 and UB2 had 100% similarity were from two different locations.

Conclusions

Controlled sonication using a probe tip was deduced to be the most efficient method for sample homogenization. The comparison of RAPD clustering suggests more association of host and origin amongst *M. anisopliae* isolates and only partial association relationship with *B. bassiana* isolates.

Table 5.1: Comparison of the different lysis procedures used for DNA extraction

	Methods *	Observation/ remarks	Average yield of DNA**
1	Hand grinding of freshly harvested mycelium	Grinding and crushing have less deleterious effect on DNA than shearing but the yield was lowest. Sample recovery is also way too lesser than the starting material.	4-10 for both *M. anisopliae* and *B. bassiana* isolates.
2	Hand grinding of lyophilized samples	Lyophilized and liquid-nitrogen-dried samples presented an optimal surface to volume ratio, thus allowing rapid contact with the extraction solution.	87.81 (*M. anisopliae*) 66.76 (*B. bassiana)*
3	Hand grinding of liquid-nitrogen-dried samples	It is critical that the tissue powder remain frozen and moisture condensation is minimized particularly when working with small samples as condensed water may contribute significantly to the weight of the tissue.	86.74 (*M. anisopliae*) 71.25 (*B. bassiana)*
4	Hand grinding of lyophilized samples with glass-wool	Inclusion of glass-wool showed to improve the yield efficiency. The amount of disruption depended on the processing time. If the cell grinding exceeds a certain threshold (which was isolate dependent) then shearing of the genomic DNA takes place, greatly hampering its quality.	94.55 (*M. anisopliae*) 71.58 (*B. bassiana)*
5	Enzymatic digestion with lytic enzymes from *Trichoderma viridi*	Digestion with lytic enzyme prior to mortar and pestle grinding gave a very good yield.	100.71 (*M. anisopliae*) 77.90 (*B. bassiana)*
6	Water-bath sonication	The vibrational shearing did not lyse cell walls for appreciative DNA isolation.	4-10 for both *M. anisopliae* and *B. bassiana* isolates.
7	Probe-tip sonication	This method implied physical contact of the probe with the sample and thus was more efficient in terms of rapidity but it calls for control in terms of amplitude and pulsar and care also called for placing the microfuge tube on ice-bath so as to minimize heat generation. The major advantage of the technique was that cells from any phase of growth in either yeast form or hyphal form could be disrupted efficiently and initial lyophilization step could be done away with.	104.06 (*M. anisopliae*) 78.86 (*B. bassiana)*

* Hand grinding was done using the conventional mortar and pistils.

** Average yield (in µg per 100mg of the starting material) of all the *Metarhizium* and *Beauveria* isolates is considered to evaluate the efficiency of the methods.

Table 5.2. The primers along with sequences and the isolates of *M. anisopliae* which responded to RAPD

Primer	**Sequence (5' to 3')**	**Isolates**
OPA-18	AGGTGACCGT	UM1 – 13 & AR1 (all 14)
OPB-12	CCTTGACGCA	UM1 – 13 & AR1 (all 14)
OPB-19	ACCCCCGAAG	UM1 – 13 & AR1 (all 14)
OPC-16	CACACTCCAG	UM1 – 13 & AR1 (all 14)
OPF-12	ACGGTACCAG	UM1 – 13 & AR1 (all 14)

Table 5.3. The primers along with sequences and the isolates of *B. bassiana* which responded to RAPD

Primer	Sequence (5' to 3')	Isolates
OPA-13	CAGCACCCAC	UB1, UB2, UB6, UB10, UB12, UB16 & UB 17
OPA-19	CAAACGTCGG	UB1, UB2, UB5, UB10, UB12, UB15 UB16 & UB17
OPB-07	GGTGACGCAG	UB1, UB2, UB6, UB10, UB11, UB14 & UB16
OPC-12	TGTCATCCCC	UB2, UB3, UB5, UB6, UB9, UB10 UB11 UB12, UB13 & UB17
OPC-20	ACTTCGCCAC	UB2, UB3, UB4, UB6, UB8, UB9, UB10, UB13, UB15, UB16 & UB17
OPF-09	CCAAGCTTCC	UB2, UB6, UB9, UB10, UB11 UB12 & UB16
OPF-17	AACCCGGGAA	UB1, UB2, UB4, UB7, UB10, UB12 UB15 & UB17

Table 5.4: Similarity coefficient of *M. anisopliae* isolates as revealed by SAHN/ UPGMA analysis

	UM1	UM2	UM3	UM4	UM5	UM6	UM7	UM8	UM9	UM10	UM11	UM12	UM13	AR1
UM1	1.00													
UM2	0.81	1.00												
UM3	0.73	0.69	1.00											
UM4	0.71	0.81	0.80	1.00										
UM5	0.41	0.50	0.58	0.55	1.00									
UM6	0.76	0.71	0.90	0.83	0.53	1.00								
UM7	0.71	0.81	0.73	0.93	0.48	0.76	1.00							
UM8	0.55	0.50	0.58	0.55	0.87	0.53	0.55	1.00						
UM9	0.48	0.58	0.44	0.64	0.54	0.46	0.64	0.54	1.00					
UM10	0.50	0.37	0.60	0.43	0.76	0.55	0.43	0.83	0.56	1.00				
UM11	0.59	0.54	0.55	0.44	0.64	0.57	0.44	0.64	0.67	0.81	1.00			
UM12	0.65	0.67	0.61	0.52	0.63	0.63	0.52	0.69	0.57	0.65	0.60	1.00		
UM13	0.59	0.54	0.48	0.52	0.79	0.50	0.52	0.93	0.50	0.74	0.62	0.67	1.00	
AR1	0.67	0.70	0.69	0.75	0.56	0.72	0.83	0.64	0.57	0.50	0.52	0.59	0.61	1.00

Table 5.5: Summary of cluster analysis and geographic and host relationship of *M. anisopliae* isolates.

Cluster no.	Clad no.	Isolate	Geographic origin	Origin similarity	Host insect	Host similarity
1	1	UM1	India	No	*Nilaparvata lugens* (Hemiptera)	To some extent (4 isolates belong to hemiptera while 2 to Lepidoptera)
		UM2	Philippines		*Spodoptera* sp. (Lepidoptera)	
	2	UM3	Indonesia		*Nephotettix virescens* (Hemiptera)	
		UM6	India		*Pyrausta machaeralis* (Lepidoptera)	
	3	UM4	Indonesia		Lepidoptera larva	
		UM7	USA		*Helicoverpa zea* (Lepidoptera)	
		AR1	India		Unknown	
2	1	UM5	India	Yes	Coleoptera	Yes (as most belong to hemiptera)
		UM8	India		*Nilaparvata lugens* (Hemiptera)	
		UM13	India		*Nilaparvata lugens* (Hemiptera)	
	2	UM10	Mexico		*Anticarsia gemmatalis* (Lepidoptera)	
		UM11	India		*Nilaparvata lugens* (Hemiptera)	
	3	UM12	India		*Nilaparvata lugens* (Hemiptera)	
3	1	UM9	India	Sole isolate so no comparison	*Nilaparvata lugens* (Hemiptera)	Sole isolate so no comparison

Table5.6: Similarity coefficient of *B. bassiana* isolates as revealed by SAHN/ UPGMA analysis.

	UB1	UB2	UB3	UB4	UB5	UB6	UB7	UB8	UB9	UB10	UB11	UB12	UB13	UB14	UB15	UB16	AB1
UB1	1.00																
UB2	0.81	1.00															
UB3	0.00	0.11	1.00														
UB4	0.35	0.30	0.50	1.00													
UB5	0.14	0.00	0.00	0.00	1.00												
UB6	0.55	0.48	0.00	0.00	0.17	1.00											
UB7	0.24	0.30	0.00	0.40	0.00	0.00	1.00										
UB8	0.00	0.00	0.80	0.57	0.00	0.00	0.00	1.00									
UB9	0.00	0.00	0.57	0.44	0.33	0.29	0.00	0.67	1.00								
UB10	0.56	0.57	0.00	0.24	0.09	0.60	0.16	0.00	0.08	1.00							
UB11	0.00	0.17	0.18	0.00	0.20	0.33	0.00	0.00	0.17	0.43	1.00						
UB12	0.67	0.67	0.11	0.30	0.12	0.64	0.20	0.00	0.21	0.69	0.35	1.00					
UB13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00	1.00				
UB14	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.18	0.40	0.00	0.00	1.00			
UB15	0.25	0.21	0.00	0.44	0.00	0.00	0.44	0.00	0.00	0.25	0.00	0.21	0.33	0.00	1.00		
UB16	0.45	0.40	0.00	0.00	0.00	0.80	0.00	0.00	0.14	0.53	0.22	0.56	0.17	0.33	0.14	1.00	
AB1	0.50	0.44	0.00	0.12	0.00	0.45	0.24	0.00	0.00	0.44	0.00	0.44	0.29	0.00	0.38	0.55	1.00

Table 5.7: Summary of cluster analysis and geographic and host relationship of *B. bassiana* isolates.

Cluster no.	Clad no.	Isolate	Geographic origin	Origin similarity	Host insect	Host similarity
1	1	UB1	Spain	To some extent	*Helicoverpa virescens* (Lepidoptera)	To some extent (as half of them belong to Lepidoptera and half to coleoptera)
		UB2	Philippines		*Cnaphalocrocismedinalis* (Lepidoptera)	
	2	UB10	USA		*Coccinella* sp. (Coleoptera)	
		UB12	India		*Chilo infuscatellus* (Lepidoptera)	
	3	UB6	India		*Spodoptera litura* (Lepidoptera)	
		UB16	India		Adult Coleoptera	
	4	AB1	India		Unknown	
	5	UB7	Denmark		*Coccinella septumpunctata* (Coleoptera)	
		UB15	India		*Spodoptera litura* (Lepidoptera)	
	6	UB11	Denmark		Coccinella *septempunctata* (Coleoptera)	
		UB14	India		*Basilepta fulvicornis* (Coleoptera)	
	7	UB13	India		*Xyloryctes jamaicensis* (Coleoptera)	
2	1	UB3	USA	To some extent	*Bemicia tabaci* (Hemiptera)	No
		UB8	Spain		*Helicoverpa armigera* (Lepidoptera)	
	2	UB9	USA		*Coccinella* sp. (Coleoptera)	
	3	UB4	India		*Emmalocera depressella* (Lepidoptera)	
3	1	UB5	India	Sole isolate so no comparison	*Hyblaea puer* (Lepidoptera)	Sole isolate so no comparison

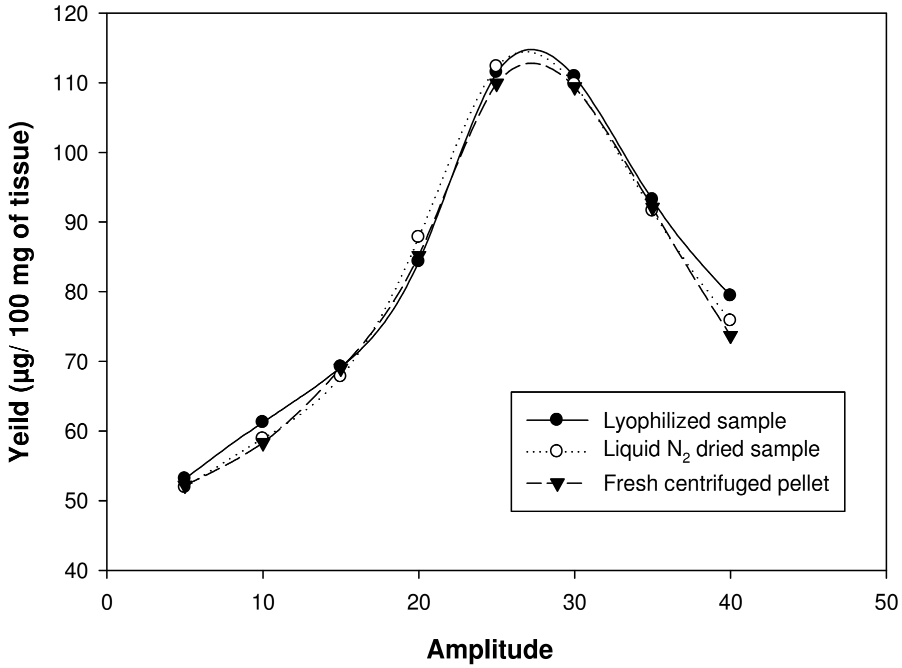


Fig. 5.1: Dependence of amplitude on DNA yield by probe-tip homogenization for mycelium-lysis

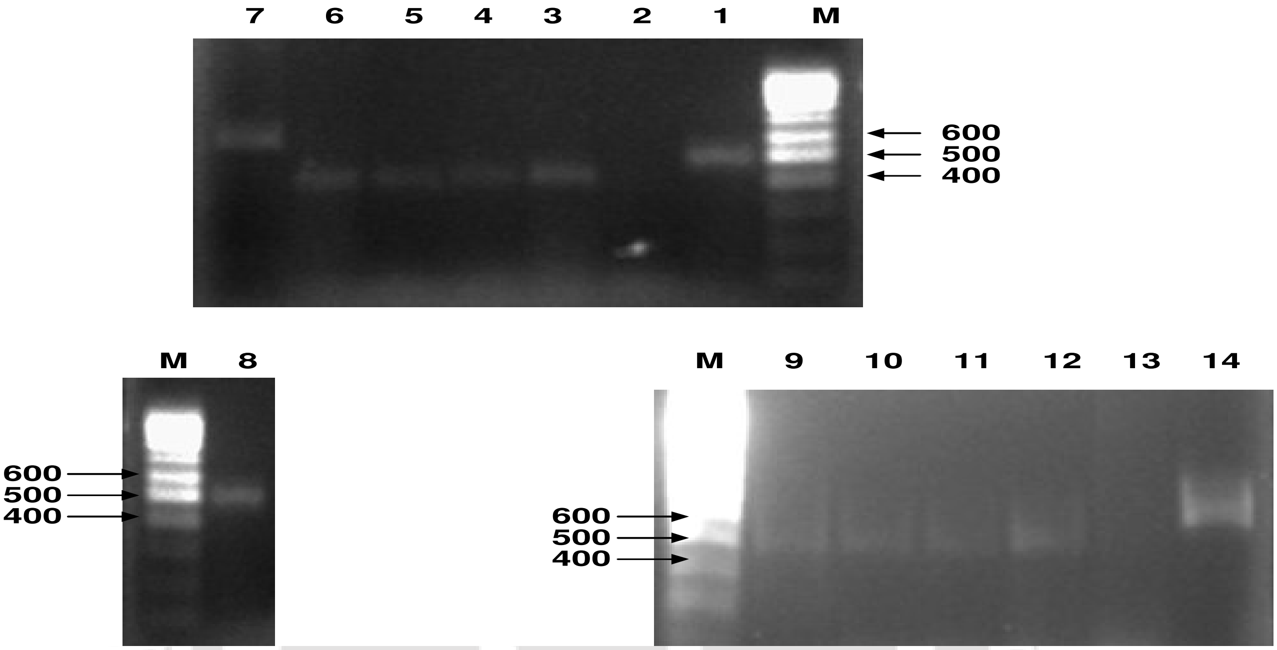


Fig. 5.2: Polymorphism in *M. anisopliae* isolates by OPA 18 as visualized on 1.5% agarose gel. M: 100bp marker; lane 1-14 are isolates UM1-13 and AR1.

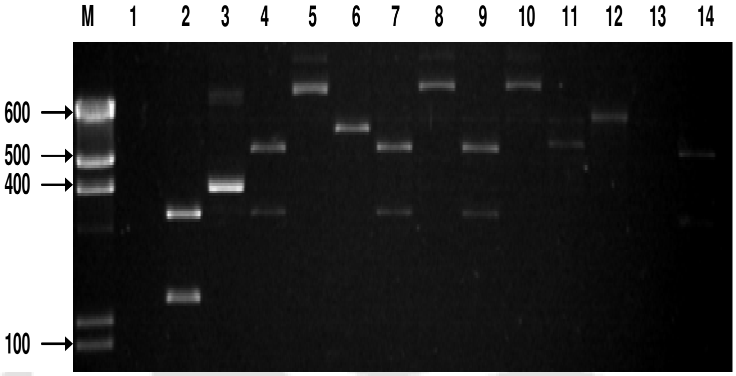


Fig. 5.3: Polymorphism in *M. anisopliae* isolates by OPB 12 as visualized on 1.5% agarose gel. M: 100bp marker; lane 1-14 are isolates UM1-13 and AR1.

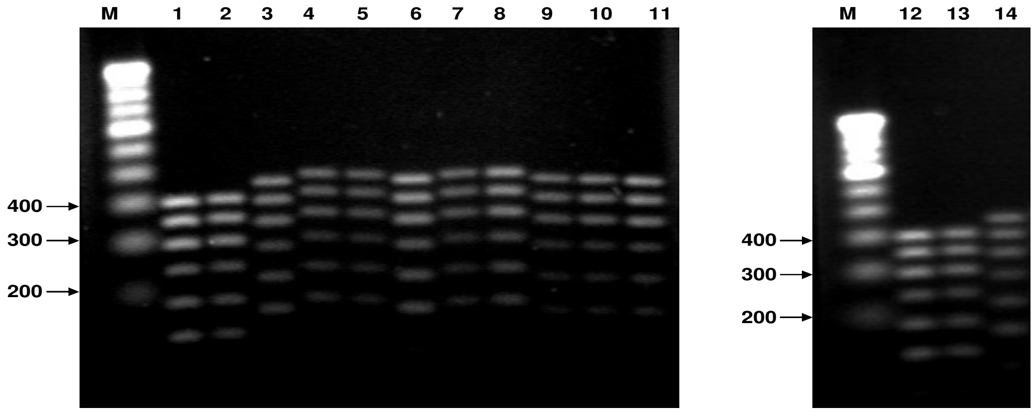


Fig. 5.4: Polymorphism in *M. anisopliae* isolates by OPB 19 as visualized on 1.5% agarose gel. M: 100bp marker; lane 1-14 are isolates UM1-13 and AR1.

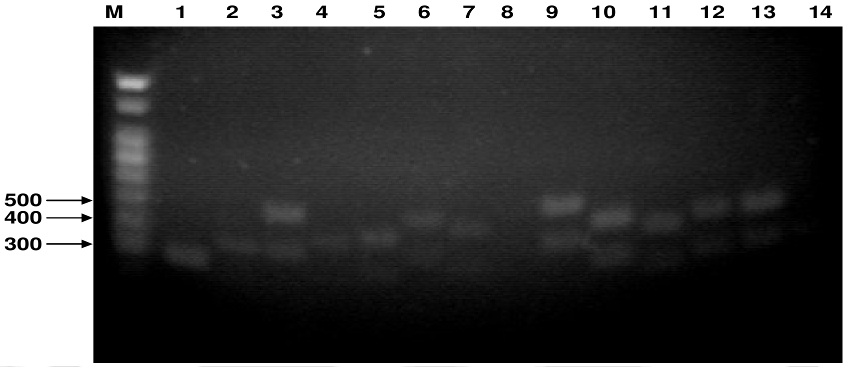


Fig. 5.5: Polymorphism in *M. anisopliae* isolates by OPC 16 as visualized on 1.5% agarose gel. M: 100bp marker; lane 1-14 are isolates UM1-13 and AR1.

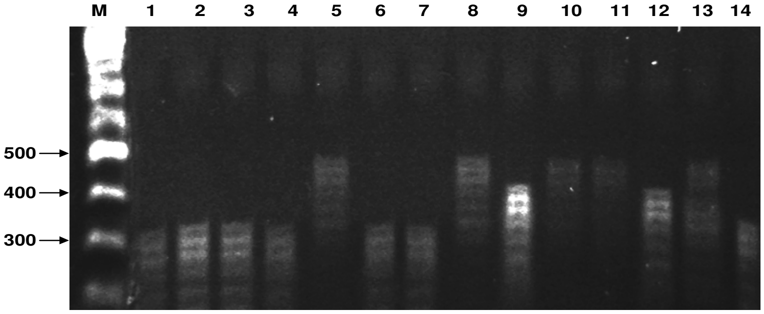


Fig. 5.6: Polymorphism in *M. anisopliae* isolates by OPF 12 as visualized on 1.5% agarose gel. M: 100bp marker; lane 1-14 are isolates UM1-13 and AR1.

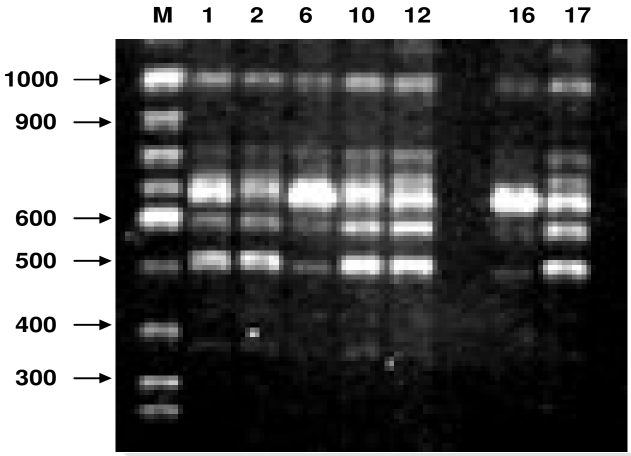


Fig. 5.7: Polymorphism in *B. bassiana* isolates by OPA 13 as visualized on 1.5% agarose gel. M: 100bp marker; the numbers on lanes represent the *B. bassiana* isolate number (UB).

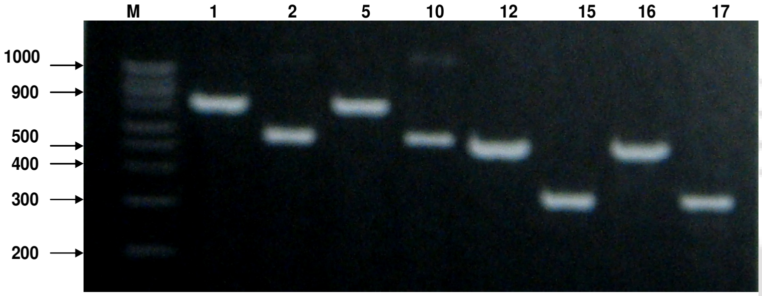


Fig. 5.8: Polymorphism in *B. bassiana* isolates by OPA 19 as visualized on 1.5% agarose gel. M: 100bp marker; the numbers on lanes represent the *B. bassiana* isolate number (UB).

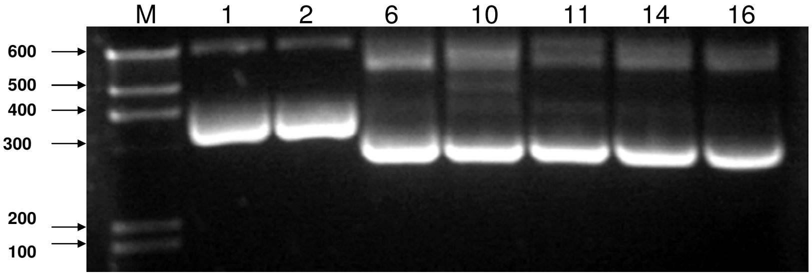


Fig. 5.9: Polymorphism in *B. bassiana* isolates by OPB 07 as visualized on 1.5% agarose gel. M: 100bp marker; the numbers on lanes represent the *B. bassiana* isolate number (UB).

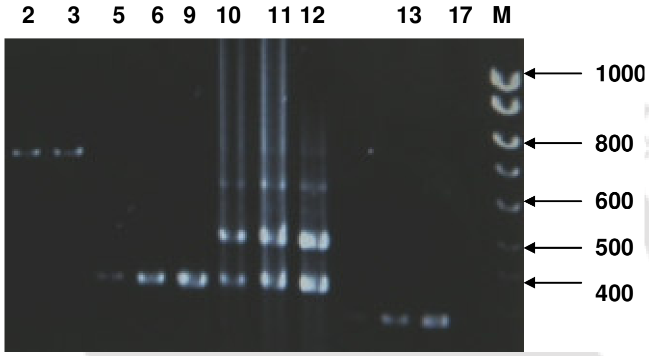


Fig. 5.10: Polymorphism in *B. bassiana* isolates by OPC 12 as visualized on 1.5% agarose gel. M: 100bp marker; the numbers on lanes represent the *B. bassiana* isolate number (UB).

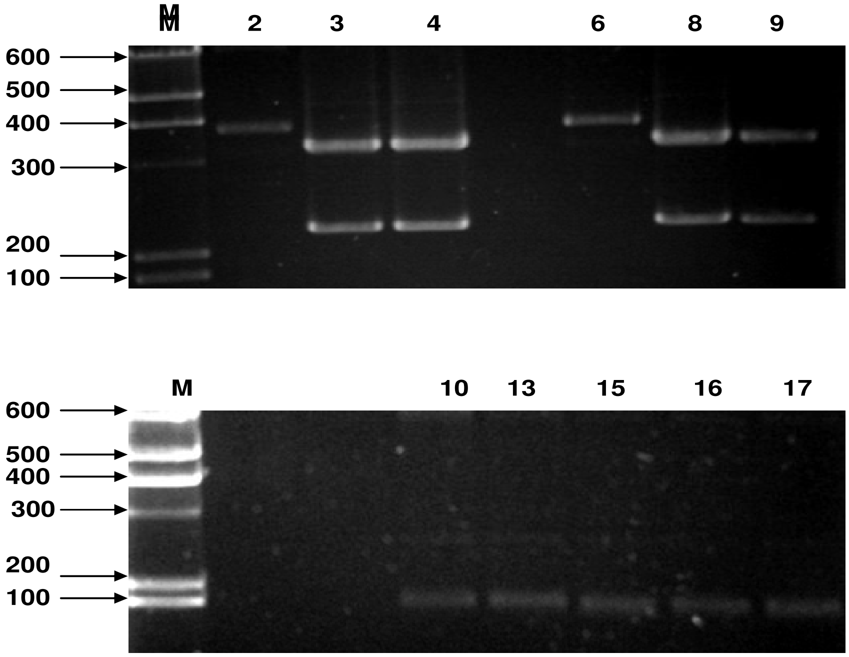


Fig. 5.11: Polymorphism in *B. bassiana* isolates by OPC 20 as visualized on 1.5% agarose gel. M: 100bp marker; the numbers on lanes represent the *B. bassiana* isolate number (UB).

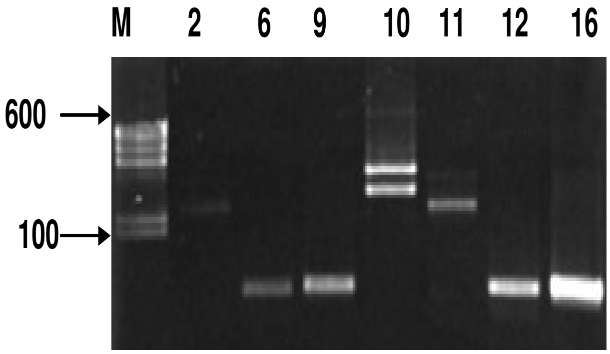


Fig. 5.12: Polymorphism in *B. bassiana* isolates by OPF 09 as visualized on 1.5% agarose gel. M: 100bp marker; the numbers on lanes represent the *B. bassiana* isolate number (UB).

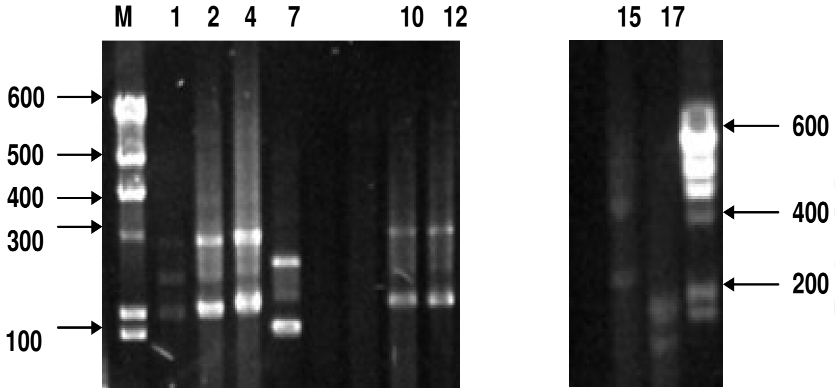


Fig. 5.13: Polymorphism in *B. bassiana* isolates by OPF 17 as visualized on 1.5% agarose gel. M: 100bp marker; the numbers on lanes represent the *B. bassiana* isolate number (UB).

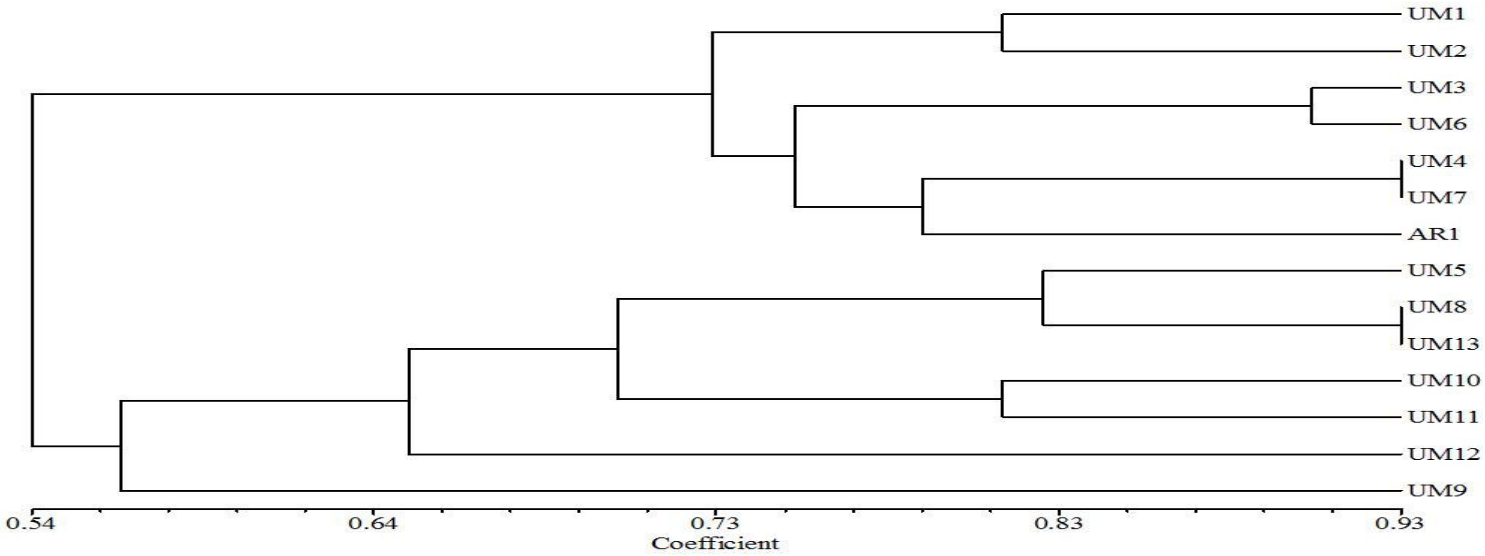


Fig. 5.14: Phenogram of 14 *M. anisopliae* on basis of performed RAPD analysis

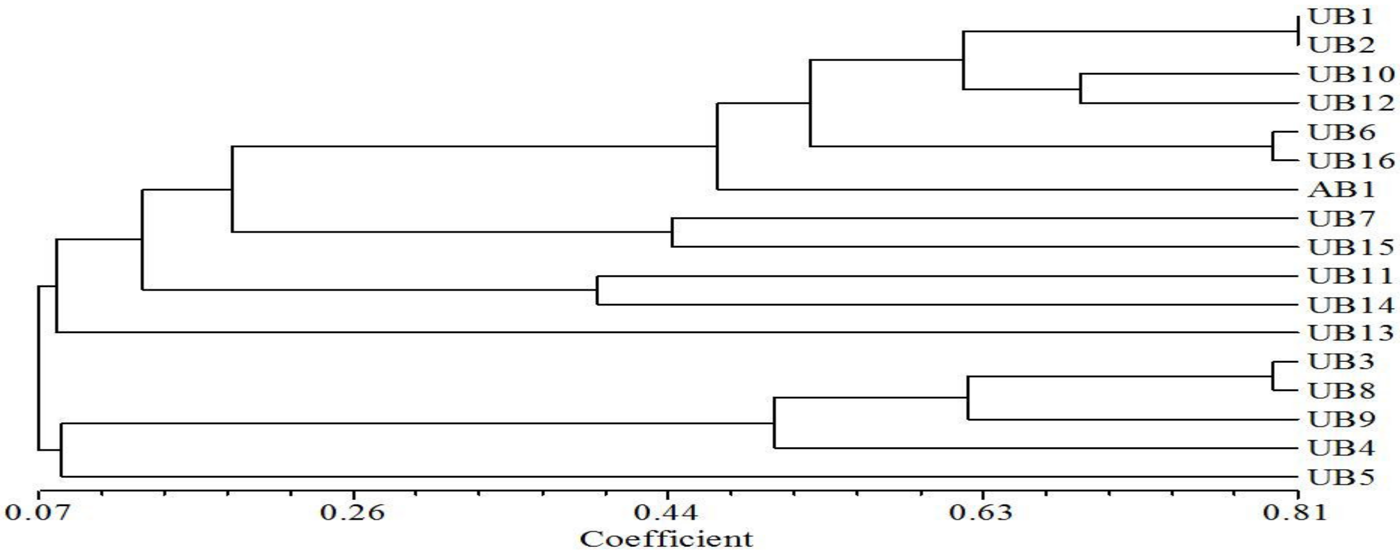


Fig. 5.15: Phenogram of 17 *B. bassiana* on basis of performed RAPD analysis

6.1 Introduction

The breakthrough development of RFLP markers has significantly revolutionized and impacted the microbial genetics, including those of entomopathogenic fungi (Destefano et al., 2004; Han et al., 2002; Mavridou et al., 2000; Couteaudier and Viaud, 1997; Neuveglise and Brygoo, 1994). The technique has been widely employed to clarify the relationship among genetic diversity, virulence and other characteristics of conidia (Sugimoto et al., 2003), with varying degree of relationship resolvement. There are 'universal' primers commercially available, which can target DNA regions that are either relatively conserved or rapidly evolving, depending on the amount of variation observed and the taxonomic level under examination. The PCR products obtained can be digested with restriction enzymes and visualized by simple staining with ethidium bromide. The approach undoubtedly aids in detection of polymorphism and variation amongst isolates but there have been evidences of major similarity too when the gene complex under study is conserved. There have been evidence of conservation of ITS1 – 5.8S – ITS2 region among *M. anisopliae* isolates (Mavridou et al., 2000). Molecular markers have been used widely to consider host pathogen relationships among microbes (Power, 1996). The cardinal proposition of all the molecular typing schemes is that isolates from an epidemiological cluster arise from a common ancestor and therefore, they will share certain characteristics that can distinguish them from epidemiologically unrelated isolates of the same species. The suitability of the techniques for determining the relationships is generally only assumed. Maurer et al. (1997) studied the genetic diversity of *B. bassiana* and their relatedness to host insect range. The fungal isolates from various geographical sites were examined by RFLP and RAPD analysis. Similar groupings were recovered from both the approaches and indicated a conspicuous relationship between the population structure of the fungal isolates and some defined host species. The objective of this chapter is to evaluate two separate populations of *B. bassiana* (17 isolates) and *M. anisopliae* (14 isolates), using the RFLP analysis of its β – tubulin gene. The isolates belong to different geographical regions and are isolated from different hosts. We have tried to correlate the nativity and host insect with the RFLP signature profiles of the isolates. Sugimoto et al. (2003) characterized 46 isolates of *Verticillium lecanii* using RFLP of the β – tubulin gene.

6.2 Materials and Methods

Fungal cultrures

Refer chapter 2 (Tables 2.1 and 2.2)

DNA extraction

Refer chapter 5.

DNA amplification

Genomic DNA was extracted from fungal mycelia as described by Lee and Taylor (1990). The β - tubulin regions were amplified using the forward and reverse primers, Bt2a ((5'GGTAACCAAATCGGTGCTGCTTT3') and Bt2b (ACCCTCAGTGTAGTGCCCTTGGC3'). The PCR reactions were carried out using a Thermal Cycler (Eppendorf) using 25 µL reaction volumes, each containing

25 ng of template DNA, 1X Taq buffer, 0.4 mM of each primer Bt2a and Bt2b, 200 mM of dNTP mix and 1U of Taq DNA polymerase (Fermentas). The selected temperature profile included initial denaturation for 3 min at 94 °C followed by 35 cycles of denaturation for 40 sec at 94 °C, annealing for 40 sec at 65°C, extension for 1 min at 72 °C, and a final extension of 5 min at 72 °C.

RFLP analysis of PCR amplified DNA

The β-tubulin regions of the two populations were amplified and examined on 1.5% agarose gels. The PCR products (3 µL) were individually digested with restriction endonucleases Alu I, Hae III, Hpa II, Pst Iand EcoR I under conditions specified by the manufacturer (Fermentas) and the restriction fragments separated by electrophoresis in 8% poly- acrylamide gels using 0.5X TAE buffer (Sambrook *et al.*, 1989). The gels were stained with ethidium bromide and then visualized under UV.

Cluster analysis

The presence and absence of bands was characterized as a binary matrix of ‘1’ and ‘0’ respectively for their being present and absent. Based on the matrix data, simple matching coefficients between all possible pair combinations of isolates was calculated (Sokal and Michener, 1958). The similarity values were subjected to the Sequential Agglomerative Hierarchical Nested (SAHN) clustering using the Unweighted Pair Group method (UPGMA), using NTSYS PC (version 2.2) to generate dendrograms.

6.2 Results and Discussion

β-tubulin amplification products

A single β – tubulin gene product was observed for all the isolates in both the populations. For *M. anisopliae* isolates, size polymorphisms of the β-tubulin amplification products were observed. All the *M. anisopliae* showed β - tubulin amplification product of 400 bp with exception of isolate UM6 and AR1 which showed amplified product of 500 bp (Fig.6.1). The *B. bassiana* isolates on the other hand showed a β - tubulin amplification product of 500 bp (Fig. 6.2).

Restriction analysis of β-tubulin amplified products

In the initial trials we used five restriction enzymes viz. Alu I, Hae III, Hpa II, Pst Iand EcoR I, but finally chose Alu I and Hpa II for restriction digestion of *M. anisopliae* β – tubulin product and Alu I and Hae III for restriction digestion of *B. bassiana* β – tubulin amplification product.

The restriction profile of *M. anisopliae* digested by Alu I is shown in Fig. 6.3 with three distinct banding patterns. The Hpa II restriction digestion also showed three distinct patterns for *M. anisopliae* isolates (Fig. 6.4). The restriction digestion pattern of β-tubulin amplified product of *B. bassiana* isolates by Alu I showed three distinct patterns (Figure 6.5), with product size ranging from < 100 to 400 bp (< 100 to 450 bp for isolate UM6 and AR1) while that digested by Hae III showed four distinct patterns, with size ranging from <100 to 280 bp. The bands lower than 100 bp mark of the molecular ladder was not the primer dimer but a conspicuous band of varying intensity amongst the isolates. The band polymorphism was scored as a binary matrix of ‘1’ and ‘0’ for the band being present and absent respectively.

Cluster analysis of *M. anisopliae* isolates

The cluster analysis phenogram of *M. anisopliae* is shown in Fig. 6.7. The cluster analysis by SAHN / UPGMA tree showed three main clusters bifurcating at 0.86 similarities. The similarity coefficient of *M. anisopliae* isolates as revealed by SAHN/ UPGMA analysis is detailed in Table 6.1 and the summary of the relationship between the experimentally observed clustering and geographic origin and host is depicted in Table 6.2. The first cluster had isolate UM1 and UM7 in 1st clad and isolate UM6 and AR1 in the 2nd clad with absolute similarity (similarity coefficient 1.00). The isolates had geographical similarity (3 out of 4 isolates belong to India) and also host similarity to an extent as 2 out of 4 isolates had order Lepidoptera as host insects while for one of the isolate the order was unknown. Cluster 2 had one clad with similarity coefficient of 1.00 for all its members and comprised of isolates UM 4, UM5, UM8, UM9, UM11, UM12 and UM13. All the isolates of cluster 2 had origin similarity (as 6 out of 7 isolates belong to India.) and host similarity (as 5 out of 7 isolates have *Nilaparvata lugens* as host). The third cluster had isolate UM2 in clad 1 and isolates UM3 and UM10 in clad 2. Isolate UM2 belongs to Philippines and isolate UM3 belongs to Indonesia, and since these two countries fall under the same geographic belt, we can consider them to have geographic similarity. Similarly, the isolates can be considered to have host similarity as 2 out of 3 isolates have order Lepidoptera as host (Table 6.2). Thus on the basis of similarity of clusters (similarity coefficient, origin and host) we can conclude that the overall phenogram of *M. anisopliae* shows a high index of similarity. The restriction profiles of the isolates shows less variation, which is suggestive of conservation of β – tubulin gene.

Cluster analysis of *B. bassiana* isolates

The cluster analysis phenogram of *B. bassiana* is shown in Figure 6.8, and it shows three main clusters bifurcating at 0.65 similarities. The similarity coefficient of *B. bassiana* isolates as revealed by SAHN/ UPGMA analysis is detailed in Table 6.3 and the summary of the relationship between the experimentally observed clustering and geographic origin and host is depicted in Table 6.4. The first cluster comprised of 5 clads. Clad 1 had isolate UB1. Clad 2 consisted of isolate UB2, UB3, UB4, UB5, UB8 and UB9, which showed absolute similarity coefficient of (1.00). The members of clad 2 did not have any origin similarity but exhibited host similarity to an extent as 4 out of 6 isolates have Lepidoptera hosts. But here, the similarity coefficient is an index of the restriction digestion pattern, and it being absolutely similar is suggestive of conservative nature of β – tubulin region. Clad 3, 4 and 5 had certain extent of host similarity (as the host insects belong to same order) and also similarity of origin as 4 out of 6 isolates have similar origin, India. Cluster 2 comprised of isolates UB12, UB15 and UB16 and exhibited similar geographic origin and host range. Cluster 3 had single isolate UB7.

Comparison between cluster and clad similarity

In both the populations, the individual clads of each cluster were compared for their possible relationship between their similarity coefficient (the basis of cladding; refer

Figure 6.7 and 6.8 for details), and geographic origin and host range. In case of *M. anisopliae*, all the clads under each cluster showed an overall similarity with the geographic origin of the fungus and its insect host from which it was isolated. In case of *B. bassiana* isolates, cluster 3 had single clad of single isolate; cluster 2 comprised of 2 clads (of three isolates) with similarity in origin and host; while cluster 1 was a big group of 5 clads (13 isolates), which had individual clad similarity of isolates but overall in this cluster, the isolates had varied geographic origin and host range. Sugimoto et al. (2003) did not observe any relationship between isolate source, location and RFLP profiles of β – tubulin region of *Verticillim lecanii*. De Maro et al. (2005) used IISR-PCR and AFLP analysis to indicate intraspecific groupings correlated with geographical origin and relative genetic diversity among some isolates of *B. bassiana* but did not observe any obvious association of the fungal isolates with the host insect. They also anticipated that in the absence of any obvious association between molecular fingerprinting profiles and hosts, there might be only casual association of the isolates with the host rather than clear association between isolates adapted to the pest species. Valderrama et al. (2000) observed some correlation between *B. bassiana* isolates and their geographical origins, but no clear correlation between the clusters (obtained by RAPD analysis) and the insect host or pathogenicity against *Hypothenemus hampei*. Bidochka et al. (2002) found that genetic groups of *B. bassiana* are associated with their geographical origin. Padmavati et al. (2003) suggested that the existence of isolates with similar DNA fingerprints in different geographic areas may not indicate that the same genotype is distributed in several geographical regions, but it is likely that similar changes in DNA sequences may have occurred independently in different genotypes.

The absolute similarity coefficient inference of *M. anisopliae* and *B. bassiana* isolates

The phenogram for *M. anisopliae* isolates in Figure 6.7 shows that isolate UM1 and UM7 (cluster 1, clad 1); isolate UM6 and AR1 (cluster 1, clad 2); isolates UM4, UM5, UM8, UM9, UM11, UM12 and UM13 (cluster 2, clad 1) and isolates UM3 and UM10 showed similarity coefficient of 1.00 i.e. 100 % similarity. For *B. bassiana* isolates UB2, UB3, UB4, UB5, UB8 and UB9 (cluster 1, clad 2); and isolates UB11, UB13 and UB14 (cluster 1, clad 3) showed similarity coefficient of 1.00. A possible explanation for this might be the highly conserved nature of β – tubulin gene. Begerow et al. (2004) evaluated evolutionary relationship among β – tubulin gene sequence of basidiomycetous fungi and their data confirmed monophyly of basidiomycete fungi. Mavridou et al. (2000) reported experimental evidence of conservation of ITS1 – 5.8S – ITS2 region among *M. anisopliae* isolates and so utilized other alternatives of isolate differentiation. In our study too, the 100 % similarity coefficient based on RFLP analysis of the restriction patters, is suggestive of possible conservation of β – tubulin gene in the two populations of entomopathogenic fungi. Hansen et al. (2005) reported the conservative behavior of β – tubulin gene in the evolutionary relationships of the cup – fungus *Peziza*. On the other hand there have been evidences which suggest evolving behavior of β – tubulin gene. Grant et al. (1996) studied variation in *Trichostrongylus colubriformis*, using β – tubulin gene based RFLP.

Conclusion

Based on the RFLP signatures of the two populations, the β – tubulin gene seems to be conserved in entomopathogenic fungi. The comparison of RFLP clustering suggests more association of host and geographic origin on *M. anisopliae* isolates than in *B. bassiana* isolate.

Table 6.1: Similarity coefficient of *M. anisopliae* isolates as revealed by SAHN/ UPGMA analysis.

	UM1	UM2	UM3	UM4	UM5	UM6	UM7	UM8	UM9	UM10	UM11	UM12	UM13	AR1
UM1	1.00													
UM2	0.84	1.00												
UM3	0.84	0.90	1.00											
UM4	0.94	0.89	0.89	1.00										
UM5	0.94	0.89	0.89	1.00	1.00									
UM6	0.95	0.80	0.80	0.89	0.89	1.00								
UM7	1.00	0.84	0.84	0.94	0.94	0.95	1.00							
UM8	0.94	0.89	0.89	1.00	1.00	0.89	0.94	1.00						
UM9	0.94	0.89	0.89	1.00	1.00	0.89	0.94	1.00	1.00					
UM10	0.84	0.90	1.00	0.89	0.89	0.80	0.84	0.89	0.89	1.00				
UM11	0.94	0.89	0.89	1.00	1.00	0.89	0.94	1.00	1.00	0.89	1.00			
UM12	0.94	0.89	0.89	1.00	1.00	0.89	0.94	1.00	1.00	0.89	1.00	1.00		
UM13	0.94	0.89	0.89	1.00	1.00	0.89	0.94	1.00	1.00	0.89	1.00	1.00	1.00	
AR1	0.95	0.80	0.80	0.89	0.89	1.00	0.95	0.89	0.89	0.80	0.89	0.89	0.89	1.00

Table 6.2: Summary of cluster analysis and geographic and host relationship of *M. anisopliae* isolates.

Cluster no.	Clad no.	Isolate	Geographic origin	Origin similarity	Host insect	Host similarity
1	1	UM1	India	Yes, 3 out of 4 isolates belong to India	*Nilaparvata lugens* (Hemiptera)	Similarity to an extent as order Lepidoptera for two host insects is similar.
		UM7	USA		*Helicoverpa zea* (Lepidoptera)	
	2	UM6	India		*Pyrausta machaeralis* (Lepidoptera)	
		AR1	India		Unknown	
2	1	UM4	Indonesia	Yes, as 6 out of 7 isolates belong to India.	Lepidoptera larva	Yes, as 5 out of 7 isolates have *Nilaparvata lugens* (Hemiptera) as host.
		UM5	India		Coleoptera	
		UM8	India		*Nilaparvata lugens* (Hemiptera)	
		UM9	India		*Nilaparvata lugens* (Hemiptera)	
		UM11	India		*Nilaparvata lugens* (Hemiptera)	
		UM12	India		*Nilaparvata lugens* (Hemiptera)	
		UM13	India		*Nilaparvata lugens* (Hemiptera)	
3	1	UM2	Philippines	Yes to an extent as Philippines and Indonesia fall under the same geographic belt.	*Spodoptera* sp. (Lepidoptera)	Yes, as 2 out of 3 isolates have order Lepidoptera as host.
	2	UM3	Indonesia		*Nephotettix virescens* (Hemiptera)	
		UM10	Mexico		*Anticarsia gemmatalis* (Lepidoptera)	

Table 6.3: Similarity coefficient of *B. bassiana* isolates as revealed by SAHN/ UPGMA analysis.

	UB1	UB2	UB3	UB4	UB5	UB6	UB7	UB8	UB9	UB10	UB11	UB12	UB13	UB14	UB15	UB16	AB1
UB1	1.00																
UB2	0.94	1.00															
UB3	0.94	1.00	1.00														
UB4	0.94	1.00	1.00	1.00													
UB5	0.94	1.00	1.00	1.00	1.00												
UB6	0.80	0.86	0.86	0.86	0.86	1.00											
UB7	0.63	0.67	0.67	0.67	0.67	0.63	1.00										
UB8	0.94	1.00	1.00	1.00	1.00	0.86	0.67	1.00									
UB9	0.94	1.00	1.00	1.00	1.00	0.86	0.67	1.00	1.00								
UB10	0.84	0.89	0.89	0.89	0.89	0.75	0.70	0.89	0.89	1.00							
UB11	0.89	0.94	0.94	0.94	0.94	0.80	0.63	0.94	0.94	0.95	1.00						
UB12	0.67	0.71	0.71	0.71	0.71	0.67	0.63	0.71	0.71	0.74	0.78	1.00					
UB13	0.89	0.94	0.94	0.94	0.94	0.80	0.63	0.94	0.94	0.95	1.00	0.78	1.00				
UB14	0.89	0.94	0.94	0.94	0.94	0.80	0.63	0.94	0.94	0.95	1.00	0.78	1.00	1.00			
UB15	0.74	0.78	0.78	0.78	0.78	0.75	0.60	0.78	0.78	0.80	0.84	0.95	0.84	0.84	1.00		
UB16	0.75	0.80	0.80	0.80	0.80	0.77	0.59	0.80	0.80	0.82	0.88	0.88	0.88	0.88	0.82	1.00	
AB1	0.90	0.84	0.84	0.84	0.84	0.71	0.67	0.84	0.84	0.95	0.90	0.70	0.90	0.90	0.76	0.78	1.00

Table 6.4: Summary of cluster analysis and geographic and host relationship of *B. bassiana* isolates.

Cluster no.	Clad no.	Isolate	Geographic origin	Origin similarity	Host insect	Host similarity
1	1	UB1	Spain	Sole isolate so no comparison	*Helicoverpa virescens* (Lepidoptera)	Sole isolate so no comparison
	2	UB2	Philippines	No	*Cnaphalocrocismedinalis* (Lepidoptera)	Similar to an extent as 4 out of 6 isolates have Lepidoptera hosts.
		UB3	USA		*Bemicia tabaci* (Hemiptera)	
		UB4	India		*Emmalocera depressella* (Lepidoptera)	
		UB5	India		*Hyblaea puer* (Lepidoptera)	
		UB8	Spain		*Helicoverpa armigera* (Lepidoptera)	
		UB9	USA		*Coccinella* sp. (Coleoptera)	
	3	UB11	Denmark	Similar to an extent as 4 out of 6 isolates belong to India.	Coccinella *septempunctata* (Coleoptera)	Yes, host belongs to same order.
		UB13	India		*Xyloryctes jamaicensis* (Coleoptera)	
		UB14	India		*Basilepta fulvicornis* (Coleoptera)	
	4	UB10	USA		*Coccinella* sp. (Coleoptera)	
		AB1	India		Unknown	
	5	UB6	India		*Spodoptera litura* (Lepidoptera)	
2	1	UB12	India	Yes	*Chilo infuscatellus* (Lepidoptera)	Yes
		UB15	India		*Spodoptera litura* (Lepidoptera)	
	2	UB16	India		Adult Coleoptera	
3	1	UB7	Denmark	Sole isolate so no comparison	*Coccinella septumpunctata* (Coleoptera)	Sole isolate so no comparison.

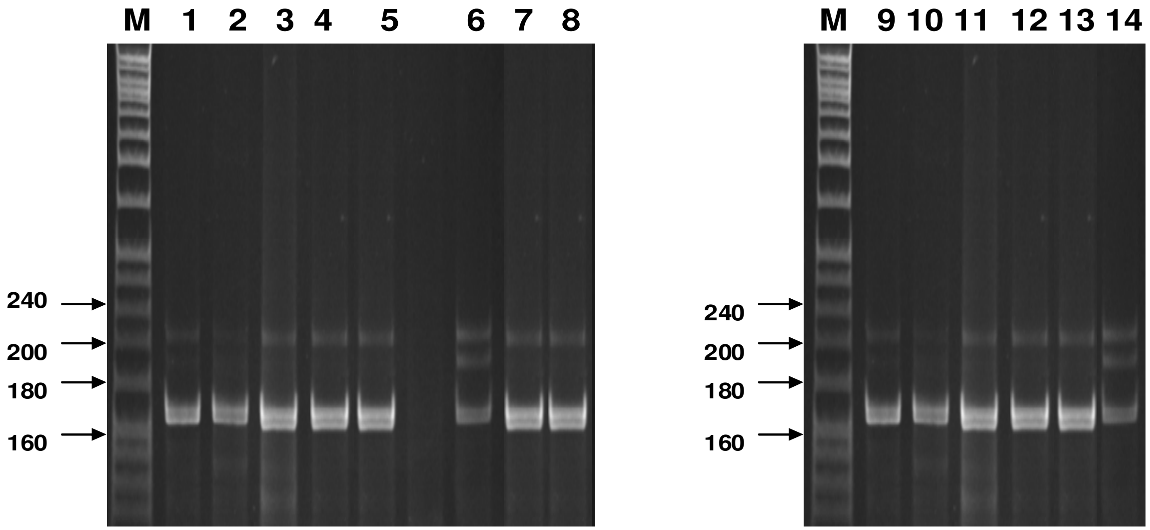


Fig.6.1: β-tubulin amplification of *M. anisopliae* isolates as visualized on 1.5% agarose gel. M: 100 bp marker; lane 1-14 are isolates UM1-13 and AR1.

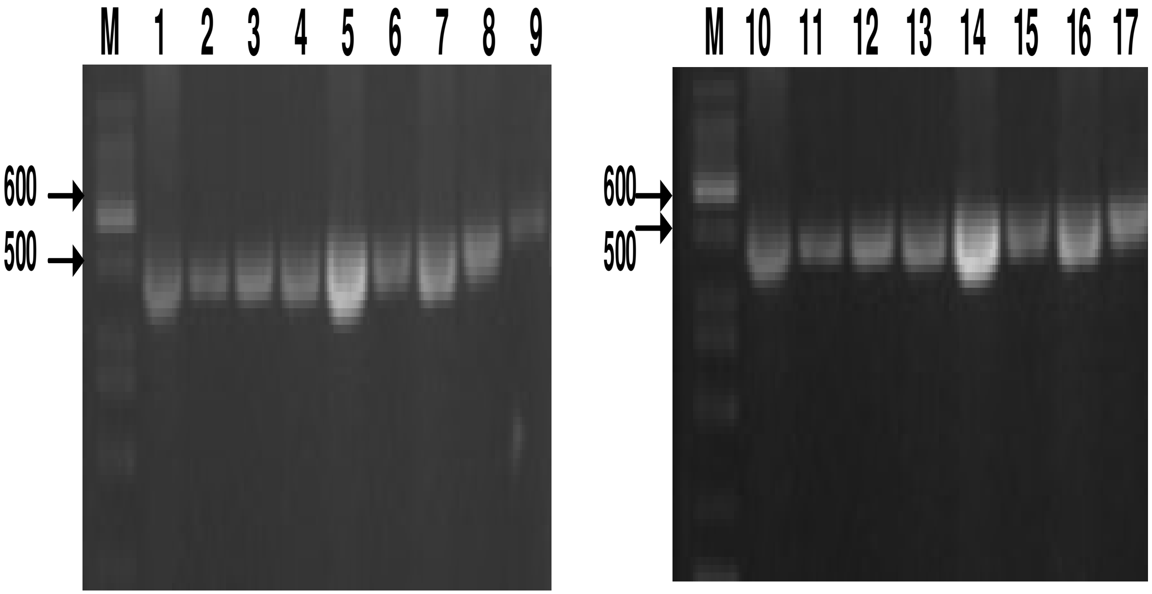


Fig.6.2: β-tubulin amplification of *B. bassiana* isolates as visualized on 1.5% agarose gel. M: 100bp marker; lane 1-17 are isolates UB1-16 and AB1.

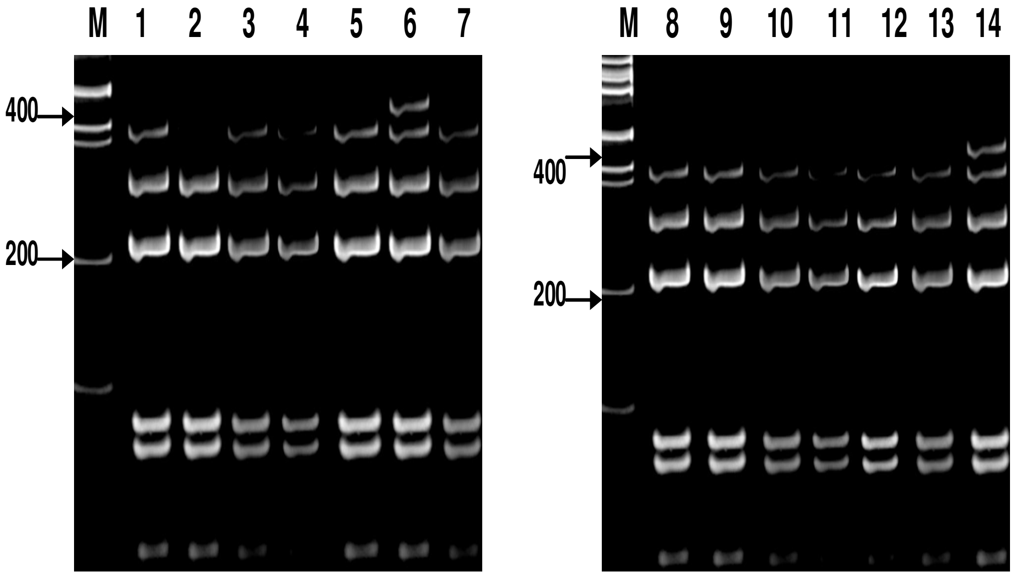


Fig. 6.3: Restriction digestion of β-tubulin amplified product of *M. anisopliae* isolates by Alu I as visualized on 8% polyacrylamide gel. M: 100bp marker; lane 1-14 are isolates UM1-13 and AR1.

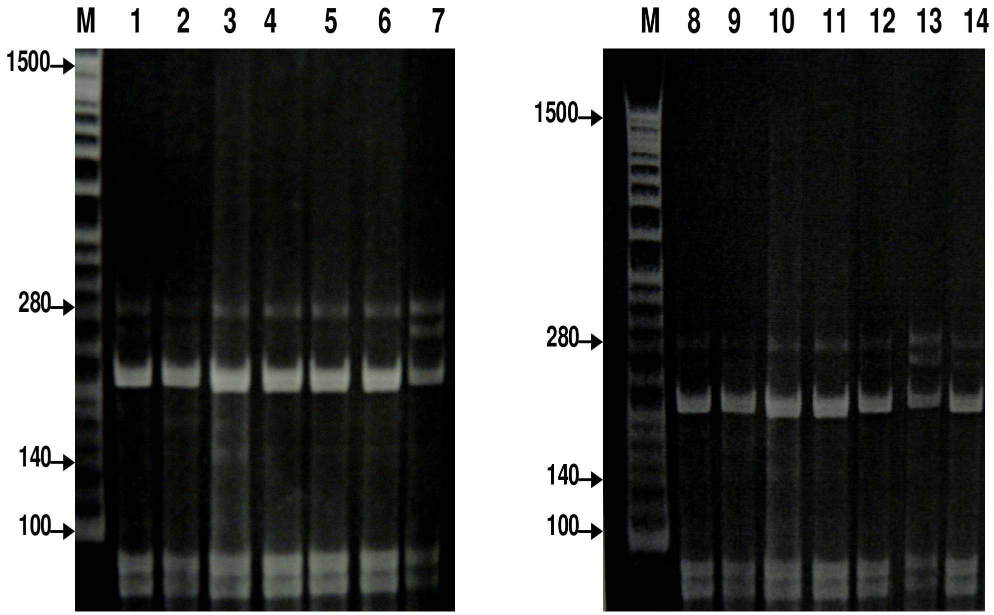


Fig. 6.4: Restriction digestion of β-tubulin amplified product of *M. anisopliae* isolates by Hpa II as visualized on 8% polyacrylamide gel. M: 20bp marker; lane 1-14 are isolates UM1-13 and AR1.

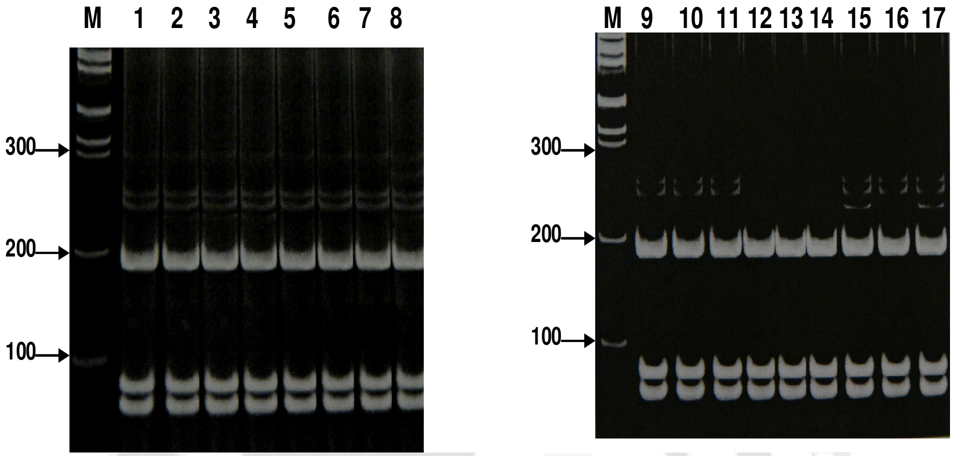


Fig. 6.5: Restriction digestion of β-tubulin amplified product of *B. bassiana* isolates by Alu I as visualized on 8% polyacrylamide gel. M: 100bp marker; lane 1-17 are isolates UB1-17 and AB1.

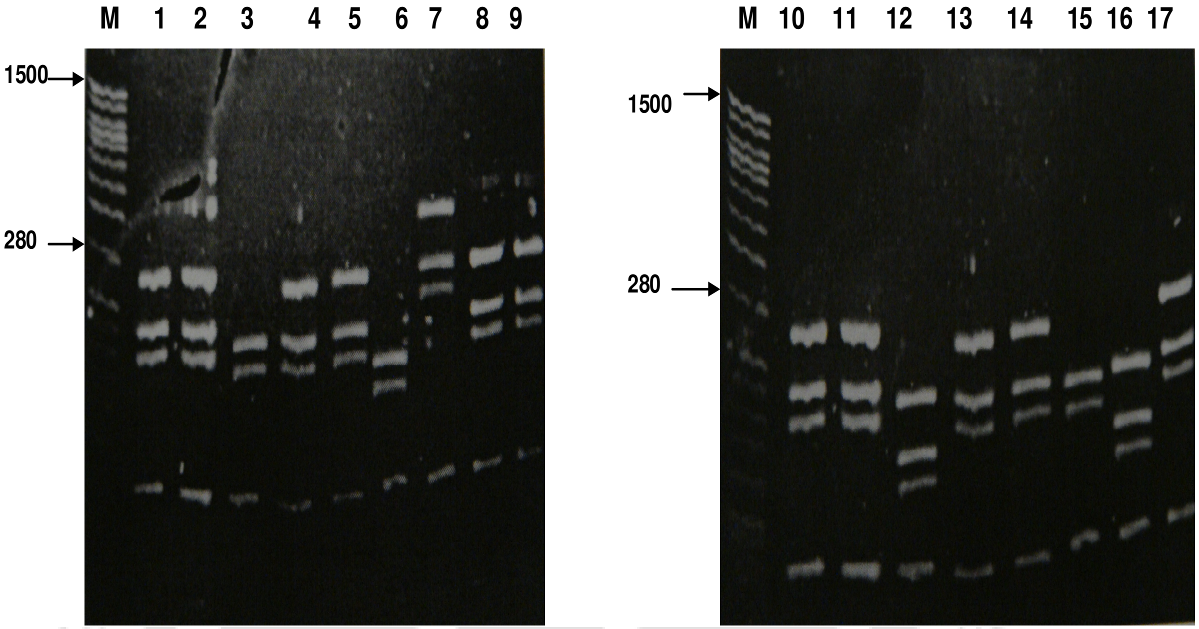


Fig. 6.6: Restriction digestion of β-tubulin amplified product of *B. bassiana* isolates by Hae III as visualized on 8% polyacrylamide gel. M: 100bp marker; lane 1-17 are isolates UB1-17 and AB1.

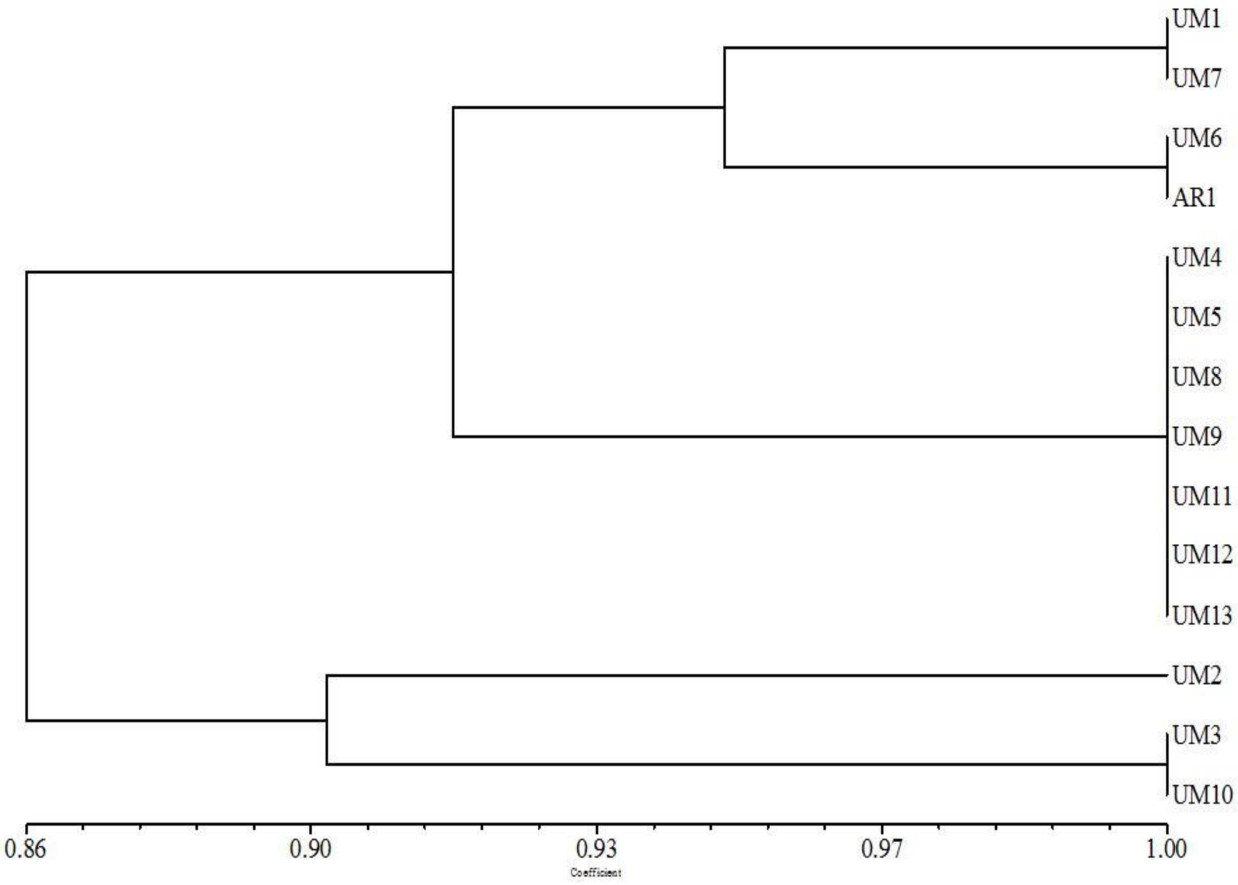


Fig. 6.7: Phenogram of 14 *M. anisopliae* isolates using restriction digestion of β-tubulin amplified product by Alu I and Hpa II restriction enzymes.

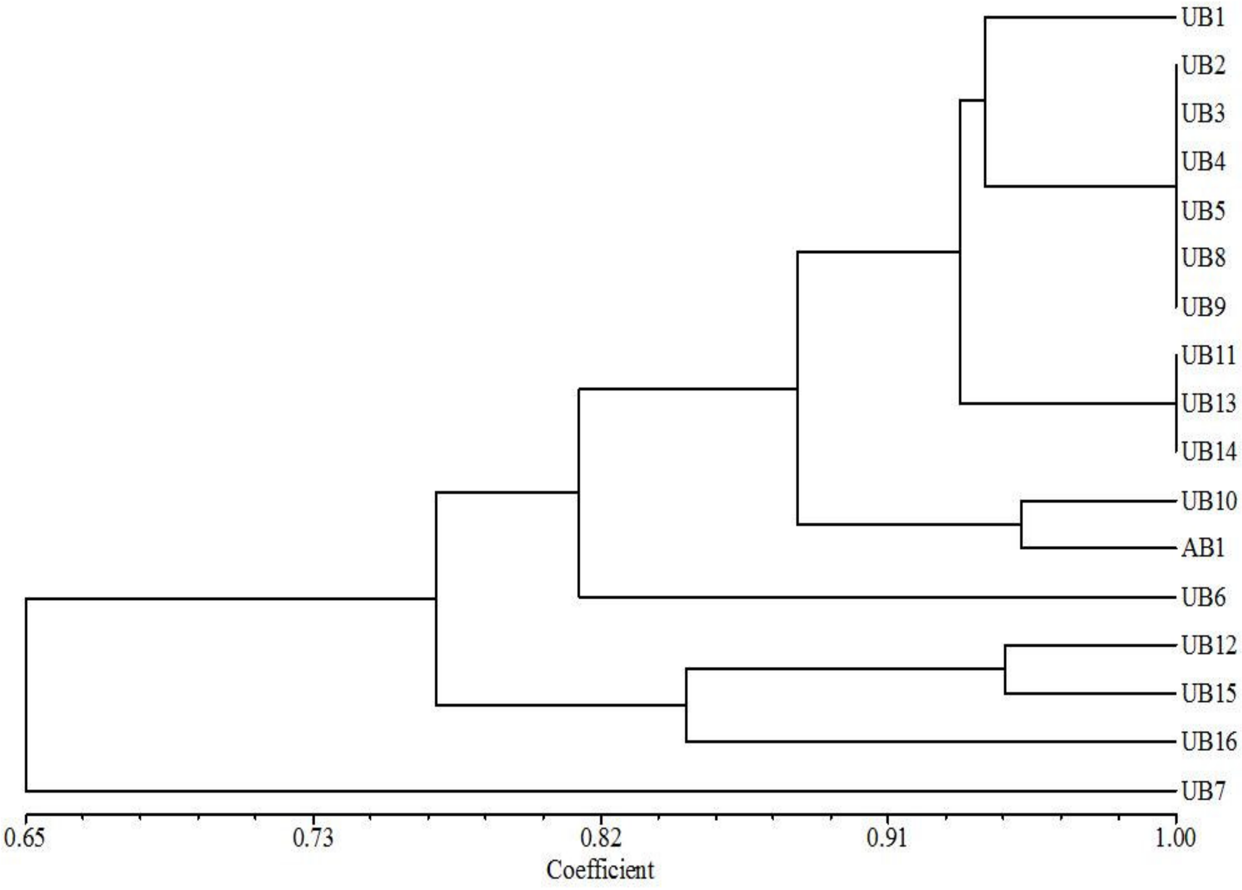


Fig. 6.8: Phenogram of 17 *B. bassiana* isolates using restriction digestion of β-tubulin amplified product by Alu I and Hae III restriction enzymes.

7.1 Introduction

The destruxin production is not only influenced by biological factors, the physicochemical environment in which the organism is grown also plays an important role in the high yield production. In particular, pH and aeration rate are the most critical parameters and plays a significant role in productivity of the process (Bing-Lan Lin *et al.,* 2007). It is reported that destruxin production is highly influenced by the component type and the CN ratio of the culture media (Liu *et al.,* 2000; Wang *et al.,* 2004). Destruxin production is reported to widely differ amongst various isolates of *M. anisopliae* (Hsiao and Ko, 2001; Kershaw et al., 1999). There are considerable evidences that suggest substantial involvement of destruxin in pathogenesis by *M. anisopliae*. Liu et al. (2000) reported variation in destruxin production with CN ratio. Wang et al. (2004) reported a correlation between pH variation and biomass change of the culture media and destruxin production. The production of one destruxin type may be significantly correlated. Feng et al. (2004) studied the response of fungal pellet size on the yield of destruxin production and concluded that the fungal pellet size is a critical factor to reach the highest yield of production of destruxin by *M. anisopliae* in the process of liquid – state fermentation. Rao et al. (2006) studied increased used of antioxidants like Menadione to enhance cultivation of destruxin E from cultivation of *M. anisopliae*. As a preliminary study of this chapter, antibiotic activity, germination growth and sporulation of the *M. anisopliae* ARSEF-2735 was estimated. The amount of crude destruxin produced by *M. anisopliae*

ARSEF 2735 was monitored in different media. The relationship between the crude destruxin production, pH and biomass variation were analysed and discussed.

7.2 Materials and Methods

Fungal culture

The *M. anisopliae* ARSEF - 2735, was routinely subcultured on Sabouraud Dextrose Agar (SDA) at 28 °C in the dark and the slants were kept at 4 ^{0}C when completely sporulated, usually after 10 days. For long term storage, conidial suspensions were prepared in 20 % glycerol solution and stored at - 20 ^{0}C. These stocks were used as inoculum for fresh culture.

Antibiotic activity

Soft agar YPD media (1% agar, yeast 1%, peptone 1% and dextrose 4%) was used for the antibiotic studies. The antibiotic activity of the *M. anisopliae* ARSEF - 2735 was tested by two rings of 20 antibiotic discs Icosa Universal 1 and Icosa Universal 2, procured from HiMedia laboratories. Around 200 µl of 1 x 10^{6} conidial suspensions was spread plated onto 25mm Petri plates and allowed to stand as such for 30 min and thereafter the antibiotic discs were introduced into them and the plates were incubated for 48 hrs before estimating the effect. The sensitivity of the fungus towards the antibiotic was denoted by a (+) sign, while the resistance against antibiotics was denoted by a (–) sign.

Culture media for destruxin production

The impact of different nutrient supplements on the level of destruxin production was evaluated by growing the fungal culture in six different media (Table 7.4). The Czapek Dox (CD) media and the Minimal Media (MM) were the two basic media used for the study. Further, we used Colloidal Chitin (CC) and Cockroach Homogenate (CH) supplements (CD + CC, CD + CH, MM + CC and MM + CH) to see if at all it has some induction effect on destruxin production. The minimal media just had the basal salts in them and did not have any carbon and nitrogen source; infact Minimal Media is Czapek Dox media without sucrose and peptone. The colloidal chitin and cockroach homogenate, both sources of chitin, a poly - sachcharide composed of β (1 - 4) linked units of the amino sugar N-acetyl-glucosamine, served basically as source of carbon but also provided nitrogen. Sucrose served as the source of carbon. Colloidal chitin was prepared with a diminutive modification of the Simhara and Takiguchi (1988) method. For preparation of cockroach homogenate, the adult cockroach *Periplanata americana*, were frozen to death for 45 min at - 80 ^{0}C and homogenized under liquid nitrogen. The homogenates were then oven - dried at 60 ^{0}C for 48 hrs. Supplementing the CD and MM with colloidal chitin and cockroach homogenate were included in the study to compare the induction effect of chitin on toxin production (Wang et al., 2004). The pH of all the media were adjusted to 7.0 using 1 M NaOH. Conidia were harvested from sporulating plates using 0.02 % Tween 80 solution and the suspension was agitated for 45 sec to reduce clumping. The concentration of conidia was determined by use of a haemocytometer. The viability of spores was assessed by spreading them onto a glass slide coated with SDA and incubated for 24 hrs at 28 ^{0}C in the dark. Around 600 x 10^{6} conidia were inoculated in each of the test media. The control flasks were not inoculated. The culture filtrates were harvested after incubation, every 5th day for 30 days post inoculation (dpi).

pH, biomass and destruxin production analysis

The fungal cultures in different media were subjected to centrifugation at 10,000 rpm for 20 min; the filtrate was used for recording the change in pH value while the mycelium was dried at 37 ^{0}C for biomass estimation. Three replicates were maintained for each treatment. The cultures were filtered and destruxins were extracted with dichloromethane. Crude destruxins were prepared by evaporating the organic solvent so as to obtain crude destruxin powder. The destruxin production was confirmed by Liquid Chromatography Mass Spectrometric analysis (Chapter 8) and their positive insecticidal activity in comparison to the control which caused zero mortality (Chapter 9 and 10).

7.3 Results and Discussion

Antibiotic activity, germination, growth and spore yield

It was observed that *M. anisopliae* ARSEF - 2735 was sensitive to the effects of Amoxicillin, Ceftazidime, Ciprofloxacin, Co-Trimoxazole, Norfloxacin and Tobramycin and resistant to Amikacin, Ampicillin, Ampicillin/ Cloxacillin, Ampicillin/ Sulbactam, Azithromycin, Cefaclor, Cefadroxil, Cephadroxil, Cephotaxime, Cefoperazone, Ceftriaxone, Cefuroxime , Clarithromycin, Chloramphenicol, Cloxacillin, Erythromycin, Gentamicin, Nalidsic Acid, Netillin, Netilmycin, Nitrofurantoin, Penicillin, Roxithromycin, Sparfloxacin and Vancomycin (Table 7.1 and 7.2). The germination growth and spore yield are shown in Table 7.3.

pH, Biomass and crude destruxin production

Destruxins have been implicated as one of the major causes of insect – pathogenesis (Dumas et al., 1994; Vey et al., 2001; Pedras at al., 2002; Skrobek et al., 2008, Sowjanya Sree and Padmaja, 2008). The pH of all the media (except MM) studied for destruxin production kept decreasing from day 5 - 30 post inoculation (Table 7.5). It was observed that the pH which was initially fixed at 7.0 at the start of the experiment was gradually lowered to acidic range. The decrease in pH was more pronounced in CD + CC and CD alone, where it reduced to 3.41 and 3.42 respectively after 30 dpi. The fungal biomass kept increasing up to day 15 in all the cases, after which it remained almost constant for all the media (Table 7.6). The biomass production was least in MM supplemented with CC and CH (0.97 mg/ 100 ml and 0.89 mg/ 100 ml); while in MM alone, the biomass was nil with negligible destruxin production. All the substrates studied, showed an increase in destruxin production from day 5 to day 10 post inoculations, and thereafter a gradual decline in the destruxin production rate (Table 7.7), and this observation is similar to that of Wang et al. (2004) who observed maximum destruxin production up to 7 – 9th day old culture incubation, followed by a noticeable decrease in destruxin E production. Maximum destruxin production of about 16.39 mg/ 100 ml was observed in the CD media supplemented with colloidal chitin (1 %), followed by CD media supplemented with cockroach homogenate (1 %) with concentration of 13.45 mg/ 100 ml, followed by CD as the sole nutrient substrate. The MM media supplemented with CC (1 %) and CH (1 %) showed a meager rate of destruxin production while the Minimal Media as the sole nutrient source produced negligible biomass and almost no toxin. The pH and biomass were significantly associated (at $\alpha = 0.01$ for CD + CC (1 %), and at $\alpha = 0.05$ for the rest of the substrates), but no significant association was observed between toxin production and variation in pH and biomass (Table 7.8). Wang et al., 2004, reported negative correlation of destruxin A and B on pH variation of the culture media, but found no significant association between pH and destruxin E production. Studies also suggest addition of supplements like carbon and nitrogen sources or cyclopeptolide (Espada and Dreyfuss, 1979) or use of antioxidants (Rao *et al.,* 2006) as destruxin production enhancers. In our study, when Sucrose (component of CD media) was used as the main Carbon source (CD, CD + CC, CD + CH), the biomass and destruxin production was more than in the absence of it (MM, MM + CC, MM + CH). Similarly, when peptone (component of CD media) was used as the main nitrogen source (CD, CD + CC, CD + CH), the biomass and destruxin production was more than in the absence of it (MM, MM + CC, MM + CH). When the two basic media (CD and MM) were supplemented with CC and CH (as in CD + CC, CD + CH, MM + CC AND MM + CH), the destruxin production as well as the biomass produced was more in the non-supplemented media. Further it was observed that destruxin production was more in the CD + CC than in CD + CH, and in the MM series, it was observed to be more in MM + CC than in MM + CH, thereby indicating that pure chitin i.e. Colloidal Chitin (CC) obtained commercially from Sigma, was a better elicitor of destruxin production than its crude counterpart i.e. Cockroach Homogenate (CH) derived chitin. We can also say that the same amount of colloidal chitin from Sigma, contributes more chitin to the media than the crude chitin obtained by homogenization of cockroach; thus we can safely conclude that more the amount of chitin, higher is the destruxin elicitation. Wang et al. (2004) on the other hand did not observe any toxin production when using insect homogenate as single nutrient or when included in the liquid culture media.

7.4 Conclusion

Carbon and Nitrogen sources have a major role to play in the destruxin production because when devoid of the same, the fungus does not support appreciable destruxin production. Another major finding was the role of chitin as positive-elicitor for destruxin production.

Table7.1: Antibiotic activity of *M. anisopliae* ARSEF - 2735 on Icosa Universal - 1 antibiotic discs*

Antibiotic	**Concentration (mcg)**	**Activity**
Norfloxacin	10	+
Gentamicin	10	-
Chloramphenicol	30	-
Cefuroxime	30	-
Ciprofloxacin	5	+
Cefaperazone	75	-
Ceftazidime	30	+
Roxithromycin	30	-
Clarithromycin	15	-
Co-Trimoxazole	25	+
Netillin	30	-
Cefaclor	30	-
Cephotaxime	30	-
Cephadroxil	30	-
Azithromycin	15	-
Ampicillin/ Cloxacillin	10	-
Penicillin	10	-
Amikacin	30	-
Sparfloxacin	5	-
Ampicillin/ Sulbactam	10/ 10	-

* The discs were procured from HiMedia laboratories.
+ represents sensitivity of the fingus and – represents resistance of the fungus to the antibiotic

Table 7.2: Antibiotic activity of *M. anisopliae* ARSEF - 2735 on Icosa Universal - 2 antibiotic discs*

Antibiotic	Concentration (mcg)	Activity
Amikacin	30	-
Ampicillin	10	-
Amoxicillin	10	+
Cefadroxil	30	-
Cefoperazone	75	-
Ceftazidime	30	+
Ceftriaxone	30	-
Chloramphenicol	30	-
Ciprofloxacin	5	+
Cloxacillin	1	-
Co-Trimoxazole	25	+
Erythromycin	15	-
Gentamicin	10	-
Nalidsic Acid	10	-
Netilmycin	10	-
Nitrofurantoin	300	-
Norfloxacin	10	+
Penicillin	10	-
Tobramycin	10	+
Vancomycin	30	-

* The discs were procured from HiMedia laboratories.
+ represents sensitivity of the fungus and – represents resistance of the fungus to the antibiotic.

Table 7. 3: Germination, growth and spore yield of *M. anisopliae* ARSEF – 2735 on SDA

Germination	76.67% at 8th hr and 100% at 16th hr of incubation
Specific growth	2.0 mmd^{-1}
Spore yield	3.83×10^7 as on 8th day of observation.

Table 7.4: Media composition for destruxin production

Media	Composition	Main Carbon source	Main Nitrogen source	Combined Carbon and Nitrogen source *
CD	Sucrose, 30 gm; $NaNO_3$, 3g; K_2HPO_4, 1 gm/ L; $MgSO_4{\cdot}H_2O$, 0.5 gm/ L; KCl, 0.5 gm/ L; $FeSO_4$, 0.5 gm/ L; mycological peptone, 2 %	Sucrose	2 % Peptone(from CD)	-
CD+CC	Sucrose, 30 gm; $NaNO_3$, 3 gm; K_2HPO_4, 1 gm/L; $MgSO_4{\cdot}H_2O$, 0.5 gm/ L; KCl, 0.5 gm/ L; $FeSO_4$, 0.5 gm/ L; mycological peptone, 2 %; Colloidal chitin, 1 %	Sucrose	2 % Peptone (from CD)	1 % Colloidal Chitin
CD+CH	Sucrose, 30 gm; $NaNO_3$,3 gm; K_2HPO_4,1 gm/L; $MgSO_4{\cdot}H_2O$, 0.5 gm/ L; KCl, 0.5 gm/ L; $FeSO_4$, 0.5gm/ L; mycological peptone 2 %, cockroach homogenate, 1 %	Sucrose	2 % Peptone (from CD)	1 % Cockroach Homogenate
MM	$NaNO_3$, 3 gm; K_2HPO_4, 1 gm/ L; $MgSO_4{\cdot}H_2O$, 0.5 gm/ L; KCl, 0.5 gm/ L; $FeSO_4$, 0.5 gm/ L	-	-	-
MM+CC	$NaNO_3$, 3 gm; K_2HPO_4, 1 gm/ L; $MgSO_4{\cdot}H_2O$, 0.5 gm/ L; KCl, 0.5 gm/ L; $FeSO_4$, 0.5gm/ L; Colloidal chitin, 1 %	-	-	1 % Colloidal Chitin
MM+CH	$NaNO_3$, 3 gm; K_2HPO_4, 1 gm/ L; $MgSO_4{\cdot}H_2O$, 0.5 gm/ L; KCl, 0.5 gm/ L; $FeSO_4$, 0.5 gm/ L; cockroach homogenate, 1 %	-	-	1 % Cockroach Homogenate

CD: Czapek Dox medium; CC: Colloidal Chitin; CH: Cockroach Homogenate; MM: Minimal Medium.
(-) indicates absence of the said component in the media.
* The Colloidal Chitin and Cockroach Homogenate, both sources of chitin, a poly - sachcharide composed of β (1 - 4) linked units of the amino sugar N - acetyl - glucosamine, served basically as source of carbon but also provided nitrogen.

Table 7.5: pH variation upon destruxin production on different media

	Media					
	CD	CD +CC	CD + CH	MM	MM + CC	MM + CH
5	6.34^{a}	6.43^{a}	6.13^{a}	Negligible variation in pH	6.34^{a}	6.11^{a}
10	5.85^{b}	5.52^{b}	5.41^{b}		5.85^{b}	5.83^{b}
15	5.17^{c}	4.79^{c}	5.11^{b}		5.78^{b}	5.60^{c}
20	4.25^{d}	4.17^{d}	4.71^{c}		5.31^{c}	5.17^{d}
25	3.43^{e}	3.67^{e}	4.64^{c}		5.13^{d}	4.94^{e}
30	3.42^{e}	3.41^{f}	3.65^{d}		4.75^{e}	4.60^{f}

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

Table 7.6: Biomass variation upon destruxin production on different media

Days	Media					
	CD	CD + CC	CD + CH	MM	MM + CC	MM + CH
5	0.45^{b}	0.65^{c}	0.25^{c}	Almost nil	0.21^{c}	0.25^{b}
10	0.54^{b}	0.95^{c}	0.73^{b}		0.63^{b}	0.44^{b}
15	1.24^{a}	1.39^{b}	1.46^{a}		0.91^{a}	0.87^{a}
20	1.37^{a}	1.89^{a}	1.70^{a}		0.97^{a}	0.93^{a}
25	1.36^{a}	1.83^{a}	1.80^{a}		0.92^{a}	0.87^{a}
30	1.31^{a}	1.90^{a}	1.68^{a}		0.97^{a}	0.89^{a}

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

Table 7.7: Destruxin production* on different media

Days	Media					
	CD	CD + CC	CD + CH	MM	MM+CC	MM + CH
5	5.06^{fC}	8.26^{fA}	6.54^{eB}	Almost nil	0.18^{dD}	0.18^{dD}
10	9.99^{aC}	16.39^{aA}	13.45^{aB}		0.84^{aE}	1.03^{aD}
15	8.78^{bC}	15.12^{bA}	12.30^{bB}		0.79^{aD}	0.67^{bE}
20	7.54^{cC}	13.60^{cA}	10.95^{cB}		0.45^{bE}	0.65^{bD}
25	7.38^{dC}	13.03^{dA}	11.03^{cB}		0.25^{cD}	0.36^{cD}
30	6.75^{eC}	12.28^{eA}	10.09^{dB}		0.07^{eD}	0.09^{eD}

*The amount of crude destruxin powder (in mg) obtained from 50 ml of the inoculated culture per 10^6 conidia concentration as the starting material.
Values followed by the same lower/ upper case alphabets in the same column/ row are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.

Table 7.8: Pearson relationships between variations in pH, biomass and destruxin concentration as observed during a period of 30 days

Media	Parameters	pH	Biomass	Destruxin concentration
CD	pH	1	-0.883*	0.051
	Biomass	-0.883*	1	0.061
	Destruxin concentration	0.051	0.061	1
CD + CC	pH	1	-0.973**	-0.286
	Biomass	-0.973**	1	0.258
	Destruxin concentration	-0.286	0.258	1
CD + CH	pH	1	-0.843*	-0.280
	Biomass	-0.843*	1	0.414
	Destruxin concentration	-0.280	0.414	1
MM + CC	pH	1	-0.838*	0.397
	Biomass	-0.838*	1	0.078
	Destruxin concentration	0.397	0.078	1
MM + CH	pH	1	-0.824*	0.389
	Biomass	-0.824*	1	-0.063
	Destruxin concentration	0.389	-0.063	1

* Correlation is significant at the 0.05 level (2 - tailed).
** Correlation is significant at the 0.01 level (2 - tailed).

8.1 Introduction

Around 35 different destruxins are reported till date and these are grouped into six major series A-F (Pedras et al., 2002). The entomopathogenic fungi are reported to secrete more than two of the destruxins at a time which act synergistically to cause mycosis (Pais et al., 1980). The ‘routine laboratory quantitative analysis’ of destruxins becomes ‘difficult’ as the pure standards of only two of its members are commercially available and so spectro-photometrical and HPLC based analysis can only quantify two of its members. Other procedures of destruxins quantification use preparative HPLC followed by collection of fractions and repeating the procedures until a single compound is obtained. But as said, at a time, a group of these toxins are secreted by the entomopathogenic fungi to act concomitantly to cause insect demise, so detail information on the types present in the culture extract would seem more knowledgeable; though not withstanding the importance of quantification of each of the destruxins present in the culture extract (which of course calls for ample time for analysis). In order to generate structural identification data by mass spectrometry for peptides, the molecule must undergo fragmentation of one or several bonds to match and correlate the m/z of the resulting fragments with the chemical structure. The most common mechanism used to obtain fragments from a peptide is collision- induced dissociation (CID) or by the technique of post- source decay when reflector time of flight instruments are coupled with MALDI, APCI or ESI. Since our main objective was to ascertain the response of crude destruxins (sans the effect of conidia) based insect-bioassays, and for that the cardinal pre-requisite was to know if at all destruxin(s) is/ are contained in the *in - vitro* culture and what are the types present, so we undertook the LCMS (Liquid Chromatography Mass Spectrometry) based estimation of the destruxin family present in the 10 day old culture extract.

8.2 Materials and Methods

Fungal culture

The *M. anisopliae* ARSEF - 2735, was routinely subcultured on Sabouraud Dextrose Agar (SDA) at 28°C in the dark and the slants were kept at 4^0C when completely sporulated, usually after 10 days. For long term storage, conidial suspensions were prepared in 20% glycerol solution and stored at – 20 ^{0}C. These stocks were used as inoculum for fresh culture.

Culture media and destruxin production

The Czapek Dox (CD) media (Sucrose, 30 g; $NaNO_3$, 3g; K_2HPO_4, 1 gL^{-1}; $MgSO_4 \cdot H_2O$, 0.5 gL^{-1}; KCl, 0.5 gL^{-1}; $FeSO_4$, 0.5 gL^{-1}; mycological peptone, 2%) used as the culture media was inoculated with 10^6 spores of *M. anisopliae* ARSEF-2735. Ten day old cultures were filtered and destruxin were extracted with dichloromethane (DCM) in one batch and ethyl acetate (EA) in another (each batch consisted of 5 replicates). Crude destruxin was prepared by evaporating the organic solvent so as to obtain crude destruxin powder. The destruxin production was affirmed by their positive insecticidal activity in comparison to the control which caused zero mortality (Chapter 9 and 10) and the destruxins present were confirmed by Liquid Chromatography Mass Spectrometric analysis.

Liquid Chromatographic Mass Spectrometric

LCMS analysis was performed using Waters Micromass Q-Tof Premier Mass Spectrometer and Waters Acquity^{TM} Ultra Performance LC using Reverse Phase C18 column. Spectra were recorded in APCI (Atmospheric Pressure Chemical Ionization) positive ion mode at 215 and 254nm. A linear gradient consisting of water and acetonitrile (0 to 50% Acetonitrile) was used as the mobile phase.

8.3 Results and Discussion

In the initial trials, we used both DCM and EA as the extracting solvent (separately as said above) and it was noticed that the yield of crude destruxin obtained when using DCM as the extracting solvent was twice that obtained by EA extraction. 100 ml of the CD – fungal culture yielded 35 mg of the crude destruxin when DCM was used as the extracting solvent; while 100 ml of the same culture yielded 17.5 mg of the crude destruxin when EA was used as the extracting solvent.

Figure 8.2 depicts the LCMS chromatogram of the crude destruxin in APCI positive ion mode. Table 8.1A- C and Figures 8.3A- C, enlists the members of the destruxin family excreted by the fungus on 10th day of its culturing in Czapek Dox broth. Published reports based on the structural analysis of the destruxin members suggests that in a reverse phase column the destruxin series elutes in the order B > A > E > C > D (Jegorov et al., 1998b). In the initial trials, we used 0 % to 100 % acetonitrile and noticed that the destruxins elute in the 1st half of the spectra and so we finally chose 0 % to 50 % acetonitrile as the mobile phase. Since water and acetonitrile was used as the mobile phase, the chromatogram in Fig 8.2 shows that it can be divided into two groups on the basis of 'polarity'. The 'first' group' can include the retention times of peaks falling between 0 to 2.5 min, while the 'second group' may include retention times of peaks falling between 2.5 to 5 min; so members excreted in the first group are more polar while the latter are less polar. We observed more than one destruxin in each of the retention peaks but are presenting only the selective data here. The compound eluted at 3.40 and 3.58 min did not show any significant destruxin member. Members of destruxin B and E series are isobaric in nature but on the basis of polarity and separation in reverse phase column, we anticipate that destruxin B series members will elute earlier than destruxin E series members. Destruxin B and E have protonated m/z value of 594. We observed a protonated member with m/z 594 at 0.608 and since this duration fall into the 'first group' *i.e.* its retention time is lower than the 'second group', we may conclude that it is more polar (as it is eluted earlier) and so it might be destruxin B and not destruxin E (this was further supported by diagnostic fragment ions). Similarly both destruxin B_1 and E_1 are isobaric in nature with m/z 608 for its protonated molecule and it eluted at 4.591 min (i.e. greater retention time as it is eluted in the 'second group') and so it is destruxin E_1, and not B_1. We chose the APCI positive ion mode for the LCMS analysis as it produces abundant protonated molecules $[M + H]^+$. Sodium and potassium adducts ($[M + Na]^+$ and $[M + K]^+$ respectively) were also seen as some of the members. The protonated members were noticed for destruxins A, Desm A, B, C_2, D, Ed, E_1 and Desm D_1. Sodium adducts were observed for destruxins A, Desm A, D, Ed, E_1 and Desm D_1. Potassium adducts were seen for destruxin Desm A, C_2, D, Ed and E_1. Parent molecule $[M]^+$ was seen for destruxin B with m/z 593.

Roseptorf and Fohlman (1984) elucidated concepts of destruxin fragmentation so as to characterize the different members and those concepts are extensively followed till date for destruxin characterization by HPLC followed by MS or LCMS and MS/ MS analysis. The loss of fragment ions improves the detection of destruxins above the background signal or base peak therefore presence of fragment ions are said to be 'diagnostic for destruxins'. Figure 8.1 is a typical representation of destruxin fragmentation. The denotions 'a' and 'b' with different subscripts are the fragments. R_1-R_5 is the R- group of the amino acid residues. Briefly, when the charge is retained on the N- terminal, then the fragments are named a_n, b_n and c_n, where 'n' is the subscript which depends on the number of residues. The internal ion fragment is denoted by D^+. Jegorov et al. (1998a) screened destruxin members in the *in-vitro* excretion of *Metarhizium anisopliae* and *Trichothecium roseum* using APCI positive ion mode LCMS and reported the protonated members with their diagnostic fragments (viz., b_5, a_5, b_4, a_4, b_3, a_3, D^+ and b_2). We primarily observed most of the fragment ions as depicted in Figure 8.1 but have reported only the conspicuous ones here. Ions a_3 and a_5 were observed for almost all the species. The diagnostic [M- 57] residue was observed for destruxin A, [M- 85] residue was observed for Ed, [M-142] was observed foe destruxin D, while [M- 169] was observed for destruxin A, Desm A, D and Desm D_1. Butt et al. (2009) used nano- HPLC / ESI- MS and reported the destruxin members for the first time in *Lecanicillim longisporum* and confirmed their presence by a_6, b_5, a_5, b_4, a_4 and internal fragment ions. In our study, we noticed internal fragments for destruxin B, C_2 and E_1. Pais et al. (1980) reported destruxins from *in- vitro* culture extract of *M. anisopliae* and observed M- 57, M-85, M-142, M- 169/ M- 170 which are characteristic of N- methyl- valyl- N- methyl- alanyl in the ring structure of destruxin. They also reported m/z 70 and m/z 84 residues from proline and pipecolic acid units, for most of them. Dudley et al. (2004) gave structural elucidation of destruxin E degradation by MS/ MS fragmentation. Apart from the confirmation of the 'known destruxin members' (with published fragmentation characteristics) in unknown source species, the destruxin fragmentation is also known to generate initial diagnostic data for possible discovery of new destruxin members (Jegorov et al., 1998a).

8.5 Conclusion

A total of 8 different destruxin members were present in the 10 day old culture broth of *M. anisopliae* ARSEF – 2735. The members present were A, Desm A, B, C_2, D, Ed, E_1 and Desm D_1.

Table 8.1A: Destruxin elution at 0.608 min.

$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	Characteristic fragment ions*	Anticipatory member	Confirmation of anticipatory member	Confirmed member
594	-	-	• a_3 (255) • D^+ (194)	B/ E	Structural analysis of the destruxin members suggests that in a reverse phase column B series members elute before E, so the member here is **B**.	**B**
596	-	-	• a_5 (455) • a_3 (271) • D^+ (211)	C_2/ DesmC	a_5 ion with m/z value of 455 is characteristic of C_2 and not DesmC, so the compound here is **C_2**.	**C_2**.

* See Figure 8.3A for details.
- The said species is absent.

Table 8.1B: Destruxin elution at 4.296 min.

$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	Characteristic fragment ions*	Anticipatory member	Confirmation of anticipatory member	Confirmed member
564	586	-	• a_5 (437)	A_2/ Desm A	a_5 ion with m/z value of 437 is characteristic of DesmA and not A_2, so the compound here is **Desm A**.	**Desm A**.
608	630	-	• D^+ (208)	B_1/ E_1	Structural analysis of the destruxin members suggests that in a reverse phase column B series members elute before E, so the member here is **E_1**.	**E_1**.
612	634	-	• b_5 (499) • M- 85 (526)	-	-	**Ed**
652	674	-	• a_5 (525) • M-169 (481)	-	-	**Desm D_1**

* See Figure 8.3B for details.
- The said species is absent.

Table 8.1C: Destruxin elution at 4.591 min.

$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	Characteristic fragment ions*	Anticipatory member	Confirmation of anticipatory member	Confirmed member
564	586	602	• a_5 (437) • M- 169 (392)	A_2/ Desm A	a_5 ion with m/z value of 437 is characteristic of DesmA and not A_2, so the compound here is **Desm A**.	**Desm A**
578	600	-	• M-57 (520) • M- 169 (409)	-	-	**A**
608	630	646	• D^+ (608)	B_1/ E_1	Structural analysis of the destruxin members suggests that in a reverse phase column B series members elute before E, so the member here is **E_1**.	**E_1**
624	646	662	• M- 142 (481) • M-169 (454)	-	-	**D**

* See Figure 8.3C for details.
- The said species is absent.

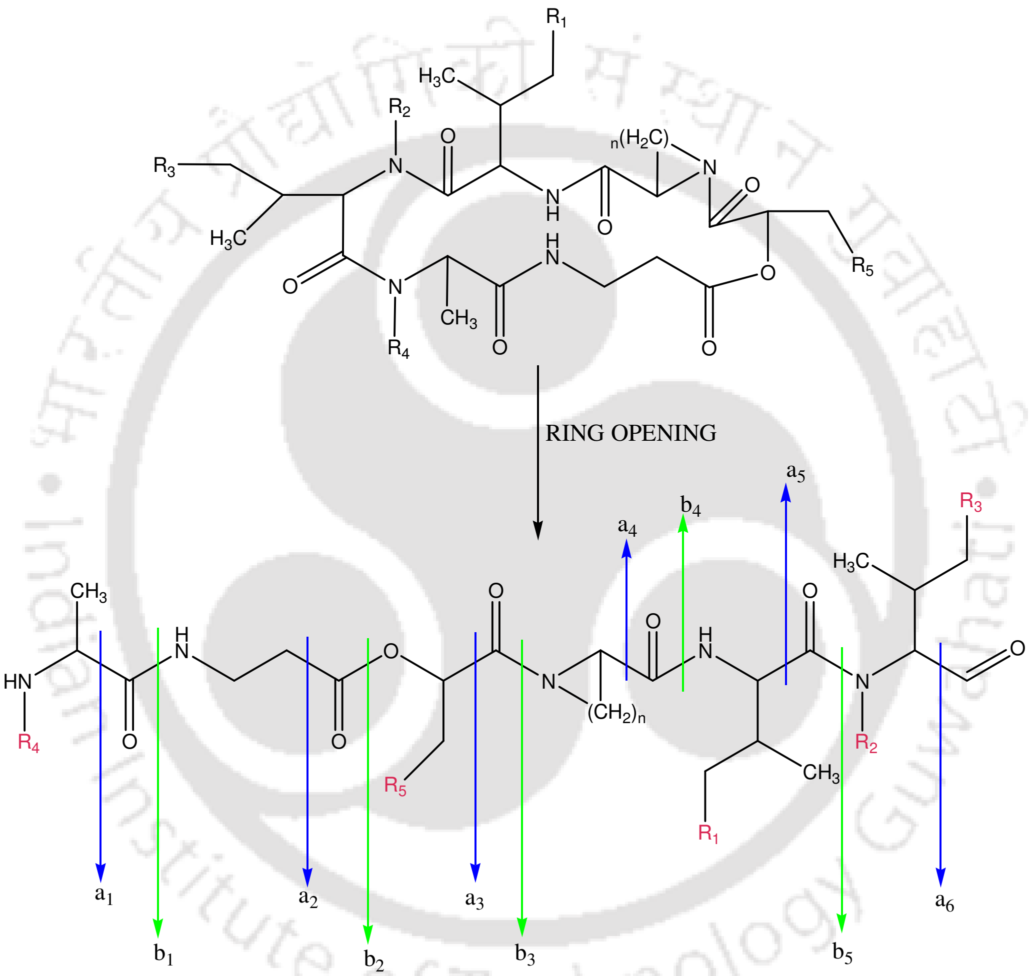


Fig. 8.1: A typical fragmentation profile of destruxin. The denotions ‘a’ and ‘b’ with different subscripts are the fragments. R_1- R_5 is the R- group of the amino acid residues.

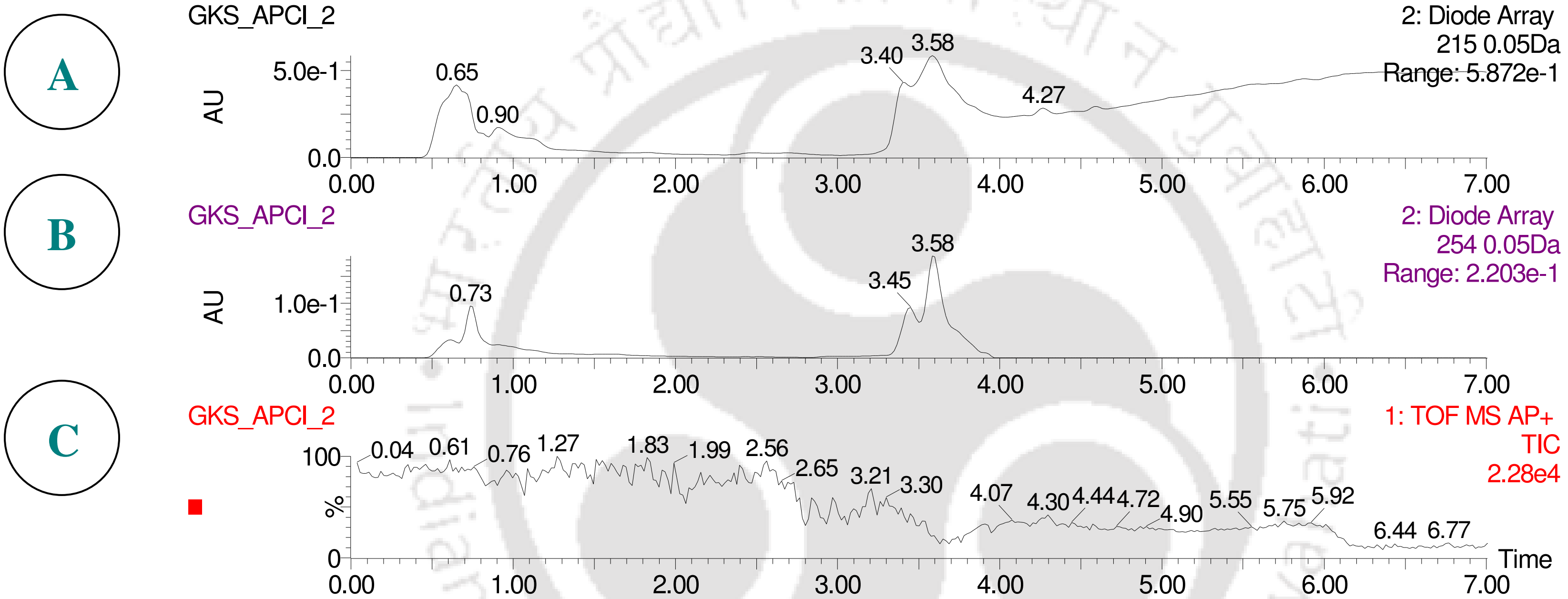


Fig. 8.2: LCMS chromatogram of destruxin analysis as observed in APCI positive ion mode.

A. The chromatogram obtained at 215nm.
B. The chromatogram obtained at 254nm.
C. The Total Ion Count

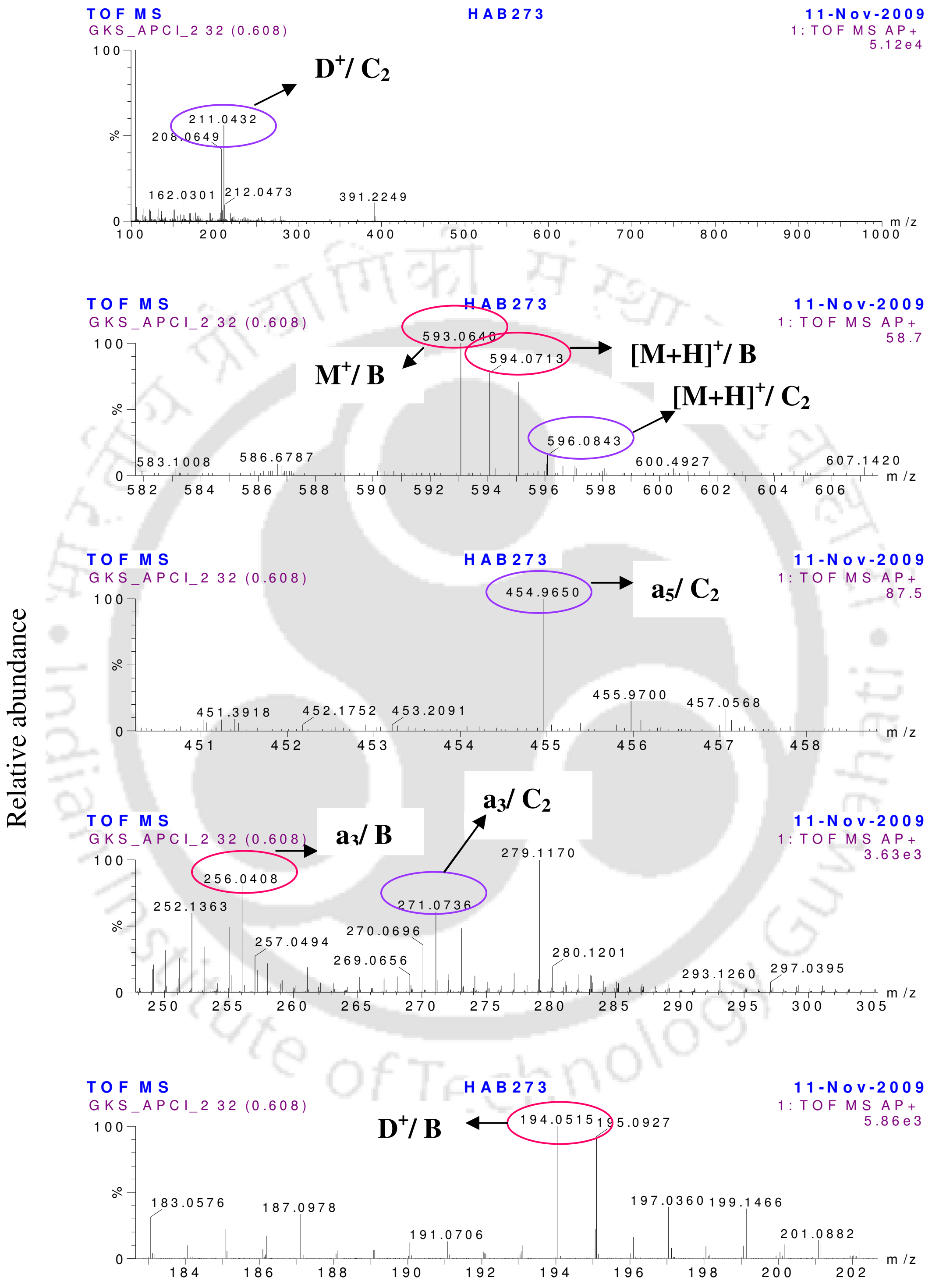


Fig. 8.3A: Destruxin profile at 0.608 min.

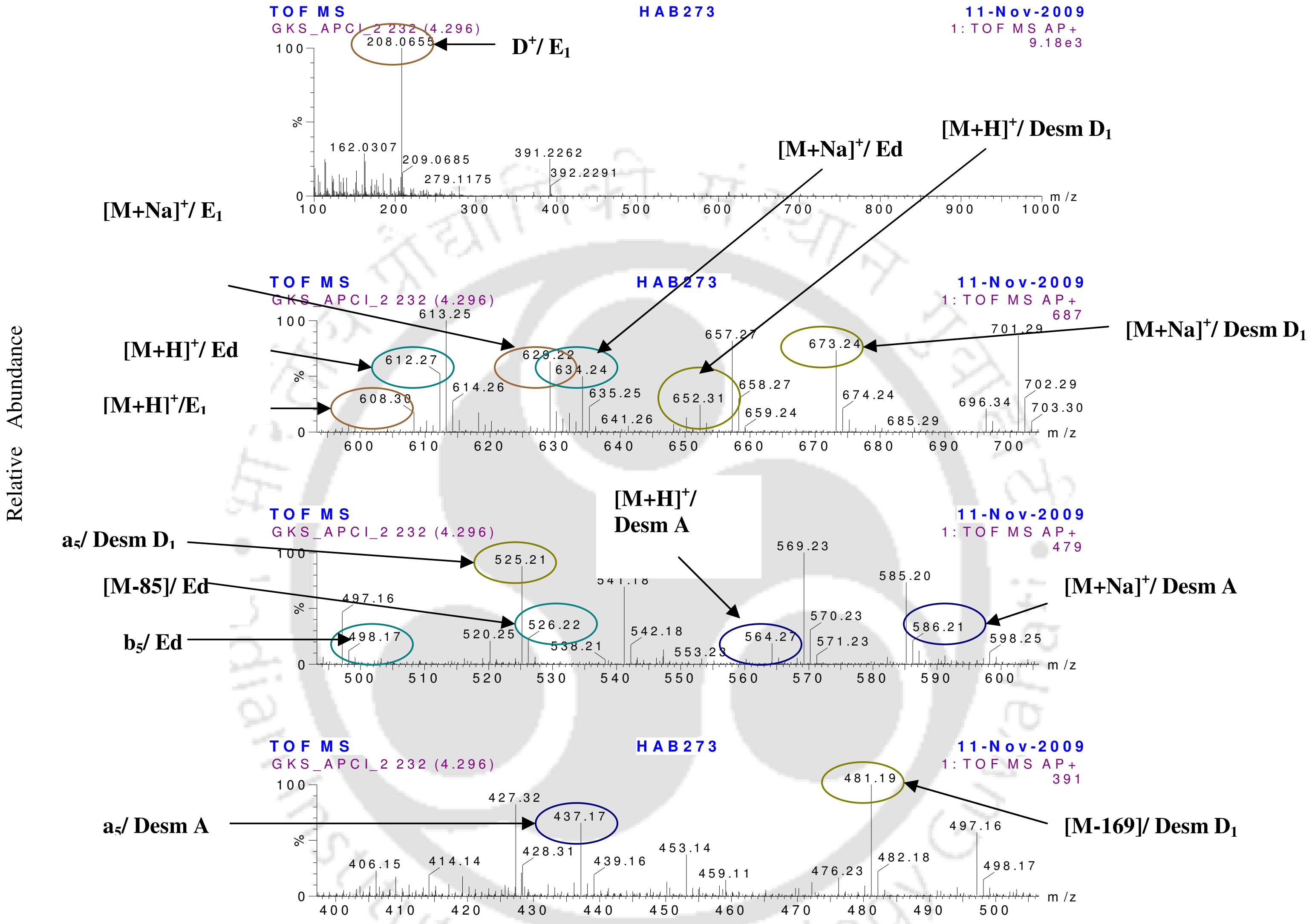


Fig. 8.3B: Destruxin profile at 4.296 min.

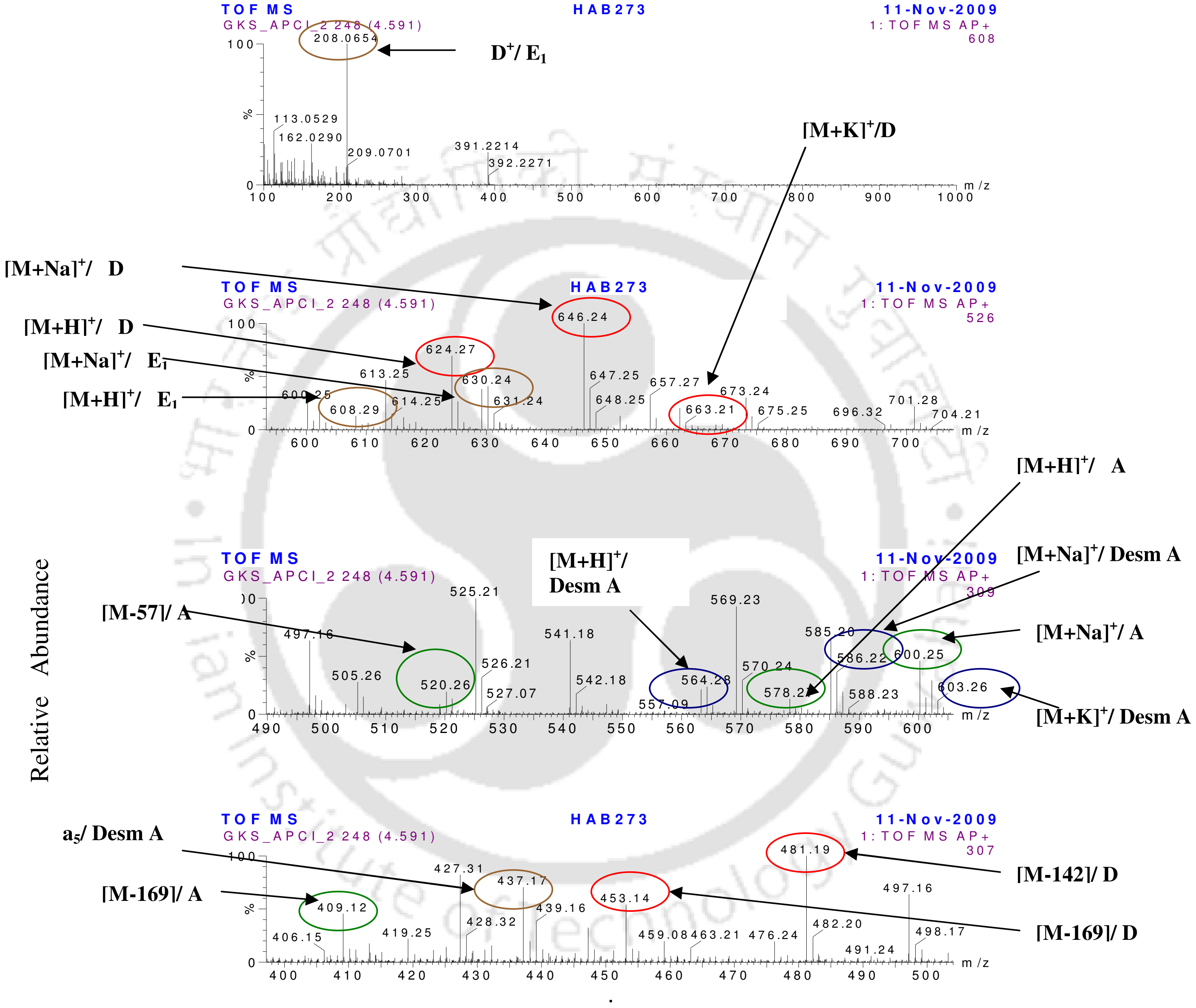


Fig. 8.3C: Destruxin profile at 4.591 min

9.1 Introduction

Almost 95 – 99 % *M. anisopliae* based insect bioassays are targeted with different (usually a range of) conidial concentrations for assessing the virulence potential of the fungus. Few reports also suggest use of commercially available destruxins (in various dilutions) for the insect bioassays. The concept of ‘conidial bioassays’ is to include the mechanical pressure and the production of lytic enzymes to depolymerize the insect cuticle but thereafter it is the toxin only which causes the insect death. The concept of ‘toxin bioassays’, more precisely the destruxins, emphasizes that it is basically the toxin which is responsible for the insect demise, while the mechanical pressure exerted by the conidia and the array of lytic enzymes only de-polymerizes the insect cuticle, but cannot cause insect death. There are considerable evidences that suggest substantial involvement of destruxin in pathogenesis by *M. anisopliae*. Destruxins are the only mycotoxins detected in the insect body at advanced stages of infection in sufficient quantities to cause mycosis (Brousseau *et al.,* 1996; Kershaw *et al.,* 1999). In the group of destruxins biosynthesized by *M. anisopliae* isolates, the destruxin A, B and E are predominant. Destruxin E is the most toxic for invertebrates, particularly insects (Dumas *et al.,* 1994; Loutelier *et al.,* 1996; Pedras *et al.,* 2002; Strasser *et al.,* 2000). Biochemical and analytical studies showed that both *in vivo* and *in vitro* diffusion of destruxin is a very rapid process from endogenous mycelia, although their ratios being different (Cherton *et al.,* 1991; Amiri-Besheli *et al.,* 2000; Butt *et al.,* 1994). Bio-pharmacological studies reveal that the paralysis induced by destruxin in insects results from a calcium-dependent effect on muscular cells (Kodaira, 1961; Samuels *et al.,* 1988a, b, c). It is suggested that destruxins also inhibit Malphigian tubule fluid secretion (James et al., 1993) as well as ecdysteroid secretion by prothoracic glands (Sloman and Reynolds, 1993). Studies show that there is an interaction between the mycotoxin and the plasma membrane and that the surface of the cells becomes modified. Electron microscopic studies reveal development of dilated vesicles at the level of brush borders. Several studies have reported immunomodulatory effects of destruxins (Huxam *et al.,* 1989; Vilcinskas *et al.,* 1997) and reveal that inhibition of cellular immune response in the haemolymph may be the key factor for destruxin induced mycosis. It is established that destruxin induce calcium influx and phosphorylation of intracellular proteins within lepidopteron cell lines (Dumas *et al.,* 1996). Based on these informations, we designed crude – destruxin – based - bioassays to evaluate the virulence potential of crude destruxins.

Materials and Methods

Experimental insects

The banded winged whitefly *Trialeurodes abutilonea* (Homoptera: Aleyrodidae) adult flies were used for determination of LD_{50}. The insects were initially fed on the flowers and foliage of *Ixora coccinia* flowers and foliage and were maintained at 25 ^{0}C – 28 ^{0}C.

Bioassay procedure

The different media studied (CD, CD+CC and CD+CH; refer Chapter 7 for details), produced maximum destruxin concentration at 10 days post inoculation. The crude destruxin powder obtained was initially dissolved in ethyl acetate to prepare stock solutions of crude destruxin by sufficient vortexing to dissolve the crude powder completely. The stock from each source was diluted 10, 100 and 1000 times to prepare five working concentrations (0X, 1X, 10X, 100X and 1000X; 0X being the control setup where only solvent was present) for each of the treatments (see Table 9.1 for details). A total of 10 adult flies were taken for each treatment and dilution and three replicates were maintained for each. The various diluents of the crude destruxin were spread evenly on the *Ixora coccinia* leaves and were allowed to dry in the laminar hood for some time to allow the solvent to evaporate. The insects being sap suckers were solely fed with the crude destruxins treated *Ixora coccinia* leaves and maintained at 12 hrs light – 12 hrs dark conditions at room temperature (25 ^{0}C – 28 ^{0}C) and were observed every 10 hrs for any mortality.

Statistical analysis

The mortality data was subjected to Probit Analysis (Finney, 1964) for calculating the LD_{50} values using the software BIOSTAT 2009, Version 5.7.4.0.

9.3 Results and Discussion

The bioactivity of destruxin to various insects has already been reported, having different mode of actions such as contact, antifeedant and growth regulation (Hu *et al.,* 2009). Kershaw *et al.,* 1999, advocated two major modes of myco-pesticidal effect to insects, viz., the ‘toxin strategy’ in which the fungus has limited growth in the haemolymph and produces destruxin in sufficient quantity to have a direct involvement in the cause of insect mortality; and secondly, the ‘growth strategy’ which basically involves mechanical pressure and results in the profuse growth of the fungus over and within the insect, thereby leading to homeostasis disruption and starvation causing insect death. This study illustrates the ‘toxin strategy’ of *M. anisopliae* ARSEF - 2735. In this study, we used the crude destruxin dilutions (sans the fungal spores and mycelium from which it was extracted) and therefore the bioassay strategy followed here was ‘toxin – strategy’.

All the three media produced varying amount of crude destruxin (Table 9.1) and 10 day old cultures of each was used for estimation of LD_{50} values. The media CD+CC produced high amount of crude destruxin (16.39 mg) followed by media CD+CH (13.45 mg) and CD (9.99 mg). The bioassay procedures for each of the destruxin sample from different media were same but the observations of each media were analyzed separately. Virulence was expressed as LD_{50} which is defined as the statistically derived exposure dosage of a pesticide expected to cause mortality in 50 % of an insect population. The LD_{50} value of CD+CC media at 30 h post treatment was the lowest (16.82) as the initial concentration of crude destruxin was also high followed by CD+CH (29.50) and CD (67.87) respectively. In all the three media it was observed that greater the dilution lesser the mortality caused as it was nil with the 1000x dilution. The other dilutions too showed the same trend where as the concentration of crude destruxin decreased, the mortality also decreased respectively. The LD_{50} values at 60 hr of observation, for each media was found to be lower than that at 30 hr of observation thus implying that lesser dose of the toxin required with increasing time of treatment (Table 9.2 to 9.4). This was true for all the media. More the destruxin concentration, lesser the lethal dose required for the desired kill rate and as the concentrations were different for all the three media studied the LD_{50} values also varied respectively.

9.4 Conclusions

Our studies shows that the Lethal Doses to ascertain the insect mortality (here whiteflies) have an inverse relationship with the time of treatment. It was observed that under similar conditions of bioassays, (like same type serial diluents, same number of insects, same bioassay procedures) more the initial concentration of the destruxin, lesser was the Lethal Dose to kill the insects. Another major finding was that the crude destruxins have commendable anti-feedant activity. Our toxin-based bioassays are unlike the majority of bioassays which are conidia-based. It is also different from other toxin-based bioassays, where usually only one type of commercially available toxin is used in various dilutions; maybe that is to find the contribution of individual destruxin member towards pathogenesis but we were more interested to prefer crude destruxin extract (which was a mixture of a number of destruxins) to see the effect of toxin – strategy on host insect, because in natural environment the destruxin family acts synergistically to cause entomopathogenesis.

Table 9.1: The details of the crude destruxins and its serial dilutions for insect bioassays#

Media*	**Extracting solvent (100 ml each)**	**Quantity of crude destruxin (mg)****	**Stock (mgml^{-1})*****	**Serial dilutions for Bioassays********				
				0X	1X	10X	100X	1000X
CD	DCM	9.99	Dissolving entire quantity in 1 ml of EA	Solvent EA only	9.99	0.999	0.0999	0.00999
CD + CC	-do-	16.39	-do-	-do-	16.39	1.639	0.1639	0.01639
CD + CH	-do-	13.45	-do-	-do-	13.45	1.345	0.1345	0.01345

DCM: Dichloromethane.
EA: Ethyl Acetate.
#The bioassay procedures for each of the destruxin sample from different media were same but the observations of each media were analyzed separately.
*Ten day old cultures from different media were used (for details refer Chapter 7).
**Quantity represents the amount of crude destruxin obtained in mg from 50 ml of the inoculated culture per 10^6 conidia concentration as the starting material. These were the values obtained when Dichloromethane was used as the extracting solvent.
***Stock was prepared by re-dissolving (in Ethyl acetate) the total amount of the crude destruxin obtained after evaporation of the extracting - solvent (Dichloromethane).
****The serial diluents are in mgml^{-1}, the same as that of stock solution

Table 9.2: Mortality data and Probit Analysis (Finney Method [Lognormal Distribution]) to estimate LD_{50}^{*} values on CD media

Serial dilution for bioassays*	**Mortality (%) at 30 h**	**Mortality (%) at 60 h**	**LD_{50}^{a}**	**LD_{50}^{b}**
0X	0	0	67.87	0.96
1X	30	80		
10X	20	40		
100X	10	30		
1000X	0	10		

*Dilutions as specified in Table 9.1 for CD media

[a] The values were recorded 30 hrs post treatment.

[b] The values were recorded 60 hrs post treatment.

Table 9.3: Mortality data and Probit Analysis (Finney Method [Lognormal Distribution]) to estimate LD_{50}^{*} values on CD+CC media at different dilutions

Serial dilution for bioassays*	Mortality (%) at 30 h	Mortality (%) at 60 h	LD_{50}^{a}	LD_{50}^{b}
0X	0	0	16.82	0.61
1X	50	10		
10X	20	50		
100X	10	30		
1000X	0	10		

*Dilutions as specified in Table 9.1 for CD+CC media

[a] The values were recorded 30 hrs post treatment.

[b] The values were recorded 60 hrs post treatment.

Table 9.4: Mortality data and Probit Analysis (Finney Method [Lognormal Distribution]) to estimate LD_{50}^{*} values on CD+CH media at different dilutions

Serial dilution for bioassays*	Mortality (%) at 30 h	Mortality (%) at 60 h	LD_{50}^{a}	LD_{50}^{b}
0X	0	0	29.50	0.68
1X	40	90		
10X	20	50		
100X	10	30		
1000X	0	10		

*Dilutions as specified in Table 9.1 for CD+CH media

[a] The values were recorded 30 hrs post treatment.

[b] The values were recorded 60 hrs post treatment.

Fig.9.1. (a).An adult whitefly (b).Plant necrosis caused by whitefly (c).The destruxin-killed whiteflies.

10.1 Introduction

Laboratory cultural practices in - context to the propagation of entomopathogenic fungi involves their *in-vitro* culture on artificial media like Sabouraud Dextrose Agar, Sabouraud Maltose Agar and Potato Dextrose Agar. On the contrary, the *in-vivo* life cycle of the entomopathogenic fungi involves the mycosis of the insects with the entomopathogenic fungi. The growth stages and events as well as the sequences and duration in which they occur can be expected to be 'similar' in the two environments *i.e.* *in-vitro* and *in-vivo*, but their 'exact simulation is very much unlikely' to happen. Some of the events of the *in-vivo* cycle may be completely missed out/ shortened/ altered in *in-vitro* culturing of the entomopathogenic fungi, and this is the basic reason of 'strain/ isolate attenuation' by *in-vitro* culturing of microbes. Strain/ isolate attenuation of entomopathogenic fungi in lab are caused by repeated sub-culturing on artificial substrates and it results in gradual loss/ weakening in intensity of virulence. Kawakami (1960) reported change in characteristics of silkworm upon successive *in-vitro* sub-culturing. Strain attenuation poses a problem when the fungus is targeted as bio-control agent (Boucias and Pendland, 2000). To overcome the problem of strain attenuation, one of the classic strategy used is to subject the entomopathogenic fungi to occasional *in-vivo* mode of life-cycle *i.e.* the enhancement of virulence by 'parasitic selection pressure' which is accomplished by passage of the *in-vitro* grown conidia through a susceptible host of the same (Veen, 1968) or an alternate (Hartmann and Wasti, 1974) insect species. Aizawa (1971) reviewed the methods of strain preservation and/ or enhancement of fungal virulence. The virulence potential of the insect passaged conidia is supposed to have an edge over that of the *in-vitro* produced conidia.

10.2 Materials and Methods

Fungal culture

The *M. anisopliae* ARSEF - 2735, was routinely sub-cultured on Sabouraud Dextrose Agar (SDA) at 28 °C in the dark and the slants were kept at 4 ^{0}C when completely sporulated, usually after 10 days. For long term storage, conidial suspensions were prepared in 20 % glycerol solution and stored at - 20 ^{0}C. These stocks were used as inoculum for fresh culture.

Production of conidia

The conidia produced on SDA were termed as 'native conidia' while those obtained after passage through grasshopper were termed 'insect-passaged conidia'. Migratory grasshoppers *Melanoiplus sanguinipes* were fed upon surface sterilized foliage sprayed with 1.00 x 10^6 conidial suspension of *M. anisopliae* ARSEF - 2735. The sporulated conidia were aseptically harvested (after 8 days) from the insect cadaver after its demise and then sub-cultured on SDA as described above. The native conidia and the insect passaged conidia were compared for chitinase and protease activity and assessed for comparative germination, growth and sporulation characteristics. The conidia from both the sources were subsequently used for the production of crude destruxins for toxicity testing against adults and nymphs of mealy-bugs, *Maconellicoccus hirsutus* and gypsy-moth caterpillar, *Lymantria dispar.*

10.2.3 Chitinase and Protease assay

Refer Chapter 4.

Experimental insects

Mealy-bugs (*Maconellicoccus hirsutus,* Pseudococcidae) or scale insects as they are commonly called were collected from China-rose (*Hibiscus rosa-sinensis*) and moss rose (*Portulaca grandiflora*). They are one of the most economically important groups of insects as they attack many cultivated food, ornamental and household plants. They go through gradual metamorphosis going through stages of eggs, nymphs and adults. Mealy-bug females feed on plant sap to the extent of causing yellowing, defoliation and ultimately complete wilting of the plant. They have piercing-sucking mouth parts and attach themselves to the plant and secrete a powdery wax layer (therefore the name mealy) used for protection while they suck the plant sap.

Gypsy moth (*Lymantria dispar*) caterpillar is one of the most damaging insect pests reported for its voracious leaf-cutter activity. They can consume tremendous amounts of leaf material, as much as one square foot of leaves per day and as a result, they produce a large amount of fecal material. The ecological and economic impact of Gypsy moth is a serious concern. When populations reach outbreak proportions, the caterpillars can completely defoliate the plants which may predispose trees to attack by opportunistic pathogens and diseases.

Both the insects were initially fed on tender foliage and were maintained at 25 – 28 ^{0}C. The adult and nymph stages of mealy - bugs, *Maconellicoccus hirsutus* and caterpillars of gypsy-moth, *Lymantria dispar,* were used for determination of LD_{50}.

Destruxin production

The Czapek Dox media (Sucrose, 30 gm; $NaNO_3$, 3 gm; K_2HPO_4, 1 gm/ L; $MgSO_4 \cdot H_2O$, 0.5 gm/ L; KCL, 0.5 gm/ L; $FeSO_4$, 0.5 gm/ L; mycological peptone, 2 %) used as the culture media was inoculated with 10^6 native as well as insect - passaged conidia of *M. anisopliae* ARSEF - 2735. Three replicates were maintained for destruxin production from both the conidial sources. Ten day old cultures were filtered and destruxin was extracted with dichloromethane. Crude destruxin was prepared by evaporating the organic solvent so as to obtain crude destruxin powder.

Bioassay procedure

The crude destruxin powder obtained as above, separately from both the sources of conidia was initially dissolved in ethyl acetate to prepare stock solutions of crude destruxin by sufficient vortexing to dissolve the crude powder completely. The stock from each source was diluted 10, 100 and 1000 times to prepare five working concentrations (0X, 1X, 10X, 100X and 1000X; 0X being the control setup where only solvent was present) for each of the treatments (refer Table 10.2 for details). The various diluents of the crude destruxin were spread evenly on the foliage (moss rose for mealy bugs and palm leaves for gypsy moth caterpillars) and the foliage was allowed to dry in the laminar hood for sometime to allow the solvent to evaporate. The insects being sap suckers (mealy bugs) and leaf - cutter (gypsy moth caterpillars) were fed upon the crude destruxins sprayed foliage, and maintained at 12 hrs light- 12 hrs dark condition at room temperature (25 - 28 ^{0}C) and were observed every 10 hrs to check for any mortality. For mealy bugs, 40 insects each for nymphs and adults were maintained per treatment and two replicates were maintained for each treatment; while for leaf- cutter caterpillar two replicates of 25 caterpillars each was maintained for each of the treatments (Figure 10.3 and 10.4).

Statistical analysis

The mortality data was subjected to Probit Analysis (Finney, 1964) for calculating the LD_{50} values at 30 hrs and 60 hrs post treatment.

10.3 Results and Discussion

Entomopathogenesis of insect pests occurs in nature by itself; but when the same involves human intervention for commercial application, it becomes imperative to check that the natural virulence potential of the prospective bio-control fungi is not compromised by its *in-vitro* laboratory maintenance (Kawakami et al., 1960). Few researches in the past have focused and emphasized upon maintaining and/ or enhancing the fungal virulence by biological, physical and chemical means (Shah et al., 2005; Aizawa, 1971; Veen, 1968). The biological enhancement of fungal virulence involves the strategy of 'parasitic selection pressure' in which the prospective fungus of interest is passed through an insect host for one or more generations. On the other hand, the physical and chemical means of fungal virulence enhancement involves its exposure to

UV- radiations and chemicals like ethyl methane sulfonate, respectively; but such results have not been very promising and perhaps demand more explorations (Al- Aidroos et al., 1978). In the present research, *M. anisopliae* ARSEF - 2735 was screened out to be the most promising candidate in terms of robustness (abiotic stress tolerance; Chapter 2 and 3) and virulence (lytic enzyme production; Chapter 4). But we anticipated some level of attenuation in its virulence potential by repetitive *in-vitro* sub-culturing on artificial media, and therefore subjected it to passage through migratory grass hopper (as detailed above) (Fig. 10.1). Luckily, as expected, we noticed an appreciable increment in its lytic enzyme production as well as its perpetuation potential, viz., its germination, growth and sporulation features (Table 10.2). The chitinase and protease activity of the insect passaged conidia were observed to be more than that of the native conidia (Figure 10.1 and 10.3). The maximum chitinase activity of the insect passaged conidia was 10.31 % more than the maximum chitinase activity of the native conidia. Similarly, the maximum protease activity of the insect passaged conidia was 20.53 % more than the maximum protease activity of the native conidia. We used mealy bugs and gypsy moth for bioassays but grasshopper to obtain insect passaged conidia. Hartmann and Wasti (1974) too used the alternate host approach to increase fungal virulence. Al- Aidroos and Roberts (1978) on the other hand were able to enhance virulence by passage of strains through the same host that was used for bioassays. Veen (1968) also noticed increase in fungal virulence during insect bioassays.

Doust and Roberts (1982) reported that the virulence of two *M. anisopliae* isolates was significantly enhanced by one passage through *Culex pipens*. Hall et al. (1980) demonstrated the effect of repeated subculturing on agar and passaging through an insect host on pathogenecity, morphology, and growth rate of *Verticillium lecanii*. In the present study, the LD_{50} values (Table 10.3) for the nymphs of mealy bugs treated with crude destruxins produced from insect passaged conidia were lower (50.73 % at 30 hrs and 30.13 % at 60 hrs of treatment) than that of the nymphs treated with crude destruxins produced from native conidia; and the LD_{50} values for the adults of mealy bugs treated with crude destruxins produced from insect passaged conidia were lower (23.16 % at 30 hrs and 29.24 % at 60 hrs of treatment) than that of the adults treated with crude destruxins produced from native conidia; so it means that the insect passaged conidia is more virulent than the native conidia (Fig.10.4). For gypsy moth caterpillars too, the LD_{50} values (Table 10.4) for destruxin obtained through insect passaged conidia was lower (77.80 % at 30 hrs and 47.53 % at 60 hrs of treatment) than that of native conidia which means that the insect passaged conidia is more virulent than the native conidia (Fig. 10.5). The insect passaged conidia germinated faster than the native conidia and also had better sporulation than the former (Table 10.1). As obvious from Table 10.2, there is only a meager difference in the quantity of crude destruxin obtained through the insect - passaged and native conidia, but the LD_{50} values by them exhibited marked difference. One probable reason to explain this could be that virulence is a polygenic-determined - trait and that there might be difference in the quantitative expression levels of the destruxin families present in the crude powder obtained by two different sources of conidia i.e. the native and the insect - passaged conidia.

10.4 Conclusion

The insect - passaged conidia had greater chitinase and protease activity than the native conidia, thereby proving an increment in virulence potential of the former. The LD_{50} values (in both the bioassays) by destruxin produced by insect-passaged conidia were lower than that of native conidia, thus highlighting the essence of virulence enhancement of the insect - passaged conidia. One more fact to be mentioned is that we used the crude destruxin to proceed with the insect bio-assays and got commendable mortality results; this is unlike the use of live fungal conidia and thus gives an insight that it is basically the toxin responsible for insect demise. The concept of conidial bioassays (as in 95 - 99 % of the bioassays cited in literature) is to include the mechanical pressure and the production of lytic enzymes to depolymerize the insect cuticle but thereafter it is the toxin which causes the insect death.

Table 10.1: Germination, growth and spore yield of *M. anisopliae* ARSEF – 2735 on SDA

Conidia stage	Parameters	Observations
Native	Germination	76.67 % at 8th hr and 100 % at 16th hr of incubation
	Specific growth	2.0 mmd^{-1}
	Spore yield	3.83 x 10^7 as on 8th day of observation.
Insect passaged	Germination	81.33 % at 8th hr and 100 % at 16th hr of incubation
	Specific growth	2.04 mmd^{-1}
	Spore yield	4.17x 10^7 as on 8th day of observation.

Table 10.2: The details of the crude destruxin obtained and its serial dilutions for insect bioassays

Conidia source	**Conidia concentration to inoculate CD Broth**$^{\$}$	**Extracting solvent (100 ml each)**	**Quantity Of crude destruxin (mg)***	**Stock solvent (1 ml each)**	**Stock (mgml^{-1})****	**Serial dilutions for Bioassays*****				
						0X	1X	10X	100X	1000X
Native	2.75 x 10^6	DCM	10.50	EA	Dissolving entire quantity in 1 ml of EA.	Solvent EA only	10.50	1.050	0.1050	0.01050
Insect passaged	2.5 x 10^6	DCM	10.56	EA	-do-	-do-	10.56	1.056	0.1056	0.01056

DCM: Dichloromethane.
EA: Ethyl Acetate.
$^{\$}$ Three replicates of 100 ml each were maintained.
* Quantity represents the amount of crude destruxin obtained in mg from 100 ml of the inoculated culture per 10^6 conidia concentration as the starting material. These were the values when DCM was used as the extracting solvent.
** Stock was prepared by dissolving (in EA) the total amount of the crude destruxin powder obtained after evaporation of the extracting-solvent (DCM).
***The serial diluents are in mgml^{-1}, the same as that of stock solution.

Table 10.3: Probit Analysis (Finney Method [Lognormal Distribution]) to estimate LD_{50}[*] values in mealybugs, *Maconellicoccus hirsutus*

Conidia source	Insect stage[a]	LD_{50}[b]	LD_{50}[c]
Native	Nymphs	6.82	2.19
	Adults	90.80	10.50
Insect passaged	Nymphs	3.46	0.66
	Adults	21.03	3.07

[*] The calculated LD_{50} values are in mgml^{-1}, the same as that of the stock solution as well as the serial diluents obtained from it.

[a] The control treatment included anti - feedant activity on foliage coated with solvent only.

[b] The values were recorded 30 hrs post treatment.

[c] The values were recorded 60 hrs post treatment

Table 10.4: Probit Analysis (Finney Method [Lognormal Distribution]) to estimate LD_{50} values in caterpillars of gypsy moth, *Lymantria dispar*

Conidia source	**LD_{50}[b]**	**LD_{50}[c]**
Native	28.38	7.91
Insect passaged	22.68	3.76

*The calculated LD_{50} values are in $mgml^{-1}$, the same as that of the stock solution as well as the serial diluents obtained from it.

[a] The control treatment included anti - feedant activity on foliage coated with solvent only.

[b] The values were recorded 30 hrs post treatment.

[c] The values were recorded 60 hrs post treatment.

Fig.10.1. (a).Migratory grasshopper cadaver showing emerged *M. anisopliae* conidia (b).The sub-cultured insect passaged conidia.

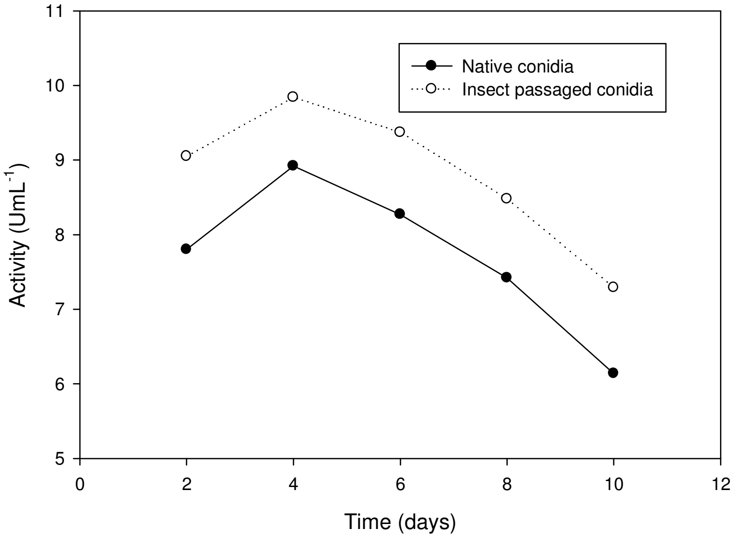


Fig. 10.2: Chitinase activity of native versus insect passaged conidia of *M. anisopliae* ARSEF - 2735.

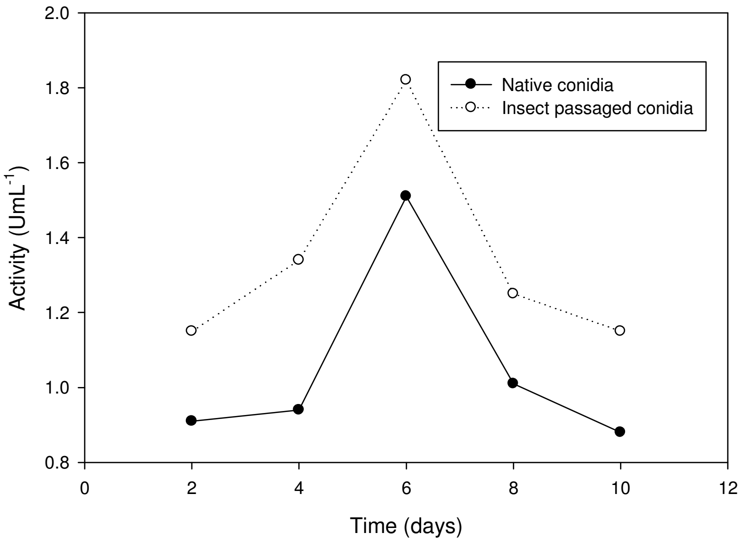


Fig. 10.3: Protease activity of native versus insect passaged conidia of *M. anisopliae* ARSEF - 2735.

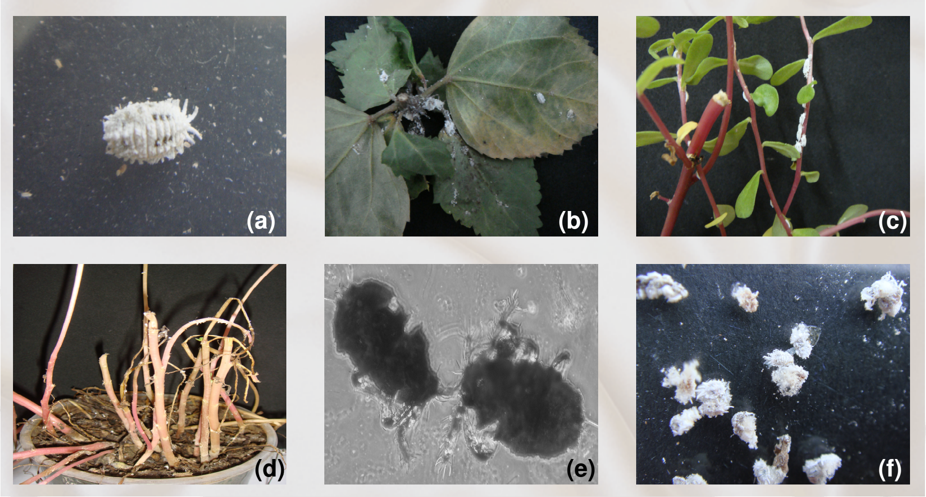


Fig.10.4: (a).An adult Mealy bug (b).Mealy bug infestation on China rose (c).Mealy bug infestation on moss rose (d).Defoliation caused by mealy bug (e).The destruxin-killed nymphs of mealy bug (10X magnification) (f).The destruxin-killed adult mealy bugs.

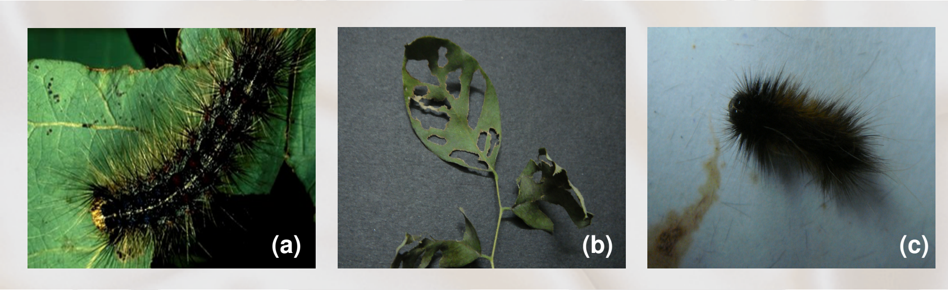


Fig.10.5: (a).A leaf-cutter caterpillar (b).Damage caused by leaf-cutter caterpillar (c).The destruxin-killed caterpillar (notice the shed bristles and loss of body fluid).

11.1 Summary and Inference

Flowcharts11.1-11.4 represents the synopsis of my thesis entitled “Studies on Abiotic Stress tolerance, Virulence Determinants and Molecular Characterization of *Metarhizium anisopliae* and *Beauveria bassiana* isolates, and Toxin Production by *M. anisopliae* (ARSEF-2735)”. Flowchart 11.1 is based on the General Introduction of pest management with emphasis on the avenues and limitations of the strategies explored. Flowchart 11.2A and 11.2B which compares the survival vigour and virulence potential of *M. anisopliae* and *B. bassiana* isolates is based on Aspect 1 of the thesis titled “Abiotic stress tolerance and extra-cellular enzyme production by *M. anisopliae* and *B. bassiana* isolates”. Flowchart 11.3 is based on Aspect 2 of the thesis titled “Establishing genetic relationships amongst the *M. anisopliae* and *B. bassiana* isolates”. Flowchart 11.4A-D is based on Aspect 3 of the thesis which is “Studies on destruxin production, characterization and toxicity testing”.

11.2 Highlights of the present research:

- The *M. anisopliae* isolates are more tolerant to abiotic stresses than the *B. bassiana* isolates
- The *M. anisopliae* isolates are better secretors of extra-cellular lytic enzymes than the *B. bassiana* isolates.
- Isolate UM2 was screened out to be the best isolate in terms of growth characteristics, tolerance to abiotic stresses and production of virulent enzymes.
- We found controlled probe-tip-sonication as an improved and efficient cell lysis methodology for rapid DNA extraction for large sample sizes.
- Maximum destruxin yield is obtained between days 5-10 of culturing.

- Insect-passaged conidia are more virulent than the native conidia.
- Destruxin has appreciable contact (immersion assays on whiteflies) as well as antifeedant activity (bioassays on mealy bugs and leaf- cutter caterpillars).

11.3 Future prospects:

- We followed one variable at a time (OVAT) strategy to study the impact of abiotic stresses on the two fungi with 31 isolates and studied growth characteristics (germinatin rate, growth rate and spore yield) at different temperatures (5 different temperatures), different UV-B exposue (at 5 different exposure duration) and different media (7 different media). So further studies on this aspect would be to screen out the weak supporters of tolerance and study the interactive effect of the abiotic stresses.
- The best protease and chitinase producing isolates from M. anisopliae and B. bassiana can be selected to proceed with strain improvement for anticipated enhanced enzyme activity and virulence.
- In the case of destruxins, we have done the bioassays with different dilutions of the crude lyophilized extract. For further studies in this area, a probable suggestion would be to isolate and purify the specific members of the destruxin family and then proceed with the bioassays.
- The basic information derived from the studies of growth characteristics with respect to the nutrient studies can be used to develop cheap mass production methodology for the commercial use of the selected isolate.

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