Influence of nutrition on the virulence and stability of the insect-pathogenic fungus Metarhizium anisopliae.

Shah, Syed Farooq Abbas

Influence of nutrition on the virulence and stability of the insect-pathogenic fungus *Metarhizium anisopliae*

Syed Farooq A. Shah

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Dedicated to My Parents

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This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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Summary

Nutrition influenced growth, sporulation, phenotypic stability and virulence of the insect pathogenic fungus, *Metarhizium anisopliae.* Virulent conidia were produced on nutrient poor or osmotic stress media, while least virulent conidia were produced on nutrient rich media. Repeated subculturing on nutrient rich media caused further attenuation of virulence, however, attenuation was strain dependant. Several strain independent parameters were identified that could be used to monitor the virulence of *M. anisopliae* conidia during normal production or when developing new inexpensive culture media. Virulent conidia typically had high levels of spore bound Pr1, a CN ratio below 5.2:1 and high germination rates. RT-PCR revealed that virulent conidia from insects contained high levels of transcripts of *pr1 A* and other pathogenicity-related genes (e.g. *ste 1, try 1 and chy 1*). Virulent conidia from 1% yeast extract media had higher levels of transcripts of these pathogenicity-related genes than the least virulent conidia from CN 35:1 medium (= SDA), however, levels were significantly lower than those in insect-derived conidia. This study shows for the first time that passaged inoculum is virulent irrespective of the original culture medium or insect host suggesting that starvation conditions, whether *in vivo* or *in vitro,* results in de-repression of Pr1 and that elevated levels of this enzyme enhance fungal virulence. Nutrition also influenced expression of other pathogenicity determinants e.g. adhesive properties of conidia (hydrophobicity, surface charge) and destruxins production, however, inconclusive relationship between these pathogenicity determinants and nutrition was observed. Nutrition also influenced fungal stability independent of strain. Under similar nutritional conditions, V275 produced fewer sectors than V245. Most sectors were sterile and produced significantly lower quantities of pathogenicity determinants. Careful selection of stable strains and manipulation of cultural conditions could be employed to enhance or stabilize virulence of *M. anisopliae*.

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Chapter 1: General Introduction

1.1 Introduction

Metarhizium anisopliae is a soil-borne entomopathogenic fungus found throughout the world (Humber, 1992; Zimmerman, 1993). It has a wide host range including major genera of insects and some medically important acarids e.g. ticks and mites (Mugnai *et al.*, 1989; Zimmerman, 1993; Kaaya *et al.*, 1995). Much progress has been made in the development of *M. anisopliae* for use in integrated pest management (IPM) programmes for the control of arthropod pests (Butt *et al.*, 2001). This biocontrol agent (BCA) offers an environmentally friendly alternative to chemical pesticides, especially to pesticides that are banned (e.g. organochlorines) or being phased out (e.g. methyl bromide) or to which pests have developed resistance (Butt *et al.* 2001).

At present more than 20 products based on entomopathogenic fungal BCAs are on the market or being developed as commercial products (Butt and Copping, 2000; Kabalouk and Gazdick, 2004; Appendix 1). However, their share in the total pesticide market is negligible and often restricted to niche markets (Butt and Brownbridge, 2001). One major factor contributing to the slow uptake of fungal BCAs such as *M. anisopliae* is the inconsistency in their performance. Attenuation of virulence during successive *in vitro* subculturing is considered to be a major factor leading to inconsistency in the efficacy of fungal BCAs (Butt *et al.*, 2001). Nutritional conditions during inoculum production of many other fungal BCAs have also been reported to influence the quality of the inoculum (Winder, 1999; Magan, 2001). These reports also indicate that nutritional conditions favouring a high yield of inoculum may adversely affect the virulence of inoculum, therefore, it is important to understand the effect of nutrition on both quantitative yield and quality of inoculum.

Studies to date have focussed on the influence of nutrition on selected attributes such conidial yield, endogenous reserves, surface carbohydrates of *M. anisopliae* where as

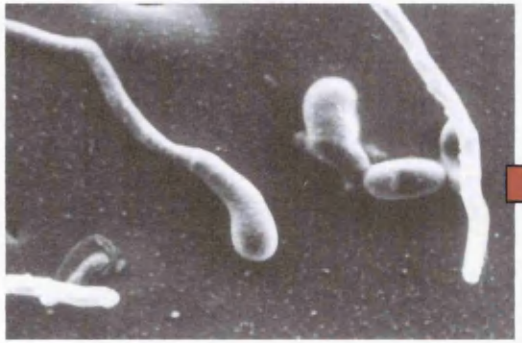
less attention has been given to study the influence of nutrition on its virulence. There is urgent need to improve production of efficacious inoculum. This will also require development of cost-effective and reliable quality control procedures to monitor the changes in *M. anisopliae* virulence during its production and storage. To date, there is no high throughput method available for monitoring changes in virulence. Current methods of determining the efficacy involve bioassays against test organisms, which is a time-consuming and expensive process.

Studies of the infection process have helped identify pathogenicity determinants of *M. anisopliae*. However, no one has conducted a detailed study to understand the relationship between nutrition and expression of pathogenicity determinants of *M. anisopliae*. To our knowledge this is the first study to addresses the influence of nutrition on both quantitative (conidial yield, phenotypic stability etc.) and qualitative attributes (virulence and its determinants) of *M. anisopliae*. The section below (1.2) describes the infection process of *M. anisopliae* and attention is drawn to factors that are considered to be virulence determinants.

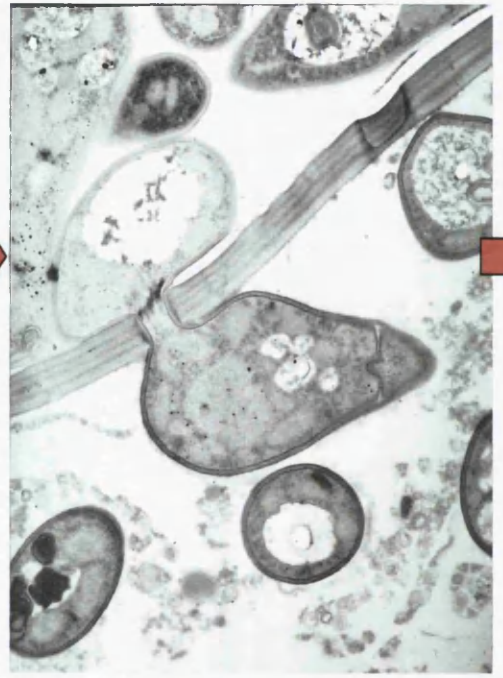
1.2 Infection process of *M. anisopliae*

M. anisopliae infects its host via a series of integrated, systematic events progressing from spore attachment to germination, penetration, growth and proliferation within the host body, interactions with host defence mechanisms and finally death of the host (Hegedus and Khachatourians, 1995). Under favourable environmental conditions, the fungus emerges from the cadaver and differentiates into conidia (Roberts and Humber, 1984). These interactions between fungus and host are extremely complex; however, certain events are necessary and will take place in any successful infection. These include attachment of conidia to host, recognition of host via signal molecules, penetration requiring a combination of enzymes and mechanical force, production of metabolites or rapid colonization of host tissues. The quantitative and qualitative differences in these attributes lead to variability in fungal virulence. Major events of the infection process are shown in fig. 1.1.

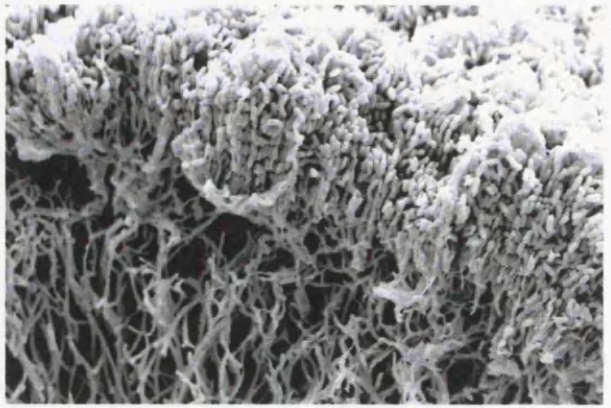
Fungus penetrates the host cuticle using a combination of enzymes and mechanical force. Cuticle provides cues for enzyme induction



Conidia attach to the host cuticle. The first step involves pre-formed hydrophobic and electrostatic forces. The second step involves secretion of mucilage and enzymes which ensure spores remain attached to the cuticle. Germinated conidia differentiate specialised infection structures called appressoria.



Pathogen multiplies inside the host in the form of blastospores and/or hyphal bodies. These cells may produce toxic metabolites e.g. destruxins which may suppress the hosts defences.



After exhausting the nutrients within the haemocoel, the fungus breaches the cuticle and differentiates conidiophores and conidia

Fig. 1.1 Key steps in the invasive and developmental processes of insect-pathogenic fungi. (Photographs kindly provided by Dr Butt)

1.2.1 Attachment of conidia to the host cuticle

The initial step in the establishment of infection is the attachment of conidia to the host surface. Conidial attachment occurs in two stages i.e. passive and active. Passive attachment involves preformed factors such as surface hydrophobicity and electrostatic forces. Secretion of mucus and spore bound enzymes during the active stage consolidates the passive adhesion (Fargues, 1984; St. Leger *et al.* 1991b; El-Sayed *et al.*, 1994).

The dry hydrophobic conidia of *M. anisopliae* have a negative charge (Boucias and Pendland, 1991). The very first step of adsorption, which occurs non-specifically, is thought to be facilitated by electrostatic and hydrophobic interactions (Boucias *et al.*, 1988; Boucias and Pendland, 1991). However, the exact mechanism by which conidia initially attach to their host remains unclear. Fargues (1984) suggested that lectins played a role in the initial attachment process whereas Boucias *et al.* (1988) reported that lectins and electrostatic force play only a minor role in adhesion of spores to insect cuticle. The latter did observe that the hydrophobicity of *M. anisopliae*, *Beauveria bassiana* and *Nomuraea rileyi* conidia did influence spore attachment. They also observed that the rodlet layer responsible for imparting hydrophobicity to the spore could be stripped off by harsh alkali treatment (1.0 M NaOH, 100°C for 1 hr), which then significantly reduced the adhesion to cuticle surfaces. In plant pathogenic fungi, the surface hydrophobicity of fungal spores has also been associated with their adhesion (Kuo and Hoch, 1996, Tuker and Talbot, 2001)

Doyle (2000) has listed several pathogens including fungi, actinomycetes and bacteria that demonstrate the role of hydrophobicity in their virulence or infection. Surface hydrophobicity has also been shown to differ between different types of fungal conidia e.g. submerged and aerial conidia of several plant and insect pathogens (Pascual *et al.*, 2000). In the plant pathogenic fungus *Magnaporth grisea*, a reduction in hydrophobicity resulted in reduction in attachment and pathogenicity (Talbot *et al.*, 1996).

Molecules other than hydrophobins are an integral part of the surface layer of fungal spores (Beever *et al.*, 1979; Cole *et al.,* 1979; Tronchin *et al.* 1997) and can therefore play a role in physiochemical properties of the spore. For instance, the conidial outer cell wall of fungal species contains lipids, which could contribute to surface hydrophobicity (Cole *et al.*, 1979; Latge *et al.,* 1988). Glycoproteins such as adhesins are distributed over the surface of the rodlet layer of *Aspergillus* conidia (Tronchin *et al.*, 1997). Differences in the composition and /or nature of these molecules may therefore, alter the adhesion potential.

Adhesion of *M. anisopliae* conidia is also influenced by the properties of the host surface. For example, Lacey *et al.*, (1988) observed that *M. anisopliae* conidia show an affinity towards the hydrophobic region i.e. the siphon tube of the mosquito. Boucias *et al.*, (1988) examined conidial adhesion on cuticle ghost preparation and observed that the presence and spatial arrangement of cuticular spines facilitates conidial adhesion. However, they concluded that the presence of these spines was not a requirement for adhesion as conidial attachment was also observed on regions void of theses structures e.g. head capsule, seta, etc. Similarly, prior work with *M. anisopliae* demonstrated that conidia bind nonspecifically over the cuticle but can be displaced from smooth sclerite epicuticle more easily than from the epicuticle folds (Zacharuk, 1970).

Studies on insect surface biochemistry revealed that cuticular lipids have a greater effect on adhesion than cuticular proteins. The removal of protein using chemical treatments did not alter the conidia adhesion, whereas cuticular lipid extraction with solvents significantly reduced the *M. anisopliae* adhesion (Sosa-Gomez *et al.*, 1997). Similarly, conidial distributions could be region specific on host cuticle (Sosa-Gomez *et al.*, 1997) and surface topography of host may influence growth of deutromycetes after adhesion (Boucias and Pendland, 1991).

As mortality is dose related (Butt and Brownbridge, 2001), any positive change in conidial properties to increase their attachment to the host would directly influence

virulence, therefore, it is vital to understand the role of nutrition on adhesion properties of the inoculum.

1.2.2 Germination

After attachment to the host cuticle, the conidia germinate using endogenous and exogenous nutrients. Osherov and May (2001) describe conidia germination as a regulated process that responds to environmental stimuli by a signalling cascade that is amenable to genetic and biochemical inquiry. The nutritional requirements for conidial germination varies inter and intra specifically, however, an exogenous source of nutrient is generally required for germination. Pre - soaking of *M. anisoplie* conidia in distilled water resulted in swelling of conidia coupled with protein synthesis but high levels of germination required a utilizable carbon source (Dillion and Charnley, 1990). Conidia of *M. anisopliae* obtained from different hosts also varied for the ability to germinate on glucose. Isolates from coleopteran hosts germinated poorly on glucose compared to isolates from hemipteran or lepidopteran sources (St. Leger *et al.*, 1994).

Smith and Grula (1981) found that, *in vitro*, a utilizable, exogenous carbon energy source was required for germination of *B. bassiana* conidia, while a nitrogen source was further required to sustain hyphal growth. Although conidia apparently possess sufficient nitrogen reserves to germinate, the supply is soon depleted and without an exogenous nitrogen source lysis of the germ tube soon occurs (Hunt *et al.*, 1984). Milner *et al* (1991) reported that simple amino acid solutions do stimulate conidial germination of *M. anisopliae* suggesting the provision of both carbon and nitrogen sources by these amino acids were sufficient for germination.

An important aspect of host specificity is thought to be associated with fungal-cuticle interactions in terms of nutrient utilization on the host surface (Boucias and Pendland, 1984; El-Sayed *et al.*, 1991). Samuels *et al.*, (1989) attributed high specificity of various strains of *M. anisopliae* against *Oryctes rhinoceros* to the

presence of intermediate nutrients on or inside the host's cuticle. Similarly, Butt *et al.*, (1995) observed that the high availability of nutrients on the cuticle of aphids stimulates the rapid germination of *M. anisopliae* conidia.

The speed of germination is important for successful infection and is an attribute of highly virulent isolates. Studies with various entomopathogenic fungi have shown that the isolate exhibiting faster speed of germination *in vitro* had higher virulence. For example, germination speed *in vitro* was correlated with the infectivity of *M. anisopliae* against mosquito larvae (Al-Aidroos and Roberts,1978; Al-Aidroos and Seifert, 1980) and of *Verticillium lecanii* isolates against adult aphids (Jackson *et al.*, 1985) and glasshouse whiteflies (Chandler *et al.*, 1993). Isolates of *Paecilomyces fumosoroseus* that germinated faster on diamondback moth cuticle were the most virulent (Alter *et al.*, 1999). Another advantage of rapid speed of germination is that environmental conditions often offer a narrow window of favourable conditions for infection, thus enabling fast germinating conidia to perform better. Similarly host defences and morphological changes could be easily tackled by fast germinating conidia as opposed to relatively slow germinating ones. For example, on immature insects, a fungal pathogen needs to penetrate before being shed with moulted cuticle (Vey and Fargues, 1977).

Hassan *et al.*, (1989) observed accelerated germination and appressorium formation *in vivo* among pre soaked conidia compared to fresh conidia. They also observed significantly higher mortality of *Munduca sexta* larvae caused by pre soaked conidia. Therefore, any change resulting in faster germination could directly influence virulence. Studies on the plant pathogenic fungus *Coletotrichum truncatum* showed that conidia produced on low CN 10:1 media germinated faster compared to those produced on moderate 30:1 or high CN 80:1 ((Jackson and Schisler, 1992). Similarly, Hallsworth and Magan (1995) manipulated endogenous reserves of conidia of *M. anisopliae*, *B. bassiana* and *P. fumosoroseus* enabling conidia to germinate faster. These modified conidia were also able to germinate at a relatively

low humidity suggesting the potential of nutritional/cultural manipulations to improve germination speed and virulence of pathogenic fungi.

1.2.3 Cuticle Penetration

Following germination, the most important step in the infection process of *M. anisopliae* is the penetration of the host cuticle (Zacharuk, 1970; Hassan and Charnley, 1989). This process is achieved by a combination of mechanical force and enzymatic activity. The relative contribution of mechanical force and enzymatic activity depends on the characteristics of host cuticle. It has also been demonstrated that the pathogenic process involving the production of cuticle degrading enzymes (CDEs) and penetration of host cuticle occurs only when it is necessary to establish a nutritional relationship with host (St. Leger *et al.*, 1989)

Insect cuticle is a complex structure comprising 25-40% of the chitin embedded in protein (up to 70%) and lipid layers (Hackman, 1964). The majority of CDEs are inductive in nature and the relative composition of polymers in the cuticle may therefore predetermine the production of individual enzymes (St. Leger *et al.*, 1986a, b). *M. anisopliae* extra cellular hydrolytic enzymes are important for the degradation of host cuticle during infection, assisting penetration and providing nutrients for further growth (Zacharuk, 1970; Hassan and Charnley, 1989). Several authors have reported that *M. anisopliae* produces a diverse and large array of enzymes during the cuticle penetration and *in vitro* growth on medium containing insect cuticle as the sole source of carbon and nitrogen (St. Leger *et al.*, 1986a, 1987; Charnley 1992; Hajek and St. Leger 1994) These enzymes are synthesized in a co-ordinated manner related to cuticle structure (Samsinnakova *et al.*, 1971: Smith *et al.*, 1981) and are induced *in vitro* when grown on insect cuticle (St. Leger *et al.*, 1986a,b; 1987; El Sayed *et al.*, 1992; Gupta *et al.*, 1991, 1992). The variability of enzymes increases the range of tools naturally available to degrade the cuticle, however, proteases and chitinases have an important correlation with virulence.

Protein is the predominant structural component of the insect cuticle and it has been shown that proteases released during the first steps of invasions are involved in the penetration of the cuticular barrier and thus constitute a crucial factor in the pathogenicity (St. Leger *et al.*, 1988). The Expressed Sequence Tag (EST) analysis of *M. anisopliae* revealed that proteases account for the 36% *M. anisopliae* of a secreted cDNA library constructed under inductive conditions (Freismore *et al.*, 2003).

M. anisopliae produces families of catalytically distinct extracellular subtilisin-like proteases (Pr1), trypsin like proteases (Pr2), and metalloproteases as well as several families of exo-acting peptidases. The only known function of secreted subtilisins is the acquisition of nutrients and the breaching of host cuticle. The sharp increase in their production during penetration and the presence of their exceptionally large number of subtilisins isoenzymes is presumably related to pathogenicity (Bagga *et al.*, 2003). Among these, subtilisin Pr1 is predominant in its role in virulence and exists as several isoenzymes (Pr1 A, B, C, D, E, F, G, H, I, J, K) with Pr1A playing a major role in cuticle penetration (St. Leger *et al.*, 1989). The EST for Pr1 A are 10 times more abundant than the second most highly expressed sequence (Pr1J) (Bagga *et al.*, 2003). These different isoforms of Pr1 are thought to play an important role in host specificity and pathogenicity (Bagga *et al.*, 2003).

Pr1 production *in vitro* is regulated by both carbon and nitrogen de-repression (St. Leger *et. al.*, 1993; Paterson *et al.*, 1994a). Once induced during infection, Pr1 is produced at high levels until penetration is complete and low molecular weight carbon and nitrogen compounds provide a feedback to stop Pr1 production. *M. anisopliae* produces Pr1 *in vitro* in liquid cultures containing insect homogenate/cuticle as the sole source of carbon and nitrogen. Simple carbon sources such as amino acids and glucose strongly repress Pr1 production (Butt *et al.*, 1998). It is possible that successive subculturing of *M. anisopliae* on artificial media with glucose as a main constituent alters the expression of the *pr1* gene. This shift in gene expression might result in a decrease or total loss of Pr1 production by attenuated

cultures. In certain cases these switched-off genes might also be lost resulting in less virulent mutants (Wang *et al.*, 2002). This hypothesis is also based on fact that all cases known for attenuation are reported from nutrient rich media and the pathogen regains its virulence with one or more passages through its host (Kawakami, 1960; Nagaich, 1973; Fargues and Roberts, 1983; Morrow *et al.*, 1989; Vandenberg and Cantone, 2004). Furthermore, St. Leger *et al.*, (1991b) showed that the spore bound enzyme profile of conidia obtained from killed insects and those obtained from culture media like Sabouraud dextrose agar (SDA) varied significantly. Higher levels of spore bound Pr1 and NAG'ase were observed on conidia obtained from insects compared to those produced on artificial media suggesting the role of nutrition to pre adapt conidia for pathogenic or saprophytic mode of action.

In addition to proteases, chitinases are required by the fungus to penetrate the cuticle as well as to emerge out of the host after its death (Coudron *et al.*1984). Hydrolysis of chitin is mediated by a series of exo (*N*-acetyleglusaminidase and chitiobiase) and endo- (chitinase) degradative enzymes. Synthesis of these enzymes is induced by the soluble monomers of chitin: glucosamine, *N*- acetyleglucosamine and chitiobiose that are present in minute quantities in the cuticle (St. Leger *et al.*1986b, 1991a, 1993; Bidochka and Khachtourians, 1993; Havukkala *et al.*, 1993). Although cuticles of insects fed with a diet containing specific chitin synthesis inhibitors were more susceptible to *M. anisopliae* hyphal penetration (Hassan and Charnley, 1989) chitinase has not been detected *in vivo* during the early stages of penetration (St. Leger *et al.*, 1987b). *In vitro* production of chitinases took place at a later stage as compared to that of proteases (Gupta *et al.*, 1992). In addition to proteases and chitinases, a variety of other enzyme are also produced by the entomopathogenic fungi, however, their role in pathogenesis and virulence is less clear but appears not to be as important as those of above enzymes.

The level and number of CDEs produced vary inter and intra specifically (Gillespie *et al.*, 1998). Many studies have shown a direct relationship between enzyme production and virulence (Gupta *et al.*, 1994). Failure to produce sufficient quantities

of enzymes delays and in most cases stops the penetration and infection process. Gillespie *et al.*, (1998) suggested that sub optimal properties of the CDEs of an isolate might contribute towards its avirulence to a particular host. Reduced virulence has been observed in protease deficient mutants of *B. bassiana* (Bidochka and Khachatourians, 1990) and *M. anisopliae* (Wang *et al.* 2002). An increase in Pr1 production was linked with high virulence in a Pr1 over expressing mutant of *M. anisopliae* (St. Leger *et al.*, 1996). Paris *et al.* (1985) and El-Sayed *et al.* (1989) observed a similar loss of virulence in chitinase deficient mutants. However, recently, a transformant of *M. anisopliae* with increased chitinase production showed no corresponding increase in virulence suggesting that chitinase is not limiting for cuticle penetration (Screen *et al.*, 2001).

The regulation of CDEs is complex, usually involving a combination of substrate induction and carbon and nitrogen repression (St. Leger, 1993; Butt *et al.*, 1998). Proteases have an additional role in providing nutrients, before and after the cuticle is penetrated. Consequently, regulation is looser, with production being triggered in response to limitation for nutrients such as carbon and nitrogen (St. Leger, 1993). However, production is enhanced when the pathogen is grown on insect cuticle (Paterson *et al.*, 1994b). The enhanced levels of enzymes activities present on *Manduca sexta* derived spores as compared to those produced on nutrient rich SDA media suggest that nutritional conditions in which spores develop can pre adapt them for a pathogenic life style (St. Leger *et al.*, 1991b). Although there is sufficient information on the regulation of CDEs both during *in vitro* and *in vivo* growth of *M. anisopliae*, very little is known about the influence of nutrition during inoculum production on the ability of the inoculum to produce these enzymes. Considering the importance of CDEs in *M. anisopliae* virulence, it is vital to understand regulation of important enzymes with special reference to *M. anisopliae* nutrition during inoculum production. Such information will help us to identify nutritional conditions, which could be used to produce large quantities of *M. anisopliae* inoculum with minimal repression of CDEs.

1.2.4 Host colonization

Once inside the host body, *M. anisopliae* either grows rapidly to colonize host tissues or secretes toxic metabolites to kill the host (Amiri *et al.*, 2000; Kershaw *et al.*, 1999). Among the metabolites produced, the destruxins (dtxs) are of particular interest because they are the dominant mycotoxins detected in the insect body at advanced stages of infection to cause death (Suzuki *et al.,* 1971). Destruxins, the cyclic depsipeptides are secondary metabolites produced by *M. anisopliae* (Roberts, 1981). At least twenty-six different destruxins have been identified so far, of which destruxins A, B and E predominate (Pais *et al.*, 1981; Wang *et al.*, 2004).

Injection of the dtxs into lepidopteran larvae or to adult Diptera resulted in immediate, titanic muscular paralysis, followed by flaccidity, which was reversible at low doses but high doses were lethal (Kodaira, 1961; Roberts, 1966; Samuels *et al.*, 1988a, b, c.). Similar symptoms together with the identification of dtxs or dtx-like material from lepidopteran larvae infected with *M. anisopliae* in quantities sufficient to have caused death proves the role of dtxs in pathogenesis and insect mortality (Roberts, 1966; Samuels *et al.*, 1988a; Suzuki *et al* 1971; Vey *et al.*, 1986). These toxins have often been implicated as the cause of death of insects infected with *M. anisopliae* (Butt *et al.*, 1994; Vestergaard *et al.*, 1995). Death occurs shortly after inoculation and often without any sign of the fungus in the haemocoel, suggesting that toxins are secreted during very early stages of infection and that the pathogen colonizes its host as a necrotroph (Butt *et al.*, 1994).

Although destruxins have been suggested to be an important virulence determinant (Suzuki *et al.*, 1971; Vey *et al.*, 1986; Samuels *et al.*, 1988b; Dumas *et al.*, 1994; Brousseau *et al.*, 1996; Kershaw *et al.*, 1999), other factors may also contribute to pathogenesis, and isolates that do not produce dtxs may have alternative virulence strategies (Amiri *et al.*, 2000; Kershaw *et al.*, 1999). Kershaw *et al.*, (1999) also reported that isolates of *M. anisopliae* varied considerably in their ability to produce dtxs *in vitro*. High titres were produced only by *M. anisopliae* sf. *anisopliae* isolates while least or no production was observed for *M. anisopliae* sf. *majus*. Similarly a

spontaneous mutant *of M. anisopliae* exhibiting a higher virulence than the wild type was also observed to produce more toxin than wild type (Al-Aidroos and Roberts 1978). However, the mutant also had denser sporulation and more rapid *in vitro* germination, therefore the link between destruxins production and virulence is not proven. Recently, Wang *et al.*, (2002, 2003) reported a *M. anisopliae* mutant, which exhibited lower virulence than the wild type against wax moth larvae and had also lost the ability to produce destruxins.

Some strains of *M. anisopliae* however, grow profusely in their hosts without inducing symptoms of toxicosis and death of the insect occurs slowly (Samuels *et al.*, 1988b). Bagga *et al.*, (2003) observed the presence of several transcripts encoding enzymes involved in the synthesis of toxic metabolites in *M. anisopliae* sf. *anisopliae* (toxin producing fungus as reported by Samuels *et al.* 1989) and the absence of counterparts in *M. anisopliae* sf. *acridium* (colonizes host tissues and no toxin detected by Inglis *et al.* 2001) and concluded that these differences are representative of the different strategies these two fungi use.

A number of other observations further suggest intra specific variation in toxin production. Destruxins A and E were observed to be more toxic than others (Dumas *et al.*, 1994), so the relative amount of these toxins could influence virulence and specificity. Amiri *et al.,* (2000) showed inter and intra specific differences in destruxin production among different isolates of *M. anisopliae*. Amiri *et al.*, (2000) suggested that the quantity and type of destruxins secreted could play an important role determining virulence and/or specificity for some strains of *Metarhizium.*

Destruxin A is the predominant toxin produced by *M. anisopliae* sf. majus, *M. flavoviride* and *M. album*, which are usually restricted to representatives of Coleoptera, Orthoptera and Hemiptera, respectively (Kaijiang and Roberst, 1986; Rombach, *et al.*, 1987). Samuels *et al.*, (1988b) reported intra-specific variation in destruxin A production in four strains and found that those which produced large quantities were highly pathogenic for *Munduca sexta*, however, such a relationship

was not observed by Amiri *et al.*, (2000). Amiri *et al.*, (2000) also suggested that destruxins presumably work in concert with other pathogenicity factors in killing the insect.

Trace amounts of destruxins A, B and E detected in the mycelium of *M. anisoliae* suggested that all three compounds are synthesized in their active form but are rapidly secreted into the medium (Amiri *et al.*, 2000). The presence of the destruxin in similar ratios in mycelia and in media suggest that destruxins are biosynthesised in mycelia and straight after, released in culture media, thereby suggesting rapid diffusion process for destruxins (Loutelier *et al.*, 1996).

Cultural conditions are also thought to influence destruxins production (Wang *et al.*, 2004). Jegerov *et al.*, (1989) reported that no selective *in vitro* production of destruxins could be achieved by addition of any common amino acids. However, they did find that addition of L- proline into Czapek Dox medium significantly affected the total production of destruxins. Using maltose as a main carbon source gave higher destruxins yield than that of the conventional Czapek Dox broth medium (Chen *et al.*, 1999). Espada and Dreyfuss (1997) reported cyclic peptolide 90-215 influenced the titre and ratios of destruxins and helvolic acids in various *M. anisopliae* strains, however little is known about the effect of successive subculturing on destruxins production.

From the above discussion it is evident that *M. anisopliae* uses multiple approaches as pathogenicity and virulence determinants. Certain pathogenicity determinants appear to be influenced by the nutritional conditions, however, further studies are required to elucidate the role of nutrition on individual pathogenicity determinants.

1.3 Genetics of *M. anisopliae*

M. ansipliae is haploid, mitosporic fungus and is known to have no teleomorphic stages (Bidochka *et al.*, 2001). It is assumed to reproduce clonally and has the potential for parasexual recombination. Modes of genetic recombination have been identified in *M. anisopliae*, but it can only anastomose with closely related, parasexually compatible isolates (Bidochka *et a*l., 2000; St. Leger *et al.*, 1993).

Several studies using a variety of molecular techniques e.g. RAPD, RFLP, suggest genetic variability among difference isolates of *M. anisopliae* (Leal *et al.*, 1994; Fegan *et al.*, 1993). The total genome size of *M. anisopliae* was estimated to vary from 23.39 to 31.88 Mb among the Brazilian isolates (Inglis and Peberdy, 1998). They also reported variation in chromosome numbers form 7-8. The smallest and largest chromosome observed in their study was 1.33Mb and 6.02 respectively, however, each chromosome varied in its size among the different isolates. In an earlier study Shimizu *et al.*, (1992) reported variability among Japanese isolates with the genome size varying from 29.6 to 32.1 Mb. They observed 7 chromosomes in each isolate and estimated the size of the chromosomes varied between 1.6 to 7.4 Mb. In a recent study, Wang *et al.*, (2003) reported loss of a conditionally dispensable chromosome (1.05 Mb) from a mutant strain of *M. ansiopliae*. This dispensable choromosome was also reported as encoding two important pathogenicity related genes Pr1 and destruxins (Wang *et al.*, 2002, 2003).

1.4. Mechanisms involved in attenuation of virulence

An important aspect in the production and development of *M. anisopliae* is to maintain its virulence during *in vitro* growth. Like most pathogenic fungi, the entomopathogenic fungi are also notorious for losing virulence upon repeated *in vitro* subculturing on artificial media but the rate and frequency of this phenomenon varies inter and intra specifically (Brownbridge *et al.* 2001). There have been many reports demonstrating the loss of virulence after repeated subculturing in isolates of *B. bassiana*, *M. anisopliae*, *P. farinosus* and *V. lecanii* (Table, 1.1; Kawakami, 1960; Schaerffenberg, 1964; Nagaich, 1973; Fargues and Roberts, 1983; Morrow *et al.*,

1989). Similar findings of decreased virulence during *in vitro* growth have also been reported for entomophthorales fungi. Hajeck *et al.*, (1990) observed an increase in incubation time for *Entomphaga maimaiga* after repeated subculturing and concluded that the rate of subculturing had an impact on virulence, while the absolute length of time in axenic culture did not influence virulence. Similarly a decline in pathogenicity and ability to produce oospores was observed after prolonged culturing of *Lagenidium gigantem* on sterol free medium (Lord and Roberts, 1986).

However, some reports contradict these findings as no attenuation was observed upon repeated subculturing of *B. bassiana*, *B. brongniartii*, *Culicinomyces clavisporus*, *M. anisopliae*, *P. farinosus* and *V. lecani* (Hall 1980; Ignoffo *et al.*, 1982; Sweeney, 1981; Brownbridge *et al.*, 2001; Vandenberg and Cantone 2004). Recently, Vandenberg and Cantone (2004)) observed that up to 30 successive serial *in vitro* transfers of *P. fumosoroseus* had no effect on its virulence against *Diuraphis noxia* or *Plutella xylostella*. Similar observations were also made by Hayden *et al.*, (1992) and Brownbridge *et al.* (2001) for *P. farinosus* and *B. bassiana* respectively, with no loss of virulence seen for up to 15 *in vitro* subcultures on artificial media. The most extreme case of stable virulence was reported for an isolate of *C. clavisporus,* which maintained its virulence against mosquitoes even after 8 years of continuous subculturing on nutrient agar (Sweeney, 1981).

In some cases, the repeated subculturing resulted in morphological changes but no noticeable effect on pathogens virulence. Sparse mycelial growth and less intense pigmentation of *P. fumosorseus* conidia were observed after 30 serial passages but no change in genetic or virulence was observed (Vandenberg and Cantone, 2004)). Similarly, changes in the colony morphology of *V. lecanii* were observed after repeated subculturing (Hall, 1980). Hajeck *et al.*, (1990) observed change in morphology (enlarged spherical protoplasts) and loss of ability to produce conidia in *E. maimaiga* after repeated subculturing.

Although, the phenomenon of attenuation is well documented the underlying mechanisms of attenuation are poorly understood. Nearly all known cases of attenuation are reported from growth on artificial media or due to cultivation in non-host media. These findings suggest a link between nutrition and attenuation of virulence. The fact that virulence can be restored by a single or multiple passage through a host further supports this hypothesis. Several studies have demonstrated recovery of virulence upon host passages; however, some reports even suggest an improvement in virulence and expansion in host range (Ferron, 1985). An increase in virulence of *P. farinosus* was observed after a single passage thorough *Stiobion avenae* and continued to increase for up to 9 passages (Hayden *et al.*, 1992). Vandenberg and Cantone (2004) observed loss of specificity to *D. noxia* by two isolates of *P. fumosoroseus* upon passage through *P. xylostella* but this ability was regained after 5 passages through *D. noxia.*

These contradictions and discrepancies serve to highlight inter and intra-species variation that exists in the effects of repeated subculturing on the virulence and genetic stability of this trait as well as the effect of approaches (media, cultivation method i.e. single spore or multispore etc) used to study attenuation (Brownbridge *et al.*, 2001). Studies by Vandenberg and Cantone (2004) suggest that stability of traits i.e. phenotype and virulence can vary by strain and therefore should be monitored in commercial production settings.

Table. 1.1 Attenuation of virulence in entomopathogenic fungi upon successive *in vitro* subculturing

Entomopathogenic fungus	**Type of attenuation**	**References**
Paecilomyces fumosoroseus	Less virulent, sparse mycelial growth, decline in sporulation	Kawakami, 1960; Vandenberg and Cantonne, 2004
Verticillum lecanii	Less virulent/variant phenotype to parent culture	Nagaich, 1973, Hall, 1980
Metarhizium anisopliae	Less virulent	Al-Aidroos and Seifert, 1980
Beauveria bassiana	Less virulent	Schaerffenberg, 1964; Samsinankova *et al.*, 1981
Entomophaga maimaiga	Less virulent, enlarged spherical protoplasts and loss of ability to form conidia	Hajek, *et al.*, 1990
Lagenidium giganteum	Less virulent, poor sporulation (unable to form oospores and zoospores)	Lord and Roberts, 1986
Nomuraea rileyi	Less virulent, altered phenotype	Morrow, *et al.*, 1989

1.4.1 Gene silencing - a potential factor involved in attenutation of virulence

Gene silencing in Eukaryotic organisms has been described by several terms or forms e.g. Post transcriptional gene silencing (PTGS), RNA – interference (RNAi), virus induced gene silencing (VIGS) and quelling (Pooggin *et al.*, 2001). Post transcriptional gene silencing is a general term for a variety of phenomena that repress gene expression by causing degradation of mRNA (Maine, 2003). Studies by several authors reported common characteristics in different PTGS phenomena

suggesting that PTGS in different organisms may be mediated by similar molecular mechanism (Fire *et al.*, 1998; Montgomery *et al.*, 1998; Fire, 1999). Gene involved in PTGS have been identified in several organsin as *qde* in *Neurospora crassa*, *rde*, *mut*, and *ego – 1* genes in soil nematode *Caenorhabditis elegans* and *sde*, *sgs* in the mustard plant *Arabidopsis thaliana* (Cogoni *et al.*, 1997; Tabara *et al.*, 1999; Ketting *et al.*, 1999; Smardon *et al.*, 2000; Elmayan *et al.*, 1998; Mourrain *et al.*, 2000; Dalmay *et al.*, 2000). These findings are consistent with a conserved mechanism operating in these diverse species (Maine, 2003).

A variety of RNA – mediated gene silencing methods that inhibits genes at the PTGS level has also been identified in different organisms. The most common forms involve the introduction of antisense RNA, double - stranded RNA also termed as RNAi or sense transgenes also called co- suppression in plants and quelling in fungi. In the case of *Neurospora*, molecular analysis indicate that gene silencing (quelling) operates at the PTGS level and is believed to be linked with possible production of sense RNA from the introduced transgene (Cogoni *et al.*, 1996). Detailed analysis of the PTGS – resistant mutant in *Neurospora*, *Arabidopsis* and RNAi resistant mutants in *C. elegans* have helped to understand the mechanisms of these forms of RNA – mediated gene silencing (Backer *et al.*, 2002). It has also been reported that homologues of the *qde 1* gene (*Neurospora*) are required in plants (SDE1/SGS 2) for PTGS and in *C. elagans* (EGO-1) for RNAi (Dalmay *et al.*, 2000; Smardon *et al.*, 2000), and also argonaute 1 (AGO 1) which is involved in co- suppression on plants (Tabara *et al.*, 1999; Catalanotto *et al.*, 2000; Fagard *et al.*, 2000).

One major prediction from the current model is that PTGS – related phenomena may involve the production of small interfering RNAs (siRNA) that act as the mediators of gene silencing (Backer *et al.*, 2002) RNA silencing is thought to be either induced by dsRNA or by the production of dsRNA by some other means e.g. over production of sense and antisense RNA, action of a cellular RNA dependent RNA polymerase (Poogin *et al.*, 2001). At the next stage, dsRNA is then chopped non-specifically into

pieces of 21-25 nucleotides by an RNase III like activity. These 21-25 nt products named as small interfering RNA (siRNA) which are found in all cases of RNA silencing are believed to act as guide that provides sequence-specificity for target RNA degradation (Poogin *et al.*, 2001). Recently siRNAs have been used effectively to regulate specific gene expression in mammalian cells (Elbashir *et al.*, 2001; Harborth *et al.*, 2001).

So far, there has been no attempt to study such mechanisms in entompathogenic fungi; however dsRNA mycoviruses have been reported from attenuated cultures of *M. ansiopliae* (Gimenez-Pecci *et al.*, (2002; Frazzon *et al.*, 2000). Further studies on *M. anisopliae* and other pathogenic fungi may reveal existence of the above mechanisms in attenuated cultures.

1.5 Link between nutrition and virulence

For fungal BCAs to be commercially viable, it has to be produced in large quantities, which is not possible to achieve by simply passaging through hosts. For mass production, fungal BCAs have to be produced on artificial media at a cost effective price. There have been many attempts to reduce the production cost of fungal BCAs by increasing yield but little attention was paid to increasing the quality of the inoculum (Schisler *et al.*, 1991; Magan, 2001). Recent studies with various biocontrol agents have suggested that the nutrition provided during the production of biocontrol agents could significantly affect their biocontrol efficacy. Cother and Van der Ven (1999) reported that the nutritional composition of liquid media significantly influenced the conidial production and virulence of *Rhynchosporium alismatis*. Similarly, Winder (1999) observed that conditions favouring abundant sporulation of *Fusarium avenaceum* did not favour virulence. On the other hand, conidial production and biocontrol efficacy of *Colletotrichum truncatum*, *Plectosporium tabacium*, *Helminthosporium solani* and *Trichoderma harzianum* were high if they were produced in media containing a CN ratio of 5-15:1 compared to those produced in medium with CN ratios of 15-80: 1 (Jackson and Schissler, 1992; Agosin *et al.*, 1997; Elson *et al.*, 1998; Zhang *et al.*, 2001)

Earlier studies on the influence of nutrition on entomopathogenic fungi have also shown that the conditions under which conidia are produced affect attributes such as endogenous reserves, surface properties and virulence (Lane *et al.*, 1991; Hallsworth and Magan 1994, 1995; Ibrahim *et al.*, 2002). However, there is a paucity of information on the effect of nutritional conditions on the inoculum quality. Most studies represent fragmented information and addressed only the conidial attributes with a passive role (if any) in virulence, whereas information on putative virulence determinants is lacking.

1.5.1 Carbon and Nitrogen repression of cuticle degrading enzymes

Filamentous fungi have the ability to grow in diverse environmental conditions and have sophisticated regulatory mechanisms which allow them to detect changes in their environment. The efficient utilization of growth substrates by filamentous fungi is under the control of both wide – domain and pathway specific regulatory genes (Hynes, 1994). Cuticle degrading enzymes of *M. anisopliae* particularly Pr1 are regulated by carbon (C) and (N) repression and de repression (Smithson *et al.*, 1995). C and N derepression of Pr1 have been shown to operate at the level of transcription (St. Leger *et al.*, 1991, 1992). Screen *et al.*, (1997) sequenced the *pr1* promoter region and identified a motif which is identical to those found in *Aspergillus nidulans* and which act in that fungus as binding sites for the regulatory proteins AREA and CREA. The presence of binding sites for the *A. nidulans* carbon-response regulator CREA in a carbon –catabolite –repressed gene from *M. anisopliae* suggests that the molecular mechanisms for C- repression may be conserved in these two organisms. More direct evidence is provided by the identification of a gene *crr1*, encoding a protein which shows significant homology with the CREA proteins of *A. nidulans*, *A. niger*, *Trichoderma reesei* and *T. harzianum* (67%, 61%, 84% and 85% similarity respectively). In addition to the structural similarity between the *M. anisopliae* CRR1 and the CREA proteins, it was observed that *crr1* can functionally complement the *A. nidulans creA* 204 mutation, in that CRR1 is capable of substituting for *A. nidulans* CREA in the repression of alcohol dehydrogenase 1 (Screen *et al.*, 1997). These findings clearly demonstrate that *crr1* encodes a carbon – response regulator protein

which is at least in part functionally homologous to the *A. nidulans* CREA (Screen *et al.*, 1997).

The prl A gene is also regulated in response to Nitrogen availability and the presence of closely spaced $5^{/-}$ GATA sequence suggested the presence of an AREA/NIT2 equivalent in *M. anisopliae* (Screen *et a*l., 1997). Screen *et al.*, (1998) designed a number of primers based on AREA from *A. nidulans* (Kudla *et al.*, 1990) and NIT2 form *N. crassa* (Fy and Mazluf, 1990) and amplified an 840-bp fragment from genomic DNA *M. ansioplaie* strain ME1. Further studies revealed that the gene existed as a single copy and was designated as nitrogen response regulator gene *nrr1* (Screen *et al.*, 1998). This gene contains two introns of 151 nd 253 bp, and encodes a protein of 944aa. The first intron disrupts a glycine residue rather than an arginine as found in all other filamentous fungal nitrogen response regulators genes. Complementation analysis further indicated that *nrr1* is functional when expressed in an *A. nidulans areA-* mutant (Screen *et al.*, 1998). However, homology comparisons indicate that the protein is more silimar to the NIT2 protein from *Neurospora crassa* than to the AREA protein from *A. nidulans* (Screen *et al.*, 1998).

Identification of similar wide domain C and N regulatory genes in several other fungi e.g. *A. nidulans*, *N. crassa*, *Penicillium chrysogenum*, *T. reesei* and *T. harzianum* suggests that the these regulatory mechanisms may be widely conserved amongst fungi (Screen *et al.*, 1997 and references there in).

1.6 Aims and justification of the present study

It is clear from the review of the literature that several attributes of *M. anisopliae* contribute to the overall virulence of this fungus. These attributes or putative virulence determinants include: adhesion, germination, production of CDEs and toxins. At present, we have very limited information on the influence of nutrition on these virulence determinants. The overall aim of this study is to understand the influence of nutrition on the virulence and stability of *M. anisopliae*. The specific aims of the study are

1. To study the influence of successive *in vitro* subculturing on the growth and virulence of *M. anisopliae* to identify its virulence determinants.
2. To study the effect of nutrition (media composition) on the putative virulence determinants of *M. anisopliae*.
3. To identify virulence determinants, which could be used as potential quality control markers.
4. To optimise assays for the quantification of putative virulence determinants e.g. adhesion of conidia. To date studies on *M. anisopliae* adhesion describe qualitative differences only.
5. To study the influence of nutrition in the production and physiology of sector formation in *M. anisopliae* to determine the possible role of phenotypic instability in attenuation.
6. To determine the influence of successive subculturing on the virulence of single spore and parent cultures.

These specific aims are specifically designed to increase our understanding of the factors involved in attenuation of virulence In *M. anisopliae*. Since all known cases of attenuation are reported from artificial or non host environments, therefore studies are particularly focused on the influence of nutrition in attenuation and virulence of *M. anisopliae*

Chapter 2: Elucidating the underlying mechanisms of attenuation in the entomopathogenic fungus *M. anisopliae*

2.1 Introduction

Successive *in vitro* subculturing of *M. anisopliae* and other entomopathogenic fungi results in the attenuation of virulence (Butt and Goettel 2000) but the underlying mechanisms of attenuation remain unclear. It is possible that maintenance of *M. anisopliae* in nutrient rich conditions affects the expression and production of its virulence determinants. To date no one has investigated the relationship between putative virulence determinants and attenuation in *M. anisopliae*. Monitoring the virulence determinants during successive subculturing could enable us to understand the role of these virulence determinants in virulence. In this study, the components of infection process that are linked with virulence (see Chapter 1) were used as tools to monitor changes in virulence when successively subcultured on artificial media. The aim of this study was to identify which components of the infection process (i.e. adhesion, CDEs, Destruxin) were affected as the culture declines in virulence. Other aspects of fungal development such as spore production, conidial carbon to nitrogen ratio were also evaluated to see the effect of successive subculturing on these aspects of *M. anisopliae*.

2.2 Materials and Methods

2.2.1 Fungi

Two strains of *M. anisopliae* V245 (isolated from soil, Finland) and V275 (isolated from *Cydia pomonella,* Lepidoptera) were used in this study. Both isolates were passaged through *Galleria melonella* larvae and isolated using a selective Oatmeal dodine agar medium and single spore colonies were transferred to Sabouraud Dextrose Agar (SDA). These cultures were maintained at 4°C and used as a source of inoculum for further

subculturing onto SDA up to 10^{th} subculture. Subcultures (1^{st}, 3^{rd}, 5^{th}, 7^{th} and 9^{th}) of both strains were selected for further studies.

2.2.2 Monitoring growth and sporulation

Sterilized SDA media was poured into 9 cm diameter Petri dishes (Bibby Sterilin, U.K.) and on solidification inoculated with a 2 mm diameter mycelial plug taken from the growing edge of a 12 day old culture of the selected subcultures grown on SDA at 25°C in the dark. Inoculated Petri dishes were sealed in polythene bags and incubated at 25°C in the dark. The colony diameter was measured at right angles at 3 days intervals until 15 days post inoculation and radial growth (mmd^{-1}) calculated from the linear portions of the curves plotted from these values. Conidial yield was determined by suspending the conidia from the whole colony in 50 ml of 0.05% aq. v/v Tween 80, and counting the number of spores using an improved Neubauer haemocytometer (Weber Scientific International Ltd. U.K.)

2.2.3 Determining virulence of inoculum after repeated subculturing on SDA

Conidia from the above treatments were assayed against $4\text{-}5^{th}$ instar larvae of *Tenebrio molitor*. Larvae were immersed in 10 ml conidial ($1x10^7$ conidia/ml) suspension or 0.03% v/v aq. Tween carrier (control) for ca. 30 sec and the excess moisture removed by filtering over a vacuum in a Buchner funnel. Larvae were incubated without food in 9cm diameter Petri dishes lined with moist Whatman No.1 filter paper and incubated at 25°C in the dark. Each treatment was replicated three times with ten larvae per replicate. Mortality was recorded daily and dead insects were transferred to Petri dishes lined with moist filter paper to encourage external sporulation of the fungus if present. The LT_{50} (time required to kill 50% larvae) were calculated from the linear portions of the curves plotted from these values (time in days vs mortality).

2.2.4 Carbon and Nitrogen (CN) elemental composition/ratio of conidia produced after repeated subcultures

The CN elemental composition of the conidia produced after repeated subculturing on SDA were determined using an Automated Nitrogen Carbon Analysis for Gas Solids and Liquids (ANCA GSL) elemental analyser interfaced with a PDZ Europa 20/20 mass spectrometer. Conidia (1mg) from each subculture were wrapped in 6x4 mm tin foil discs (Elemental Microanalysis, Ltd. U.K.) that had first been washed in acetone (Sigma) and allowed to dry to ensure they were oil free. Samples were then kept in sealed tubes and kept in an airtight desiccator until analysis. Isoleuecine (Sigma) at different concentrations ranging from 25 µg to 150 µg was used as standard. Tin foil discs without conidia were used as negative control. Each treatment was replicated and whole procedure was repeated twice.

2.2.5 Determination of conidial adhesion using Radial Flow chamber

Conidial attachment studies were carried out in a Radial Flow Chamber (RFC) as described by Tegoulia *et al.*, (2002), however, RFC assays were optimized for *M. anisopliae* as described in Chapter 4. Briefly, *M. anisopliae* conidia obtained from different subcultures were suspended in 0.03% aq.Tween 80 to a final concentration of $1x10^9$ conidia/ml. The reservoir of the RFC contained 95 ml of deionised water. Conidial suspension (5 ml) of a single treatment was introduced into the reservoir. The suspension was allowed to run at flow rate of 0.2 l/min into RFC for 15 minutes prior to adhesion observations. These conditions were kept constant for all the experiments. In order to compare the adhesion strength of conidia different successive subcultures, shear strength of 1.33 N M^2 (15 mm from point on injection) was used for all studies. Conidial adhesion was recorded simultaneously on stainless and cuticular lipid coated glass surface.

2.2.6 Measurement of zeta potential (to quantify electrostatic forces/surface charge involved in adhesion)

The zeta potential of the conidia was calculated from experimentally determined electrophoretic mobility using the Smoluchowski equation. Smoluchowski (1918) considered the effect of an electric field on the movement of a liquid adjacent to a flat charged surface. The velocity of the liquid varies from zero (plane of shear) to a maximum value, some distance from the wall where it remains constant. A force balance on an elemental volume of the liquid results in the electro-osmotic mobility, defined in equation 2.1

$$\mu E = - (D \zeta) / \mu \qquad \textbf{Eq. 2.1}$$

Where μE is electro-osmotic mobility (m^2/SecV); D is the dielectric constant (dimensionless); ζ is the zeta potential in mV and μ represents the viscosity of water (Kg/ms).

This theory can also be applied to large particles moving within relatively thin double layers in a stationary liquid, taking into consideration that the particle is moving in the opposite direction, and therefore, the sign reversed as shown in Equation 2.2

$$\mu E = (D \zeta) / \mu \qquad \textbf{Eq. 2.2}$$

Where μE now represents the electrophoretic mobility, which could then be used to determine zeta potential.

To determine the electrophoretic mobility, conidia from different subcultures were suspended in distilled water to the final concentration of $1x10^7$ conidia/ml. The electrophoretic mobility was then measured using a Malvern Zetasizer 2000/3000 (Malvern Instruments, U.K.). The mean of 10 measurements was then used to calculate

the zeta potential. Distilled water was used as the reference sample and the system was thoroughly flushed with deionized water after each reading.

2.2.7 Surface hydrophobicity measurements

The surface hydrophobicity of the conidia was assessed by aqueous–solvent partitioning assays. The test assesses the distribution ratio of cells between water and an organic phase. The organic phase used was n-Hexadecane (Sigma). *M. anisopliae* (V245, V275) conidia from different subcultures were suspended in 0.1 M KNO_3 solution to a final concentration of $1x10^7$ conidia /ml. The optical density of the spore suspension was determined at 660 nm (Lightwave, UV/VIS Diode–Array Spectrophotometer, WPA, U.K.), referred to as 'total density' (OD_{total}). Conidial suspension (6 ml) of each sample was then transferred to a universal bottle containing 2 ml of hexadecane and agitated for twenty seconds. The suspension was held for 30 minutes to allow for phase separation. Following phase separation, the aqueous phase was collected and the $OD_{(660\ nm)}$ recorded. Relative hydrophobicity was then determined as follows:

$$\textbf{Relative hydrophobicity (\%)} = 100\ \{1 - (OD_{aq} / OD_{total})\}$$

Where the $OD_{(total)}$ and the $OD_{(aq)}$ represent the values of the start sample and resultant aqueous phase respectively.

2.2.8 Germination assays

The germination rate of inoculum from the different subcultures was assessed by inoculating SDA with 10 µl of conidial suspension ($1x10^6$ conidia/ml) of each subculture, then counting the number of germlings following 10 hours post inoculation at 25°C. Conidia with germ tubes equal to or greater than the conidial width were

considered to have germinated. For each treatment, three separate fields were observed for germination and 100 conidia observed randomly in each field.

2.2.9 Effect of repeated subculturing on the production of Pr1 and non-specific proteases

The production of CDEs was investigated by inoculating an inductive medium consisting of homogenised cockroach cuticle (10mg/ml) in minimal medium with conidia from different subcultures. The minimal medium consisted of (g/l) $0.3K_2HPO_4$, 0.3 $MgSO_4.7H_2O$, 0.15 NaCl, 0.3 $CaCl.6H_2O$, 0.008 $MnSO_4.6H_2O$, 0.0002 $CuSO_4.5H_2O$ and 0.003 $FeSO_4.7H_2O$

The inductive medium (18ml) was poured into 100 ml conical flasks and autoclaved at 120°C for 20 minutes before inoculation with 2 ml of $1x10^7$ conidia/ml. Cultures were incubated at 24°C, 120 rpm, in a Gallen-Kamp orbital incubator (Sanyo). Each treatment and the whole experiment were repeated twice.

Samples for the enzyme assays were prepared as follows: (1) 1.5ml culture filtrate was collected in an Eppendorf tube and centrifuged at 12000 g (in Sanyo Harrier 18/80 centrifuge) for ten minutes, (2) 1ml of the supernatant was stored at -80°C until required for protein and enzyme assays. All samples were collected at 72 hours post inoculation.

2.2.10 Protein assays

Protein concentrations in culture filtrates were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.2.11 Enzyme assays:

2.2.11.1 Determination of non-specific protease activity

The assays for non-specific proteolytic activity were carried out as described by Segers *et al.*, (1995). Azocoll (Calbiochem) was dissolved in 0.1 M Tris-HCl buffer (pH 7.95) and stirred gently for 2 hours at 37°C. After stirring, the solution was centrifuged at 5000g (in Sanyo, Harrier 18/80 centrifuge) for 10 minutes and the supernatant was then poured off to remove any unbound dye. The pellet was re-suspended in buffer to a final concentration of 10 mg/ml. This substrate was then used for the protease assay. Each assay was performed by incubating buffer (200 µl), enzyme (50 µl) and azocoll substrate (250 µl) for 30 minutes at 37°C in a rotary shaker (160 rpm). In controls, the enzyme was replaced with buffer. After incubation, the mixture was centrifuged at 12000 g (in Sanyo, Harrier 18/80 centrifuge) for 5 minutes and the supernatant (200 µl) were transferred to individual wells of 96 well microtitre plate (Dynatec) and absorbance was read at 492 nm wavelength. Each treatment and the whole experiment were replicated twice.

2.2.11.2 Subtilisins (Pr1) and Trypsin like (Pr2) activity

Pr1 and Pr2 activities were assayed against Succinyl-Ala-Ala-Pro–Phe-*p*-nitroanilide and N-Benzyol–Phe–Val–Arg-*p*-nitroanilide respectively (Sigma) as described by St. Leger *et al.*, (1994b). Assays were conducted using 96 well microtitre plates (Dynatec). The reaction mixture contained 50 µl of 0.1M Tris–HCl buffer (pH 7.95), 50 µl of crude enzyme and the reaction was started by adding 50 µl of 3 mM substrate dissolved in Tris–HCl buffer (pH 7.95) to each well. Absorbance was read immediately for 3 minutes with 12-second intervals between each reading in a Lab System microplate reader. Absorbance was read at wavelength of 405 nm wavelengths and enzyme activity was calculated using the Ascent software. Each sample and the whole experiment were replicated twice. Controls consisted of buffer and substrate only.

2.2.11.3 NAG'ase activity

β– N–Acetyleglucosaminidase (NAG'ase) activity was determined as described by St. Leger *et al.*, (1986); briefly 0.5 ml of culture filtrate was incubated with *p*NP-N- acetyle-*β*-D-glucosaminide solution (1mg/ml) in 0.1M-citrate buffer for 1 hour at 37°C. After incubation, the reaction was stopped by adding 1ml of 1N NH_4OH containing 2mM disodium EDTA. Samples (200 µl) from each treatment were transferred to each well of 96 well microtitre plate (Dynatec) and read for changes in absorbency. Absorbance was measured at 405 nm wavelengths and enzyme activity was calculated using the Ascent software. Each sample and the whole experiment were replicated twice. Controls consisted of buffer and substrate only. Activities were expressed as µ mol *p*-nitrophenol (*p*NP) released/ml/hr.

2.2.11.4 Direct measurement of spore bound Pr1

Conidia from different subcultures were used to study the spore bound Pr1 as described by St. Leger *et al.*, (1991b) with minor modifications. Briefly, 10 mg of conidia were incubated in 1ml of 0.1M Tris-HCl (pH 7.95) containing 1 mM Succinyl- Ala-ala-Pro–Phe-*p*-nitroanilide for 5 minutes at room temperature. After incubation the conidia were clarified by centrifugation at 12000g (in Sanyo, Harrier 18/80 centrifuge) for 5 minutes. The supernatant (200 µl) was transferred to wells of a microtitre plate (Dynatec) and absorbency was measured at 405 nm. Substrate and buffer without conidia were used as control. Each treatment and experiment was replicated twice.

2.2.11.5 Semi quantitative enzyme profiling (API-ZYM test) of the un germinated conidia from different subcultures

The enzymatic activities of 19 different enzymes (listed in tables 2.3-2.4) were determined with the semi–quantitative API-ZYM system (bio Merieux, U.K.) as described by the manufacturer. Briefly, 65 µl of conidial suspension ($1x10^7$ conidia/ml) of each treatment was added to individual cupules (lined with buffer and enzyme

substrate) of each strip followed by incubation at 37 °C for 4 hours. The pre supplied colour reagent (Fast Blue BB, laural sulphate, methoxyethnol and Tris) was added to determine enzymatic activity. Enzyme activities were scored from 0-5, with 0 representing no enzyme activity and 5 representing maximum activity.

2.2.12 Production of destruxin

2.2.12.1 Inoculation of toxin induction medium

Czapek dox broth (Sucrose, 3%, sodium nitrate, 0.2%, K_2HPO_4, 0.1%, $MgSO_4$, 0.05% KCl 0.05% and $FeSO_4$, 0.001%) supplemented with 0.5% peptone (Oxoid) was used as inductive medium. Conidia harvested from different subcultures were used to inoculate the inductive medium for destruxin production. Conidial suspension from each subculture was adjusted to a concentration of $1x10^7$ conidia /ml. The inductive medium (250 ml) in conical flask (500 ml) was then inoculated with 2.5 ml of spore suspension of the respective subcultures. Following inoculation, cultures were incubated at 24°C, 120 rpm for 7 days. Each treatment and the whole experiment were repeated twice.

2.2.12.2 Crude toxin extraction and HPLC analysis

Crude toxins were extracted from culture filtrates with dichloromethane: ethyl acetate (1:1) and analysed by HPLC as described by Wang *et al.* (2003). Briefly, each sample was diluted to 1mg/ml of crude extract in HPLC grade Methnol, 25 µl of each sample injected in Dionex HPLC system, equipped with a C18 reverse phase column (Acclaim™, silica, particle size: 5 µm, pore diameter: 120 Å, length: 4.6 x 250 mm, column temperature 30 °C) and a UVD 340U diode array detector. Samples were analysed at a flow rate of 1 ml/minute with the following gradient: eluent A = H_2O with 5 % acetonitrile, eluent B = acetonitrile with 10 % H_2O: a linear gradient from 10 % to 50 % eluent B for 17 minutes, a linear gradient from 50 % to 100 % eluent B for eight minutes and a linear gradient from 100 % to 30 % eluent B for five minutes; the column was left to re-equilibrate at 30 % eluent B for 10 minutes between runs. All the reagents

were from Sigma except for solvents (Dicholoromethane, Methnol and Acetonitrile), which were obtained from Fisher scientific, U. K.

2.2. 13 Statistical analysis

The whole study was repeated with each treatment replicated three times unless stated otherwise. Data was subjected to one-way ANOVA (Tukey test) for determining significant differences. For all statistical analysis SPSS 11 software was used.

2.3 Results

2.3.1 Effect of repeated subculturing on the growth of *M. anisopliae*

In general, non-significant ($P < 0.05$, Tukey Test) differences in radial growth were observed after repeated subculturing on SDA, however conidial yield varied significantly (Tables 2.1, 2.2). Conidial yield was high in 1st and 3rd subcultures of V245 and V275 respectively. No significant difference in conidia yield was observed among 3rd to 9th subcultures of V245, whereas in the case of V275, conidia yield was erratic among subcultures (Table 2.2).

2.3.2 Effect of repeated subculturing on the virulence of *M. anisopliae*

Virulence of both strains fluctuated among different subcultures but the net result was a decline in virulence with increasing number of subcultures (Tables 2.1, 2.2). *M. anisopliae* V245 was most virulent as 1st and 3rd subculture with LT_{50} values of 4.79 and 4.63 days post inoculation (dpi) respectively. The least virulent inoculum of V245 was that of the 9th subculture with LT_{50} value of 5.57 dpi (Table 2.1). A similar trend was also observed for V275, where, 1st and 3rd subculture were significantly ($P < 0.05$, Tukey Test) more virulent than those of 5-9th. Comparatively, there was more loss of virulence in V245 as it lost almost one day between 1st and 9th subculture (Table 2.1).

2.3.3 Effect of repeated subculturing on conidial carbon and nitrogen ratios

Conidial carbon to nitrogen ratios of both strains varied among the different subcultures (Tables 2.1, 2.2). In all the treatments, carbon to nitrogen ratios were above 6:1. In the case of V245, highest conidial CN ratio was observed in the conidia from subcultures 1st, 5th and 9th. These treatments varied significantly ($P < 0.05$, Tukey Test) with those of the 3rd and 7th subcultures (Table 2.1). Overall conidial CN ratios of V245 varied from 6.52:1 (3rd subculture) to 7.06:1 (1st subculture). In the case of V275, conidial CN ratios varied from 6.12:1 (7th subculture) to 7.17:1 (5th subculture). Significantly ($P < 0.05$, Tukey Test) higher conidial CN ratios were observed in conidia from 1st and 5th subculture, followed by intermediate conidial CN observed in 3rd and 9th subcultures (Table 2.2). Lowest conidial CN ratios were observed in the 7th subculture (Table 2.2).

2.3.4 Effect of repeated subculturing on the speed of germination of *M. anisopliae*

Except for the 9th subculture of both strains, germination speed varied non-significantly ($P < 0.05$, Tukey Test) among the subcultures. Conidia from all the subcultures of V245 germinated relatively slowly, as maximum germination recorded at 10 hours post inoculation was 38.66%. There was significantly ($P < 0.05$, Tukey Test) low germination speed for conidia from the 9th subculture of V245, which had only reached 4.66% at 10 hours post inoculation.

Although V275 conidia from different subcultures had a similar trend to that of V245, there was relatively faster germination in all subcultures of V275. Fastest germination was observed for 3rd subculture but it varied non-significantly ($P < 0.05$, Tukey Test) with rest of subcultures except with the slowest germinating conidia from 9th subculture.

Table 2.1 Vegetative growth, conidial yield, CN ratios, germination and virulence of different subculture of *M. anisopliae* V245. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated three times. Values in parenthesis represent standard errors.

Subculture	Radial growth (mm/day)	Conidial yield/l ($x10^{11}$)	Conidial Carbon to nitrogen ratio	Germination (%)	Virulence LT_{50} (Days post inoculation)
1st	8.03^{ab} (0.34)	1.67^{a} (0.01)	7.06^{a} (0.06)	38.66^{a} (1.45)	4.79^{bc} (0.12)
3rd	7.16^{b} (0.42)	0.96^{b} (0.02)	6.52^{b} (0.00)	37.66^{a} (1.20)	4.63^{c} (0.18)
5th	8.04^{ab} (0.28)	0.87^{b} (0.11)	6.99^{a} (0.01)	31.66^{a} (2.18)	5.22^{ab} (0.10)
7th	7.76^{ab} (0.32)	0.92^{b} (0.01)	6.60^{b} (0.01)	33.00^{a} (3.60)	5.19^{ab} (0.02)
9th	8.73^{a} (0.20)	0.99^{b} (0.15)	7.03^{a} (0.00)	4.66^{b} (0.88)	5.57^{a} (0.09)

Table 2.2 Vegetative growth, conidial yield, CN ratios, germination and virulence of different subculture of *M. anisopliae* V 275. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated three times. Values in parenthesis represent standard errors.

Subculture	Radial growth (mm/day)	Conidial yield/l ($x10^{11}$)	Conidial Carbon to nitrogen ratio	Germination (%)	Virulence LT_{50} (Days post inoculation)
1st	8.48^{a} (0.21)	1.79^{ab} (0.18)	6.76^{ab} (0.08)	80.33^{a} (2.33)	3.74^{c} (0.06)
3rd	9.47^{a} (0.53)	2.21^{a} (0.35)	6.32^{bc} (0.04)	82.00^{a} (4.58)	4.01^{bc} (0.16)
5th	10.22^{a} (0.29)	2.08^{a} (0.20)	7.17^{a} (0.20)	66.33^{ab} (3.17)	4.45^{a} (0.03)
7th	8.45^{a} (0.52)	1.19^{ab} (0.01)	6.12^{c} (0.01)	69.33^{ab} (2.40)	4.20^{ab} (0.08)
9th	8.48^{a} (0.26)	0.86^{b} (0.15)	6.52^{ab} (0.02)	62.00^{b} (5.13)	4.46^{a} (0.06)

2.3.5 Effect of repeated subculturing on the adhesion properties of *M. anisopliae*

Adhesion profile varied significantly among the different subcultures of both strains. On the stainless steel surface highest adhesion was observed for 1st subcultures. No significant differences in adhesion were observed for 3rd, 5th and 9th subcultures. Conidia from 7th subculture were more adhesive than these three subcultures but less than 1st subculture (Fig. 2.1). On culticular lipid coated glass surface, conidial adhesion was observed only from 5th, 7th and 1st subcultures. No adhesion was observed for conidia from 3rd or 9th subculture (Fig. 2.1).

In the case of V275, adhesion decreased with increasing number of subcultures (Fig. 2.2). Significantly higher adhesion was observed for the 1st subculture on both steel and lipid coated glass surface (Fig. 2.2). On the steel surface, no major differences in adhesion were observed among the conidia from 3rd, 7th and 9th subcultures. These subcultures had intermediate adhesion on steel. Conidia from 5th subculture were least adhesive on steel surface. On lipid coated glass surface, adhesion was observed only for the 1st and 3rd subcultures (Fig. 2.2).

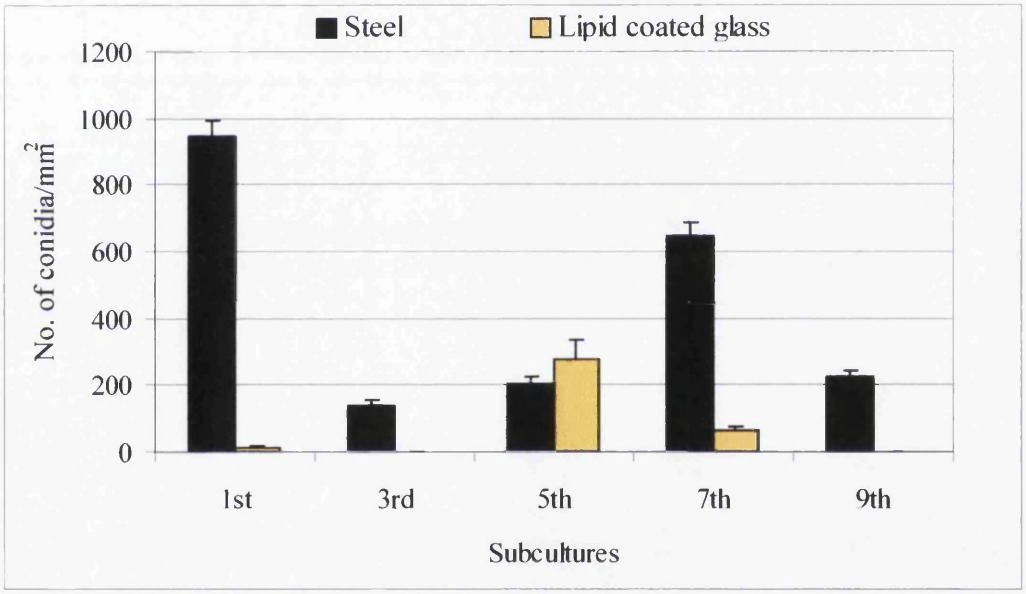


Fig. 2.1 Effect of repeated subculturing on the adhesion of *M. anisopliae* V245 conidia to stainless steel and cuticular lipid coated glass surface under a flow rate of 0.2 l./min.

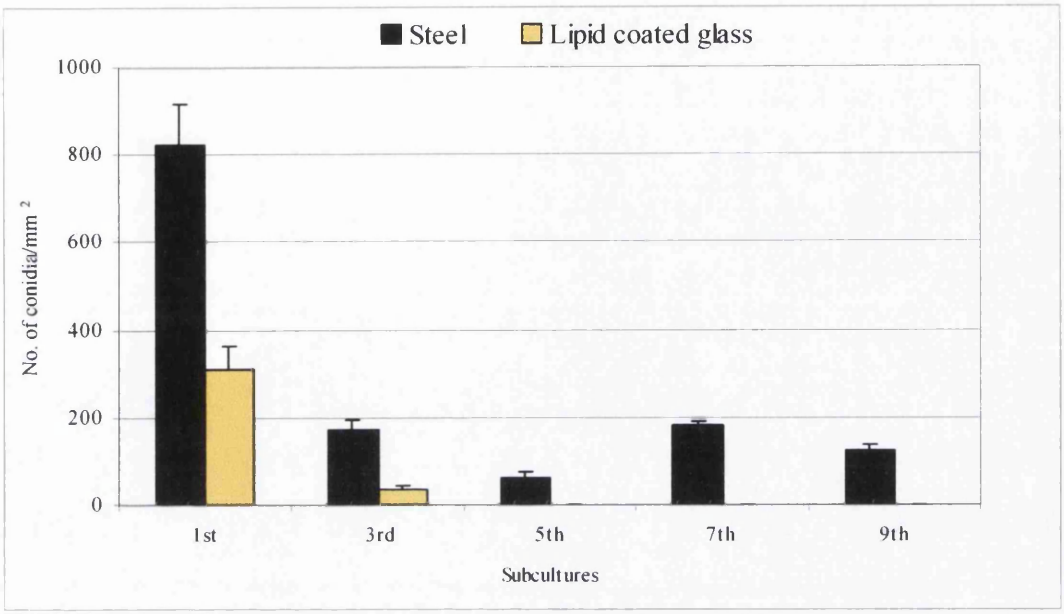


Fig. 2.2 Effect of repeated subculturing on the adhesion of *M. anisopliae* V275 conidia to stainless steel and cuticular lipid coated glass surface under a flow rate of 0.2 l./min.

2.3.6 Effect of repeated subculturing on the zeta potential of the *M. anisopliae* conidia

Significant ($P < 0.05$, Tukey Test) differences in the zeta potential of the conidia from different subcultures were also observed (Table 2.3). In the case of V245, the conidia of the 1st subculture had the least zeta potential (-27.69) which varied significantly ($P < 0.05$, Tukey Test) with all other treatments (Table 2.3). The conidia of 3rd, 5th and 7th subculture varied non-significantly ($P < 0.05$, Tukey Test) with each other and had intermediate zeta potential (Table 2.3). The conidia of the 9th subculture had the highest zeta potential (-42.42), which varied significantly ($P < 0.05$, Tukey Test) with all other treatments (Table 2.3).

The conidia of V275 from different subcultures also varied significantly ($P < 0.05$, Tukey Test) but no clear-cut pattern was observed (Table 2.3). The conidia of 1st, 5th,

and 9th subculture varied non-significantly ($P < 0.05$, Tukey Test) and had least zeta potential (Table 2.3). The conidia of 3rd and 5th varied non-significantly ($P < 0.05$, Tukey Test) with each other and constituted the middle range of zeta potential. The highest zeta potential was observed among the conidia of 7th subculture that varied significantly ($P < 0.05$, Tukey Test) with all other treatments (Table 2.3).

2.3.7 Effect of repeated subculturing on the hydrophobicity of the *M. anisopliae* conidia

Relative hydrophobicity varied significantly ($P < 0.05$, Tukey Test) among the conidia from different subcultures (Table 2.3). In the case of V245, conidia from the first subculture were significantly ($P < 0.05$, Tukey Test) more hydrophobic (92.84%) than rest of the subcultures. Subcultures 5th, 7th and 9th were intermediate in their hydrophobicity while the 3rd subculture was least hydrophobic (77.90%).

In the case of V275, conidia of the 1st subculture were more hydrophobic than the rest of the subcultures (Table 2.3). It had 93.90% hydrophobicity, which varied significantly ($P < 0.05$, Tukey Test) with all other its subcultures ranging from 86.22 to 89.25%. Except for the 1st subculture, all the subcultures of V275 varied non-significantly ($P < 0.05$, Tukey Test) among themselves (Table 2.3).

Table 2.3 Effect of repeated subculturing on the zeta potential and relative hydrophobicity of *M. anisopliae* conidia. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated three times. Values in parenthesis represent standard errors.

Subculture	Zeta Potential (mv^2)		Relative hydrophobicity (%)	
	V245	V275	V245	V275
1st	-27.69[a] (0.46)	-34.56[a] (0.26)	92.84[a] (0.35)	93.90[a] (0.66)
3rd	-34.47[b] (0.67)	-44.49[b] (0.42)	77.90[d] (0.62)	89.19[b] (0.51)
5th	-32.75[b] (0.28)	-38.15[ab] (2.66)	82.00[bc] (0.93)	89.25[b] (0.15)
7th	-34.24[b] (0.52)	-54.38[c] (2.28)	87.47[b] (0.39)	89.23[b] (1.07)
9th	-42.42[c] (0.28)	-32.80[a] (0.27)	84.26[b] (1.31)	86.22[b] (1.63)

2.3.8 Effect of repeated subculturing on the production of CDEs

Erratic results were observed for total protein, non-specific protease, inductive Pr1 and NAG'ase activity among the different subcultures of V245. Total protein and NAGase was significantly ($P < 0.05$, Tukey Test) higher in 3rd subcultures. Inductive Pr1 was higher in 1st, 3rd and 9th subcultures and least in 7th and 5th subcultures (Table 2.4). No significant ($P < 0.05$, Tukey Test) difference in Pr2 activity was observed.

Spore bound Prl activity of V245 varied significantly ($P < 0.05$, Tukey Test) among the different subcultures. Highest activity was observed for 1st and 3rd subcultures (1.30 and 1.25 µmol/ml/min respectively), which varied significantly ($P < 0.05$, Tukey Test) with rest of subcultures. Subcultures 5th and 7th had lowest spore bound activity while subculture 9th was intermediates (Table 2.4). Significant (($P < 0.04$, Pearson Correlation) negative correlation was observed between spore bound Prl and LT_{50} suggesting an increase in spore bound pr 1 would result im reduction of LT_{50} i.e. high virulence.

In the case of V275, protein and non-specific protease activity varied non-significantly ($P < 0.05$, Tukey Test) among the different subcultures. Inductive Prl activity was significantly higher in the 1st-7th subcultures than that of the 9th subculture. While Pr2 activity was higher in the 1st subculture than the rest of subcultures but varied non-significantly ($P < 0.05$, Tukey Test) with that of 3rd and 5th subcultures (Table 2.5).

NAG'ase activity also varied significantly ($P < 0.05$, Tukey Test) among different subcultures of V275, highest activity was observed in 1st subcultures and least in the 9th subculture.

Spore bound Prl activity was significantly ($P < 0.05$, Tukey Test) higher in the 1st subculture of V275. Remaining subcultures varied non-significantly ($P < 0.05$, Tukey Test). Generally the protein and enzyme activities were lower in V245 subcultures than those of V275 (Tables 2.4-2.5). Significant (($P < 0.02$, Pearson Correlation) negative correlation was observed between spore bound Prl and LT_{50} suggesting an increase in spore bound pr 1 would result im reduction of LT_{50} i.e. high virulence.

Table 2.4 The total protein, non-specific proteases, Pr1, Pr2 and NAG'ase activities of different subcultures of V245 in the inductive media in comparison with their spore bound Pr1 activity. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated two times. Values in parenthesis represent standard errors.

Subculture	Total protein concentration (µg/ml)	Non-Specific proteases (U/ml)	Inductive Pr1 (µ mol/ml/min)	Inductive Pr2 (µ mol/ml/min)	Exo - Chitinase NAG'ase (µ mol/ml/hr	Spore bound Pr1 (µ mol/ml/min)
1st	20^{b} (0.03)	1.23^{b} (0.01)	2.63^{a} (0.08)	0.52^{a} (0.02)	0.15^{b} (0.01)	1.30^{a} (0.04)
3rd	77.5^{a} (0.01)	1.33^{ab} (0.26)	2.88^{ab} (0.00)	0.59^{a} (0.03)	0.20^{a} (0.01)	1.25^{a} (0.14)
5th	10^{b} (0.00)	1.39^{a} (0.01)	2.64^{bc} (0.00)	0.66^{a} (0.05)	0.13^{b} (0.00)	0.44^{c} (0.00)
7th	5^{b} (0.00)	1.41^{a} (0.02)	2.47^{c} (0.08)	0.56^{a} (0.04)	0.13^{b} (0.00)	0.48^{c} (0.08)
9th	20^{b} (0.01)	1.32^{ab} (0.03)	2.90^{a} (0.02)	0.52^{a} (0.03)	0.15^{b} (0.00)	0.64^{b} (0.12)

Table 2.5 The total protein, non-specific proteases, Pr1, Pr2 and NAG'ase activities of different subcultures of V275 in the inductive media in comparison with their spore bound Pr1 activity. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated two times. Values in parenthesis represent standard errors.

Subculture	Total protein concentration (μg/ml)	Non-Specific proteases (U/ml)	Inductive Pr1 (μ mol/ml/min)	Inductive Pr2 (μ mol/ml/min)	Exo - Chitinase NAG'se (μ mol/ml/hr)	Spore bound Pr1 (μ mol/ml/min)
1st	85.0[a] (0.03)	1.20[a] (0.03)	3.05[a] (0.01)	0.77[a] (0.09)	0.28[a] (0.00)	1.53[a] (0.11)
3rd	72.5[a] (0.00)	1.23[a] (0.01)	2.86[ab] (0.04)	0.58[ab] (0.00)	0.16[bc] (0.00)	0.98[bc] (0.04)
5th	65.0[a] (0.02)	1.28[a] (0.06)	2.89[a] (0.04)	0.61[ab] (0.04)	0.18[bc] (0.00)	0.74[c] (0.12)
7th	82.5[a] (0.01)	1.14[a] (0.07)	2.82[ab] (0.03)	0.55[b] (0.01)	0.15[cd] (0.00)	0.57[c] (0.03)
9th	42.5[a] (0.01)	1.34[a] (0.05)	2.52[b] (0.17)	0.55[b] (0.00)	0.12[d] (0.00)	0.56[c] (0.05)

2.3.9 Effect of repeated subculturing on the semi quantitative enzyme profile (API-ZYM test) by the conidia from different subcultures

For V245 no major differences among the conidial enzyme activities were observed (Table 2.6). The API-ZYM test showed highest acid phosphatase, naphthol–As–Bi–phosphohydrolase and leucine arylamidase activities. Intermediate enzyme activities were observed for esterase C4, esterase lipase C8 and β–glucosidase. Low enzyme activities were observed for alkaline phosphatase and N-acetyl–β glucosaminidase activities (Table 2.6). No enzyme activities were observed for rest of the evaluated enzymes (Table 2.6).

In contrast to V245, subcultures of V275 had higher enzyme activities for acid phosphatase, naphthol–As–Bi–phosphohydrolase, leucine arylamidase, valine arylamidase, β-glucosidase and α- mannosidase (Table 2.7). However, no N- acetyl–β glucosaminidase activity was observed for any of the subculture of V275 (Table 2.7). Two of its subcultures showed low α-chymotrypsin and α- galactosidase activity but no activity was observed in remaining treatments (Table 2.7). For remaining enzymes no major differences were observed among the treatments of both isolates (Table 2.7).

Table 2.6 Semi quantitative enzyme profile (API-ZYM test) of *M. anisopliae* V245 conidia from different subcultures. Each treatment was replicated two times.

Enzymes	Subcultures				
	1st	3rd	5th	7th	9th
Alkaline phosphatase	1	0.5	1	1	0
Esterase (C 4)	3	3	3	3	3
Esterase Lipase (C 8)	3	3	3	3	3
Lipase (C 14)	0	0	0	0	0
Leucine arylamidase	4	4	4	5	4
Valine arylamidase	2	2	1	2	2
Cystine arylamidase	0	0	0	0	0
Trypsin	0	0	0	0	0
α-chymotrypsin	0	0	0	0	0
Acid phosphatase	5	5	5	5	5
Naphthol -AS-BI- phosphohydrolase	4	3	4	4	4
α- galactosidase	0	0	0	0	0
β – galactosidase	0	0	0	0	0
β – glucuronidase	0	0	0	0	0
α- glucosidase	0	0	0	0	0
β – glucosidase	4	3	4	3	3
N- acetyl – β glucosaminidase	1	1	1	1	1
α- mannosidase	0	0	1	0	0
α- fucosidase	0	0	0	0	0

Table 2.7 Semi quantitative enzyme profile (API-ZYM test) of *M. anisopliae* V275 conidia from different subcultures. Each treatment was replicated two times.

Enzymes	Subcultures				
	1st	3rd	5th	7th	9th
Alkaline phosphatase	1	3	3	3	3
Esterase (C 4)	3	3	3	3	3
Esterase Lipase (C 8)	3	4	4	4	4
Lipase (C 14)	0	0	0	0	0
Leucine arylamidase	5	5	5	5	5
Valine arylamidase	2	2.5	2	2	2
Cystine arylamidase	0	0	0	0	0
Trypsin	0	0	0	0	0
α-chymotrypsin	0	1	0	1	0
Acid phosphatase	5	5	5	5	5
Naphthol -AS-BI- phosphohydrolase	4	3	4	4	4
α- galactosidase	0	1	0	1	0
β – galactosidase	0	0	0	0	0
β – glucuronidase	0	0	0	0	0
α- glucosidase	0	0	0	0	0
β – glucosidase	4	4	4	3	4
N- acetyl – β glucosaminidase	0	0	0	0	0
α- mannosidase	0	3	2	3	3
α- fucosidase	0	0	0	0	0

2.3.10 Destruxin production by different subcultures

Destruxin production also varied significantly ($P < 0.05$, Tukey Test) in different subcultures. Generally, the higher total crude extract was observed in 1st to 5th subcultures of V245. Lowest crude extracts were observed in the 7th subculture, which was just 104.8 mg/l in comparison to the highest level of 262.65 mg/l, observed in the 3rd subculture. The total crude extract production in the 9th subculture was higher than the 7th subculture but was significantly ($P < 0.05$, Tukey Test) less than other subcultures. The HPLC analysis indicated least dtx A and E in 7th subcultures of V245, which varied non-significantly with 5th and 9th subcultures. The rest of the subcultures varied non-significantly for dtx A and E production. No significant difference was observed for dtx B in all the subcultures of V245 (Table 2.8).

The crude extract was significantly ($P < 0.05$, Tukey Test) higher in 1st and 3rd subcultures of V275 than its other subcultures. The highest crude extract was observed in the 1st subculture, which yielded 150.8 mg/l while the 9th subculture had the lowest with 117.40 mg/l. The crude toxin production was relatively lower in the subcultures of V275 compared to those of V245. The HPLC analysis revealed significantly higher quantities of dtx A and E in the crude extract of 1st and 3rd subcultures of V275 than its other subcultures. Dtx B production was erratic among subcultures of V275 (Table 2.9).

The ratio of dtx A, B, and E also differed between different treatments of both isolates. The dtx E was relatively more expressed in all subcultures of V245 with the exception of the 7th subculture, where dtx A was slightly higher than E. In the case of V275 subcultures, the dtx E production was higher in the 1st, and 3rd subculture. In rest of the subcultures, dtx A was produced higher than B and E. Dtx B was produced in the lowest quantities in all the subcultures of both strains.

Table 2.8 Crude extract and destruxins profiles of different subcultures of V245. Each treatment was replicated two times. Values in parenthesis represent standard errors.

Subculture	Crude extract mg/l of medium	Dtx A mg/l of medium	Dtx B mg/l of medium	Dtx E mg/l of medium	Ratio of A:B:E
1st	232.4[a] (33.6)	13.08[a] (1.56)	2.09[a] (0.25)	16.42[a] (3.43)	6.22:1:7.8
3rd	262.65[a] (36.0)	13.70[a] (2.69)	2.08[a] (0.45)	18.23[a] (4.11)	6.60:1: 8.76
5th	220.2[a] (25.0)	11.18[ab] (0.03)	1.69[a] (0.08)	14.69[ab] (0.04)	6.75:1: 8.85
7th	100.8[b] (0.00)	3.52[b] (0.00)	1.46[a] (0.00)	2.77[b] (0.00)	2.20:1: 1.91
9th	156.8[ab] (2.4)	8.53[ab] (0.43)	1.47[a] (0.05)	12.14[ab] (0.20)	5.92: 1: 8.40

Table 2.9 Crude extract and destruxins profiles of different subcultures of V275. Each treatment was replicated two times. Values in parenthesis represent standard errors.

Subculture	Crude extract mg/l of medium	Dtx A mg/l of medium	Dtx B mg/l of medium	Dtx E mg/l of medium	Ratio of A:B:E
1st	150.80[a] (4.0)	25.15[a] (0.08)	7.76[a] (0.02)	30.73[a] (0.08)	3.23:1:3.96
3rd	149.0[a] (3.0)	22.58[a] (0.51)	6.77[ab] (0.06)	27.54[a] (0.58)	3.33:1:3.80
5th	120.20[b] (1.40)	14.21[b] (1.38)	4.37[c] (0.49)	10.68[b] (1.15)	3.25:1:2.44
7th	123.0[b] (3.0)	16.85[b] (0.81)	5.29[bc] (0.24)	13.02[b] (0.78)	3.18:1:2.46
9th	117.40[b] (1.40)	15.54[b] (0.91)	5.73[bc] (0.51)	13.85[b] (1.10)	2.71:1:2.41

2.4 Discussion

The present study provides for the first time an insight of attenuation in *M. anisopliae*. Several important pathogenicity determinants appeared to be affected during successive *in vitro* subculturing. Virulence fluctuated between different subcultures but the overall trend was a decline in virulence upon successive subculturing.

Although no major differences in vegetative growth was observed after repeated subculturing, decline in conidia production suggested a shift towards sparse mycelial

growth with partial or no sporulation with the increasing number of subcultures. As sporulation is triggered by the exhaustion of nutrients (Li and Holdom, 1995), repeated subculturing on nutrient rich conditions might act as a trigger to grow vegetatively, thereby, causing a reduction in total conidial yield. Several other reports also suggested morphological changes upon repeated subculturing of *Verticellium lecanii* and *Paecilomyces fumosoroseus* (Hall, 1980; Vandenberg and Cantone, 2004).

Although erratic differences in the conidial CN ratios were observed, ratios were considerably high in all the treatments. Subsequent studies (see Chapter 3) show that virulent conidia consistently have a CN ratio below 5.2:1. These high conidial CN ratios observed in different subcultures suggest a maximum threshold level of around 7:1 beyond which no increase in conidial CN ratios was observed. However, further studies are required to prove this hypothesis.

The decline in total adhesion and adhesive forces i.e. surface charge and relative hydrophobicity indicates modifications at the conidial surface. Significantly higher adhesion by conidia with significantly higher hydrophobicity and electrostatic charge explains the role of these two forces in adhesion. Hydrophobins induction in *M. anisopliae* is regulated by starvation stress (St. Leger *et al.*, 1992), which suggests that by repeated growth on nutrient rich media, the gene encoding hydrophobins may be down regulated or switched off. Our studies indicate that by repeatedly growing under nutrient rich conditions *M. anisopliae* conidia may undergo physiological modifications leading to decline in hydrophobins expression and deposition at the conidia surface. Another possible mechanism in the decline of adhesion and hydrophobicity could be by alteration in the surface carbohydrates of conidia. Changes in the surface carbohydrates of *M. anisopliae* conidia have been shown to influence adhesion and hydrophobicity (Jeffs *et al.* 1999; Ibrahim *et al.* 2002).

The hydrophobicity of conidia of other fungi has also been shown to vary due to growth media, cell starvation and growth phases (Doyle 2000 and references therein). According to Wessels (1997), hydrophobins are deposited on the walls of aerial structures like conidia. The nature and number of hydrophobins deposited at the surface of conidia could therefore, depend on the nutritional composition and availability during conidia formation.

Exactly how subculturing could influence the surface charge remains unclear, however, it could be suggested that the type of molecules deposited at the conidia surface may vary in their surface charge and therefore influence the overall surface charge of the conidia. Repeated growth on the same nutritients may limit the number and types of molecules deposited on the surface, which could also result in reduction in electrostatic charge.

Speed of germination appeared to be less affected by repeated subculturing as only conidia produced after the 9^{th} subculture germinated slowly. Differences in the germination speed of the two strains suggest the differential ability of the strains to germinate.

Many studies have shown a direct relationship between enzyme production and virulence (Gupta *et al.*, 1994). Our studies indicated that repeated subculturing on nutrient rich media influences the ability of *M. anisopliae* to produce these enzymes. This reduction in cuticle enzyme production explains another possible mechanism by which repeated subculturing could cause an overall reduction in virulence. Reduced virulence was observed in protease deficient mutants of *Beauveria bassiana* (Bidochka and Khachatourians, 1990) and *M. anisopliae* (Wang, *et al.*, 2002) but here we report reduction in enzyme production in the same culture but in its attenuated state. Repression of Pr1 by simple sugars and amino acids (St. Leger *et. al.*, 1988, 1991;

Paterson *et al.*, 1994) explains the decline in its activity during *in vitro* subculturing on nutrient rich media.

Despite its role in digesting cuticle at very early stages of infection, spore bound Pr1 may also influence over all adhesion by influencing active phase of adhesion. The presence of spore bound Pr1 enables the attached conidia to digest the cuticle and get nutrients for further growth and ultimately influencing the virulence (St. Leger *et al.*, 1991). In certain cases, higher levels of spore bound Pr1 appeared to compensate for low passive adhesion e.g. conidia from 3rd subculture of V245 had low passive adhesion but higher spore bound Pr1 and virulence. Decline in the spore bound Pr1 therefore, may influence secondary adhesion and over all virulence as observed in this study. Our studies also clearly showed that with each subculture, the spore bound Pr1 levels declines and it showed a direct relationship with decrease in virulence

Loss or reduction in spore formation in fungi is often linked with decline in secondary metabolite production (Adams and Hu 1998; Gao and Nuss, 1996; Kale *et al.*, 1992). The simultaneous reduction in spore yield and destruxin production explains another possible mechanism of attenuation in *M. anisopliae*.

The present study is unique as it examines the effects of attenuation for the first time by using same genotype (strain) at differential levels of virulence. The comparative decline in virulence and some of virulence determinants e.g. spore adhesion, spore bound Pr1, and destruxins confirm their role as virulence determinants. The erratic changes in virulence and some of the pathogenicity determinants further suggest that these attributes fluctuate between different subcultures but the net result is a decline in virulence. The interesting pattern of gradual shift from pathogenic mode to saprophytic mode (i.e. less expression of pathogenicity determinants) further suggests that the environment and in particular nutrition causes adaptive modifications in *M. anisopliae*.

Chapter 3: Influence of nutrition on the growth and virulence of *M. anisopliae*

3.1 Introduction

Conidia constitute the infective unit of insect-pathogenic fungi like *M. anisopliae* (Butt, 2002). Inoculum produced on naturally infected arthropods is usually highly infective to susceptible hosts whereas that produced on artificial media can lose virulence (Butt, 2002). For *M. anisopliae,* and other fungal biocontrol agents (BCAs), to be commercially viable, mass production has to be done on artificial substrates. This requires an understanding of the relationship between nutrition and virulence, which currently remains obscure. Nutritional studies to date reveal tenuous links between virulence and spore endogenous reserves (Hallsworth and Magan, 1994, 1995) and surface carbohydrates (Ibrahim *et al.*, 2002). Specific conidial traits have been identified which are considered to be good indicators of virulence including spore size, adhesion, and germination speed (Altre *et al.*, 1999; Jackson *et al.*, 1985; Chandler *et al.*, 1993; Lane *et al.*, 1991). However, these traits are peculiar to some species or strains of fungi and, consequently, have limited value for quality control (i.e. to ensure the inoculum is virulent) of insect pathogenic fungi in general. In contrast, all entomogenous fungi are dependent on the production of CDEs (lipases, chitinases, proteases) to help penetrate the host cuticle (Butt, 2002). One much studied protease, Pr1, is an important virulence determinant which is induced by insect cuticle, de-repressed under starvation conditions and repressed in the presence of excess nutrients (Butt *et al.,* 1998; Wang *et al.*, 2002). Fungal pathogenicity is not determined by one single factor but is dependent on a coordinated interplay between many, disparate pathogenicity determinants. This study provides the first detailed analysis of the relationship between nutrition and virulence of conidia produced *in vitro* and *in vivo*. Most studies usually focus on inoculum produced on artificial media. Our study shows that nutrition influences the carbon and nitrogen composition of conidia, germination

rate and levels of spore bound Pr1 and that these parameters can to some extent predict the virulence of the inoculum. The significance of these findings as regards the production and quality control of inoculum in commercial systems is discussed.

3.2 Material and Methods

3.2.1 Fungi

Cultures are same as described in section 2.2.1; however the following culture media were evaluated to determine their effect on *M. anisopliae* growth and virulence.

3.2.2 Culture media

Culture media representing disparate carbon and nitrogen sources and ratios were used in this study. They included: 1. Potato Dextrose Agar (PDA), 2. High CN (75:1) medium consisting of 9.1% glucose and 1% peptone, 3. Low CN (10:1) medium consisting of 0.6% glucose and 1% peptone, 4. Intermediate CN (35:1) medium consisting of 4% glucose and 1% peptone (nutrition and CN equivalent to Sabouraud dextrose agar media), 5. Nutrient poor media consisting of either 2% peptone (2P) or 1% yeast extract (1Y) and 6. "Osmotic stress" medium consisting of 8% glucose, 2% peptone, 5.5% KCl. All the media were prepared using 2% agar except the osmotic stress medium, which required 5.5% agar to solidify.

All media were sterilized at 121°C at 15 psi for 15 minutes, and then poured (15ml) into each 9cm diameter Petri dishes. Glucose, yeast extract and KCl were obtained from Sigma, while mycological peptone, agar and PDA were obtained from Difco.

3.2.3 Monitoring growth and sporulation

Growth and sporulation of *M. anisopliae* on different media was recorded as described in section 2.2.2

3.2.4 CN ratio of conidia produced on different media

The CN elemental composition of the conidia produced on different media were determined as described in section 2.2.4

3.2.5 Germination of conidia produced on different media

Conidial germination was determined as described in section 2.2.8, however, conidia of V275 germinated relatively faster on certain media therefore its germination was recorded 8 hours post inoculation.

3.2.6 Determining virulence of inoculum produced on different culture media

The inoculum from the above treatments was assayed against 4-5th instar larvae of *Tenebrio molitor* as described in section 2.2.3.

3.2.7 CDEs by conidia produced on different media

The production of CDEs was investigated as described in section 2.2.9. Conidia produced on different media were used as inoculum.

3.2.8 Protein assays

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

3.2.9 Enzyme assays.

All enzyme assays including API-ZYM test were done as described in section 2.2.11, however, conidia produced on different media was used as inoculum.

3.2.10 Effect of host passage on virulence and its determinants

In order to confirm above findings and to prove that host passage or growth on nutrient poor media up regulate the virulence, conidia produced either on intermediate CN (representing low virulence) and those produced on 1% yeast extract (representing high virulence) were passaged through 4-5th instar larvae of *G. mellonela* and *T. molitor.* All the dead insects were transferred to moist chambers to allow fungal emergence and sporulation. Conidia was harvested from cadavers, washed with distilled water to remove any traces of insect and used for virulence, conidia CN analysis, conidial germination, spore bound Pr1 determination (as described above) and gene expression studies as described below.

3.2.11 Quantitative RT-PCR analysis of CDEs in conidia from selected media.

Insect passaged conidia as well as those from intermediate CN (35:1) and 1% yeast extract media were selected for more detailed analysis of the expression of *pr1A* and three other genes (esterases, trypsin, chymotrypsin) linked to the infection process.

3.2.11.1 RNA extraction

Total RNA was isolated from the conidia using the RNAeasy kit from Qiagen (Valencia, CA), according to the manufacturer's recommendations, which also allowed removal of DNA contamination using DNase I (Qiagen). The quantity and quality of RNA was assessed on a 1.2 % formaldehyde agarose gel.

3.2.11.2 cDNA synthesis

Total RNA from each sample was used to generate cDNA using *Reverse* iT ^{TM} 1st strand synthesis kit from AB gene (UK) with oligo (dT) primers according to the manufacturer's protocol. Briefly, 1 µg of DNase I treated total RNA was used as template, to which 2 µl of oligo (dT), 1 µl of 5mM dNTPs, 4 µl of 5x first strand buffer,

1 µl of *Reverse* iT ™ blend and 13 µl of sterile water were added. The reagents RNA, oligo (dT) and water was mixed first and heated to 70 °C for 5 minutes and chilled on ice to remove secondary structure. The samples were then incubated at 47 °C for 30 minutes followed by incubation at 75 °C for 10 minutes.

3.2.11.3 Real time PCR

The primers used for the target and house keeping genes (5.8 S rRNA genes) were designed using the program Primer3 (http://www.broad.mit.edu/cgibin/primer/primer3_www.cgi) and obtained from MWG-Biotech (Germany). The primer sequence and annealing temperatures for these genes were as follows:

Gene	Primer	Sequence	Annealing temperature
Protease *(prlA)*	Prl A U	CAC TCT TCT CCC AGC CGT TC	56 °C
	Prl A L	TCG GCT TTG GAG GTA AGA GC	
Chymotrypsin (*chyl*)	Chy U	AGA TCC TCC TTG GCC TTT TC	59 °C
	Chy L	GTT CGC TGG TGC TTG GAT TG	
Esterase (*ste 1*)	Est1 U	TCT ACC ACG TTC TTC TCG CC	60 °C
	Est1 L	GGC CCA GGT CCA AGG CTA CT	
Trypsin (*try 1*)	Tryp U	GCT GAC GAT GAA GGG GAA T	56 °C
	Tryp L	GCT CTT TAT CTG CCC CTT TG	
SSU (rDNA)	SRT1	CGA AAC TGC GAA TGG CTC A	-
	SRT2	CCG AAG TCG GGA TTT TTA GC	

Real–time quantitative PCR (RT-PCR) amplification was carried out in an I cycler (Biorad). The reaction mixture (20 µl) consisted of 2X PCR buffer containing SYBER-Green; 100mM KCl, 40mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), iTaq DNA polymerase, 50units/ml, 6 mM $MgCl_2$, SYBER Green 1,

20 nM fluorescein, stabilizers, 50 ng cDNA template and 100nM of the appropriate primer. The PCR conditions were 95°C for 4 minutes; followed by 42 cycles of 95°C for 20 sec. and annealing temperature according to the target gene (see above) for 30 sec., one cycle at 95 °C and annealing temperature each lasting for 30 sec., followed by 40 cycles of annealing temperature with each cycle lasting for 10 sec. The fluorescent spectra were recorded during the elongation phase of each PCR cycle. Standard curves were also generated for house keeping and target genes in a serial dilution using V275 cDNA.

3.2.12 Statistical analysis

The whole study was repeated twice with each treatment replicated three times unless stated otherwise. Data was subjected to one-way ANOVA followed by the Tukey test. Wherever required, linear regression for the calculation of growth rate/day, LT_{50}, and enzyme activities was also done. For all statistical analysis SPSS 11 software was used.

3.3 Results

3.3.1 Growth of *M. anisopliae* on different media

Vegetative growth of *M. anisopliae* varied significantly ($P < 0.05$) on the different media ranging from 1.44 to 6.35 mm/day for V245 and 1.75 to 6.95 mm/day for V275 respectively (Tables 3.1, 3.2). For both isolates, radial growth was highest on media with C:N ratio of 35 and 75:1 and was least on the high osmolarity medium (Tables 3.1, 3.2). Intermediate growth was observed on the remaining media for both isolates. In most instances, V275 grew marginally faster and produced more conidia than V245.

Conidial production of *M. anisopliae* varied significantly ($P < 0.05$) on the different media (Tables 3.1, 3.2). Highest yields were obtained on CN 35:1 for both V245 (1.13 X 10^{11} conidia/l) and V275 (3.56 X 10^{11} conidia/1) and the least on OSM. Intermediate conidial production (0.38 – 0.88 x 10^{11} conidia/l) was observed on the rest of the media, which varied non-significantly between the treatments (Tables 3.1, 3.2).

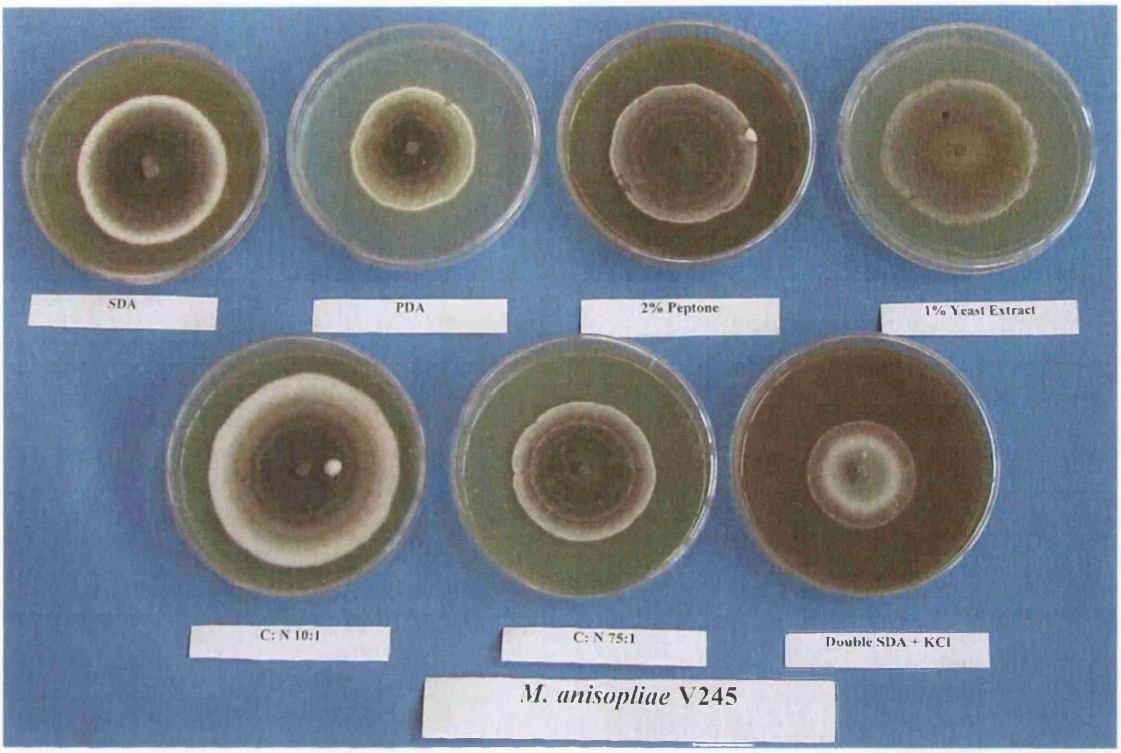


Fig. 3.1. Phenotypic variation and growth of *M. anisopliae* V245 on different growth media. Note the conidial colour varies from typical greyish green conidia on CN 35:1 (SDA) medium to light green colour conidia observed on KCl amended (OSM) medium.

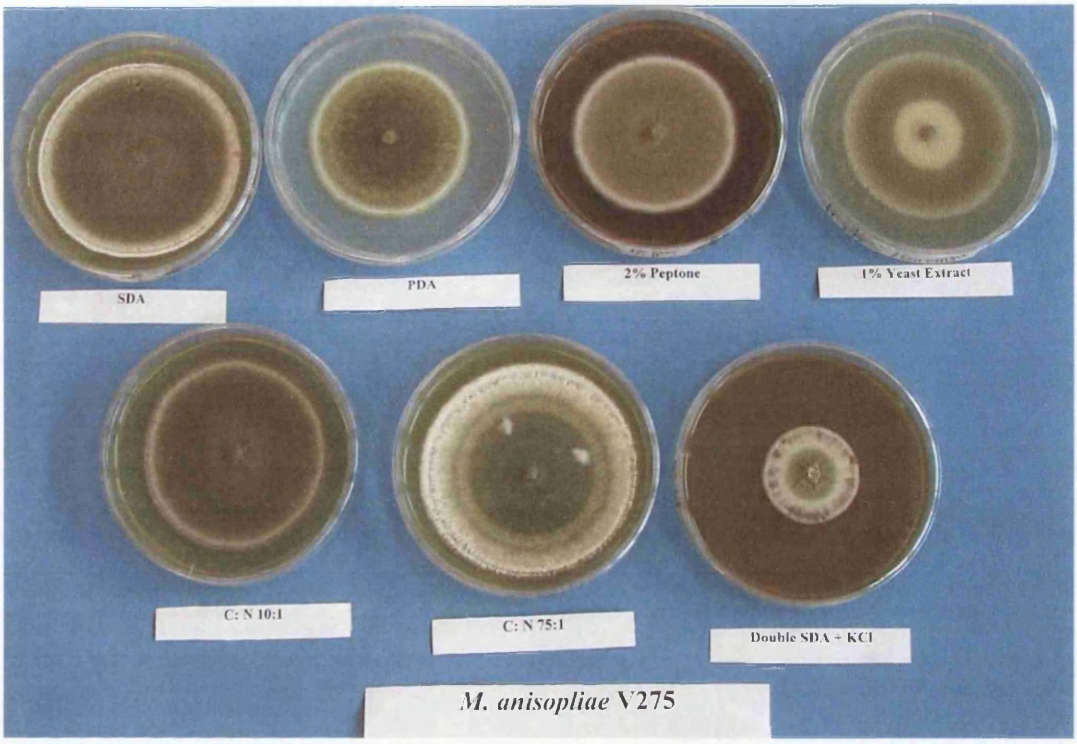


Fig. 3.2. Phenotypic variation and growth of *M. anisopliae* V275 on different growth media. Note the conidial colour varies from typical greyish green conidia on CN 35:1 (SDA) medium to light green colour conidia observed on KCl amended (OSM) medium. Also note that V275 growth rate is higher on all media than that of V245.

3.3.2 Virulence of inoculum produced on different media

Nutrition influenced conidial virulence for *T. molitor* larvae (Tables 3.1, 3.2). The most virulent conidia of V245 and V275 were produced on the OSM and/or 1% yeast extract media whilst the least virulent conidia were produced on PDA and/or C:N 35:1 (Tables 3.1, 3.2). Conidia of V275 were marginally more virulent than those of V245 when produced on the same medium (Tables 3.1, 3.2).

3.3.3 Influence of nutrition on the conidial CN ratios

The CN ratio of the conidia produced on different media varied significantly ($P < 0.05$) between treatments. V245 and V275 conidia had similar CN profiles. For example, the CN ratios were significantly higher in conidia produced on C:N 35:1, PDA, C:N 75:1 and OSM but were comparatively lower in 1% yeast extract, 2% peptone and C:N 10:1 (Tables 3.1, 3.2). The highest CN ratios were in PDA and C:N 75:1 and the least in 1% yeast extract.

Table 3.1 Vegetative growth, conidial yield, CN composition and virulence of *M. anisopliae* V245 conidia produced on different media. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated three times except for CN analysis which was replicated twice. Values in parenthesis represent standard errors.

Media	Radial growth (mm/day)	Conidial yield/l (X 10^{11})	Carbon to Nitrogen ratio of conidia	Virulence LT_{50} (Days post inoculation)
PDA	4.19^{b} (0.12)	0.50^{b} (0.11)	7.12^{a} (0.00)	3.9^{b} (0.03)
1% Yeast extract	4.51^{b} (0.08)	1.07^{a} (0.02)	4.70^{b} (0.13)	3.72^{c} (0.03)
2% Peptone	4.04^{b} (0.09)	0.63^{b} (0.03)	4.82^{b} (0.07)	3.70^{c} (0.00)
CN 10:1	4.82^{b} (0.36)	0.56^{b} (0.00)	4.87^{b} (0.01)	3.63^{cd} (0.03)
CN 35: 1	6.37^{a} (0.09)	1.13^{a} (0.00)	6.35^{a} (0.47)	4.12^{a} (0.02)
CN 75:1	5.55^{b} (0.20)	0.38^{b} (0.01)	7.06^{a} (0.07)	3.66^{cd} (0.04)
KCl	1.44^{c} (0.13)	0.02^{c} (0.00)	6.49^{a} (0.1)	3.51^{d} (0.03)

Table 3.2 Vegetative growth, conidial yield, CN composition and virulence of *M. anisopliae* V275 conidia produced on different media. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated three times except for CN analysis which was replicated twice. Values in parenthesis represent standard errors.

Media	Radial growth (mm/day)	Conidial yield/l (X 10^{11})	Carbon to Nitrogen ratio of conidia	Virulence LT_{50} (Days post inoculation)
PDA	4.17^{c} (0.06)	1.05^{b} (0.15)	7.14^{e} (0.06)	3.93^{a} (0.00)
1% Yeast extract	4.59^{c} (0.21)	0.88^{bc} (0.00)	4.05^{a} (0.00)	3.46^{d} (0.02)
2% Peptone	3.60^{c} (0.11)	0.87^{bc} (0.02)	4.39^{b} (0.06)	3.58^{cd} (0.02)
CN 10:1	4.98^{b} (0.13)	0.25^{cd} (0.00)	5.11^{c} (0.06)	3.66^{bc} (0.02)
CN 35: 1	6.95^{a} (0.09)	3.56^{a} (0.33)	6.84^{d} (0.02)	3.86^{ab} (0.09)
CN 75:1	6.36^{a} (0.26)	0.70^{bcd} (0.04)	7.39^{f} (0.04)	3.89^{a} (0.03)
KCl	1.75^{d} (0.28)	0.06^{d} (0.01)	6.67^{d} (0.01)	3.49^{cd} (0.02)

3.3.4 Influence of nutrition on the speed of germination of *M. anisopliae* conidia

Germination speed of conidia produced on different media varied significantly ($P < 0.05$) between treatments and between the two isolates (Table 3.3). At 8 hours post inoculation significant germination was observed for V275 but ranged between 2-10% for V245. The percentage germination increased for both isolates at 10 hours post-inoculation but germlings were more developed in some media than others. For example, over 80% of V275 conidia had germinated irrespective of the substrate but the germ tube of germlings produced on 1% yeast extract or high C:N were over 4-5 times conidial length whereas germlings on CN 35:1 media had germ tubes equal to or less than the conidial length. The germination rate for both isolates was lowest on PDA and C:N 35:1 (Table 3.32). The highest germination rates for V275 (>90%) were noted for conidia produced on 1% yeast extract and C:N 10:1, while those of V245 (89%) were noted for conidia produced on OSM (Table 3.3).

Table 3.3 Influence of nutrition on the germination speed of *M. anisopliae* conidia. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated three times. Values in parenthesis represent standard errors.

Media	Germination (%)	
	V245 (10 hrs post inoculation)	V275 (08 hrs post inoculation)
PDA	54.33^{c} (4.25)	32.66^{c} (2.33)
1% Yeast extract	74.66^{b} (2.40)	93.0^{a} (1.73)
2% peptone	78.66^{ab} (1.73)	87.0^{a} (1.73)
CN 10:1	69.0^{b} (0.57)	93.0^{a} (0.57)
CN 35: 1	47.0^{c} (3.78)	44.66^{b} (2.96)
CN 75:1	67.0^{b} (1.73)	84.33^{a} (2.84)
KCl	89.33^{a} (0.88)	83.66^{a} (2.02)

3.3.5 Production of CDEs by the conidia produced on different media

Total protein production varied non-significantly ($P < 0.05$, Duncan Test) among the different treatments in case of V245 samples but it varied significantly ($P < 0.05$, Tukey Test) for those of V275 (Tables 3.4, 3.5). Generally higher protein concentration was observed in the samples of V275 as compared to those of V245. In case of V275, conidia produced on media with relatively low levels of nutrients e.g. 1% yeast extract, medium with CN of 10:1 exhibited higher protein concentration (Table 3.5).

Non-specific protease activity also varied non-significantly ($P < 0.05$, Duncan Test) in the samples of V245 (Table 3.4). In case of V275, generally non-significant differences were observed but two treatments CN 10:1 (highest activity i.e. 1.24) and OSM (lowest activity i.e. 0.94) varied with each other significantly ($P < 0.05$, Tukey Test). No significant differences were observed in remaining treatments (Table 3.5).

No significant differences were observed for inductive Pr1 or Pr2 activities among the various treatments of either V245 or V275; however, all the treatments of V275 had slightly higher Pr1and Pr 2 activity than those of V245 (Tables 3.4, 3.5).

Significant differences were observed for inductive NAG'ase activity. NAG'ase activity was higher in samples inoculated with V245 conidia produced on PDA, while all other treatments of V245 varied non-significantly (Tables 3.4). In case of V275, conidia from CN 35:1 media had significantly higher in NAG'ase activity while conidia from OSM yielded lowest activity (Table 3.5). Remaining treatments varied non-significantly among them selves but varied significantly with these two extremes (Table 3.5).

3.3.6 Influence of nutrition on the spore bound Pr1 activity

Spore bound Pr1 activities varied significantly ($P < 0.05$) depending upon the medium on which conidia were produced (Tables 3.1, 3.2). Highest activities for both isolates were observed for conidia produced on 1% yeast extract and C:N 10:1. V275 also appeared to have more spore bound Pr1 when produced on 2% peptone. V275 generally produced more Pr1 than V245 except for conidia produced on the C:N 10:1 and OSM (Tables 3.4, 3.5). Non significant ($P < 0.05$) corelation was observed for both isolates between spore bound Pr1 and virulence. However, correlation analysis was highly significant ($P < 0.00.$, Pearson Correlation) for V275 when analysed by excluding high osmolarity media.

Table 3.4 The total protein, non- specific proteases, Pr1, Pr2 and NAG'ase activities of V245 conidia produced on different media in the inductive media in comparison with their spore bound Pr1 activity. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated two times. Values in parenthesis represent standard errors. * Duncan test used for statistical analysis

Media	Total protein concentration (µg/ml)	Non-Specific proteases (U/ml)	Inductive Pr1 (µ mol/ml/min)	Inductive Pr2 (µ mol/ml/min)	ExoChitinase NAG'ase (µ mol/ml/hr	Spore bound Pr1 (µ mol/ml/min)
PDA	35.0^{a*} (0.02)	0.72^{a} (0.09)	2.74^{a} (0.02)	0.39^{ab} (0.06)	0.33^{a} (0.04)	1.33^{c} (0.14)
1% Yeast extract	35.0^{a} (0.03)	0.71^{a} (0.13)	2.90^{a} (0.01)	0.60^{a} (0.01)	0.18^{b} (0.00)	2.92^{a} (0.03)
2% Peptone	$< 20^{a}$	1.03^{a} (0.04)	2.77^{a} (0.11)	0.54^{ab} (0.01)	0.15^{b} (0.00)	1.49^{bc} (0.05)
CN 10:1	52^{a} (0.06)	0.82^{a} (0.07)	2.83^{a} (0.02)	0.59^{a} (0.03)	0.16^{b} (0.00)	3.27^{a} (0.05)
CN 35: 1	42.0^{a} (0.01)	1.13^{a} (0.08)	2.78^{a} (0.01)	0.54^{ab} (0.05)	0.13^{b} (0.00)	0.70^{d} (0.03)
CN 75:1	$< 20^{a}$	0.84^{a} (0.14)	2.73^{a} (0.09)	0.60^{a} (0.03)	0.13^{b} (0.000)	0.81^{d} (0.02)
KCl	$< 20^{a}$	1.2^{a} (0.16)	2.76^{a} (0.00)	0.48^{ab} (0.01)	0.18^{b} (0.00)	1.79^{b} (0.09)

Table 3.5 The total protein, non- specific proteases, Pr1, Pr2 and NAG'ase activities of V275 conidia produced on different media in the inductive media in comparison with their spore bound Pr1 activity. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated two times. Values in parenthesis represent standard errors. * Duncan test used for statistical analysis

Media	Total protein concentration (µg/ml)	Non-Specific proteases (U/ml)	Inductive Pr1 (µ mol/ml/min)	Inductive Pr2 (µ mol/ml/min)	ExoChitinase NAG'ase (µ mol/ml/hr	Spore bound Pr1 (µ mol/ml/min)
PDA	43^{a*} (0.01)	1.08^{ab} (0.16)	2.87^{a} (0.03)	0.59^{a} (0.02)	0.20^{bc} (0.0)	1.50^{c} (0.04)
1% Yeast extract	120^{bc} (0.02)	1.22^{ab} ((0.04)	2.92^{a} (0.02)	0.59^{a} (0.01)	0.24^{ab} (0.01)	3.24^{a} (0.02)
2% Peptone	60^{ab} (0.03)	1.12^{ab} (0.01)	2.91^{a} (0.01)	0.67^{a} (0.02)	0.21^{bc} (0.00)	3.15^{a} (0.03)
CN 10:1	140^{c} (0.01)	1.24^{b} (0.07)	2.89^{a} (0.00)	0.68^{a} (0.05)	0.24^{ab} (0.01)	2.51^{b} (0.03)
SDA	85^{ab} (0.03)	1.20^{ab} (0.05)	2.90^{a} (0.01)	0.62^{a} (0.03)	0.28^{a} (0.02)	1.59^{c} (0.02)
CN 75:1	45^{a} (0.01)	1.18^{ab} ((0.03)	2.79^{a} (0.01)	0.57^{a} (0.04)	0.22^{b} (0.01)	1.51^{c} (0.12)
KCl	50^{a} (0.01)	0.94^{a} (0.11)	2.90^{a} (0.04)	0.56^{a} (0.01)	0.16^{c} (0.00)	1.17^{d} (0.03)

3.3.7 Effect of culture media on the semi quantitative enzyme profile of the conidia

API-ZYM studies showed that enzyme activities varied between treatments and isolates. Both isolates varied in their enzyme profiles, however, none of the V245 and V275 treatment showed enzyme activity for cystine arylamidase, trypsin, α – chymotrypsin, α- fucosidase. Weak β-galactosidase activity was observed in only one treatment i.e. V245 conidia produced on 1% yeast extract media. Similarly, alkaline

phosphatase activity was observed in conidia of V275 if produced on 1% yeast extract, 2% peptone and CN 10:1 media (Table 3.7). Conidia of both strains secreted certain enzymes irrespective of the growing medium. These enzymes included: C4 and C8 esterase, leucine and valine arylamidase, acid phosphatase, Naphthol-AS- BI - phosphohydrolase and β-glucosidase (Table 3.6). The C14 lipase was produced only by V245 conidia irrespective of the growing media on which they were produced (Table 3.6).

Table 3.6 Semi quantitative enzyme profiles of *M. anisopliae* V245 conidia produced on different media. No enzyme activity is represented by 0, while 5 represents the highest enzyme activity. Each treatment was replicated two times.

Enzymes	**SDA**	**PDA**	**1% Yeast extract**	**2% Peptone**	**CN 10:1**	**CN 75:1**	**KCl**
Alkaline phosphatase	0	0	0	0	0	0	0
Esterase (C 4)	2	3	3	3	2	2	2
Esterase Lipase (C 8)	2	3	3	3	2	2	1
Lipase (C 14)	1	1	1	0.5	0	0.5	0.5
Leucine arylamidase	3	5	5	5	3	4	4
Valine arylamidase	2	3	3	1.5	0.5	1	1
Cystine arylamidase	0	0	0	0	0	0	0
Trypsin	0	0	0	0	0	0	0
A-chymotrypsin	0	0	0	0	0	0	0
Acid phosphatase	5	5	5	5	5	5	4
Naphthol -AS-BI-phosphohydrolase	3	4	4	3	3	3	4
A- galactosidase	0	1	0.5	0	0.5	0	1
B - galactosidase	0	0	0.5	0	0	0	0
B - glucuronidase	0	0.5	0.5	0	0	0	0
A- glucosidase	0	0.5	0.5	0	0	0	0
B – glucosidase	4	4	5	4	4	3	3
N- acetyl – β glucosaminidase	2	3	0.5	3	2	4	2
A- mannosidase	0	1	0.5	0	0	0	0
A- fucosidase	0	0	0	0	0	0	0

Table 3.7 Semi quantitative enzyme profiles of *M. anisopliae* V275 conidia produced on different media. No enzyme activity is represented by 0, while 5 represents the highest enzyme activity. Each treatment was replicated twoe times.

Enzymes	SDA	PDA	1% Yeast extract	2% Peptone	CN 10:1	CN 75:1	KCl
Alkaline phosphatase	0	0	2	3	3	0	0
Esterase (C 4)	3	2	2	1	2	3	2
Esterase Lipase (C 8)	3	2	3	3	3	3	2
Lipase (C 14)	0	0	0	0	0	0	0
Leucine arylamidase	4	3	5	5	5	5	5
Valine arylamidase	1	1	3	3	3	3	3
Cystine arylamidase	0	0	0	0	0	0	0
Trypsin	0	0	0	0	0	0	0
A-chymotrypsin	0	0	0	0	0	0	0
Acid phosphatase	5	5	5	5	5	5	5
Naphthol -AS-BI-phosphohydrolase	3	3	3	2	4	3	4
A- galactosidase	0	0	0.5	2	1	0	0.5
B – galactosidase	0	0	0	0	0	0	0
B - glucuronidase	0	0	0	0	0	0	0
A- glucosidase	0	0	0	0	0	0	0
B – glucosidase	4	1	4	4	5	4	4
N- acetyl – β glucosaminidase	2	2	0	0	0	2	4
A- mannosidase	0	0	1	2.5	2	0	0
A- fucosidase	0	0	0	0	0	0	0

3.3.8 Effect of host passage on virulence

Both insect passaged and conidia produced on 1% yeast extract media were significantly ($P < 0.05$, Tukey Test) more virulent than those produced on intermediate CN media. *M. anisopliae* V245 conidia passaged from *T. molitor* were significantly more virulent than its conidia produced on either 1% yeast extract or intermediate CN media. In the case of V275, no significant difference between insect passaged and conidia produced on 1 % yeast extract media were observed but they varied significantly with those produced on intermediate CN media. No significant

differences in virulence was observed for each strain whether they were passaged through *Galleria* or *Tenebrio* (Tables 3.8, 3.9)

3.3.9 Effect of host passage on conidial carbon and nitrogen ratio

Conidial CN ratios of all insect passaged conidia were significantly lower than those produced on media with intermediate CN (Table 3.8). Conidial CN ratio of conidia produced on 1% yeast extract varied non-significantly with insect passged conidia of both strains. Insect passaged conidia also varied significantly with all media treatments for their total contents, which were only 50-70% to those present in conidia produced on artificial media. The carbon content of conidia recovered from mycosed insects and artificial media ranged between 250-350 µg/mg of conidia and 450 - 550 µg/mg of conidia, respectively.

3.3.10 Effect of host passage on conidia germination

Significantly ($P < 0.05$, Tukey Test) fast conidial germination was observed for the conidia produced either on 1% yeast extract media or passaged through insect hosts (Table 3.8). For both strains, conidia produced on intermediate CN had a significantly slow speed of germination (Tables 3.8, 3.9). *M. anisopliae* V245 conidia produced on 1% yeast extract had intermediate speed of germination and varied significantly with both fast and slow germinating conidia (Table 3.8).

3.3.11 Effect of host passage on spore bound Pr1

Spore bound Pr1 was significantly ($P < 0.05$, Tukey Test) higher in insect passaged conidia than those produced on artificial media. Conidia of both strains produced on 1% yeast extract had intermediate spore bound Pr1 activity. For V245, they varied significantly with all insect passaged treatments, however, two of the V275 treatments varied non-significantly with it (Tables 3.8, 3.9). Least spore bound Pr1 activity was observed in conidia produced on intermediate CN media. Spore bound Pr1 activities varied significantly between the two strains for conidia produced on artificial media but non-significantly for insect passaged conidia (Tables 3.8, 3.9).

Table 3.8 Effect of host passage on the conidial CN, germination, spore bound Prl and virulence of *M. anisopliae* V245. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated three times except for CN analysis and spore bound Prl which were replicated twice. Values in parenthesis represent standard errors.

Source of conidia	Conidial CN	Germination (%)	Spore bound Prl (μ mol/ml/min)	Virulence LT_{50} (Days post inoculation)
CN 35:1	6.35^{a} (0.37)	48.66^{c} (2.02)	1.08^{e} (0.00)	4.22^{a} (0.05)
1% yeast extract	4.47^{bc} (0.03)	80.33^{b} (2.33)	1.94^{c} (0.01)	3.65^{b} (0.03)
CN 35:1 passed through *G. mellonela* larvae	5.12^{b} (0.03)	91.66^{a} (0.88)	2.64^{a} (0.03)	3.56^{bc} (0.03)
1% yeast extract passed through *G. mellonela* larvae	5.08^{b} (0.00)	93.0^{a} (2.64)	2.71^{a} (0.03)	3.61^{bc} (0.05)
CN 35:1 passed through *T. molitor* larvae	4.92^{b} (0.02)	92.66^{a} (2.02)	2.75^{a} (0.06)	3.44^{c} (0.02)
1% yeast extract passed through *T. molitor* larvae	5.15^{bc} (0.11)	92.0^{a} (1.73)	2.75^{a} (0.01)	3.42^{c} (0.04)

Table 3.9 Effect of host passage on the conidial CN, germination, spore bound Prl and virulence of *M. anisopliae* V275. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated three times except for CN analysis and spore bound Prl which were replicated twice. Values in parenthesis represent standard errors.

Source of conidia	Conidial CN	Germination (%)	Spore bound Prl (μ mol/ml/min)	Virulence LT_{50} (Days post inoculation)
CN 35:1	6.84^{a} (0.02)	47.33^{b} (2.02)	1.67^{d} (0.09)	3.96^{a} (0.02)
1% yeast extract	4.05^{c} (0.00)	93.33^{a} (2.18)	2.43^{b} (0.00)	3.47^{b} (0.02)
CN 35:1 passed through *G. mellonela* larvae	4.66^{bc} (0.02)	91.0^{a} (2.08)	2.69^{a} (0.01)	3.40^{b} (0.03)
1% yeast extract passed through *G. mellonela* larvae	4.64^{bc} (0.01)	92.33^{a} (0.66)	2.59^{ab} (0.01)	3.43^{b} (0.04)
CN 35:1 passed through *T. molitor* larvae	4.47^{bc} (0.02)	93.33^{a} (2.18)	2.61^{ab} (0.03)	3.44^{b} (0.02)
1% yeast extract passed through *T. molitor* larvae	4.47^{bc} (0.03)	94.0^{a} (2.30)	2.75^{a} (0.02)	3.32^{b} (0.02)

3.3.12 RT-PCR analysis of cuticle-degrading enzymes in conidia

Gene expression was significantly ($P < 0.05$, Tukey Test) higher in insect passaged conidia than those produced on artificial media (Fig. 3.1-3.8). Irrespective of strain or host, generally all the insect passaged conidia varied non-significantly ($P < 0.05$, Tukey Test) among them. Expression of *prl A*, *try 1* and *chy1* was 4- 16 fold higher in insect passaged conidia than those produced on artificial media. However, *ste 1* expression was only 1-3 fold higher than the conidia produced on artificial media.

Conidia produced on 1% yeast extract media had significantly ($P < 0.05$, Tukey Test) higher expression of *pr1A* than those produced on intermediate CN media. For all other genes, these two treatments varied non-significantly ($P < 0.05$, Tukey Test) with each other (Figs. 3.1-3.8). Melt curve observations of each well for each Real time PCR amplification revealed single curve confirming specific amplification of the target gene. Gel photographs of the amplified gene products also showed a single band for each gene (Appendix 2).

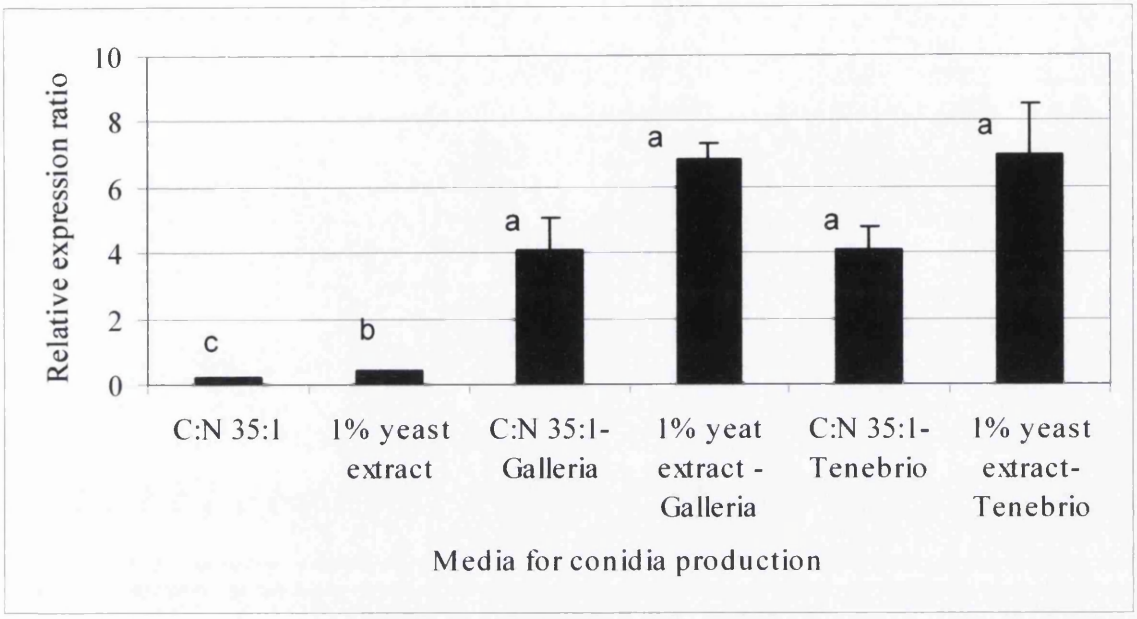


Fig 3.3. Relative expression of *pr1 A* in un-germinated conidia of *M. anisopliae* V245. Sources of conidia include, conidia produced on intermediate CN (35:1), conidia produced on 1% yeast extract media (1% yeast extract), conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (1% yeast extract -*Tenebrio*). Bars labelled with same letter differ non-significantly ($P < 0.05$, Tukey test).

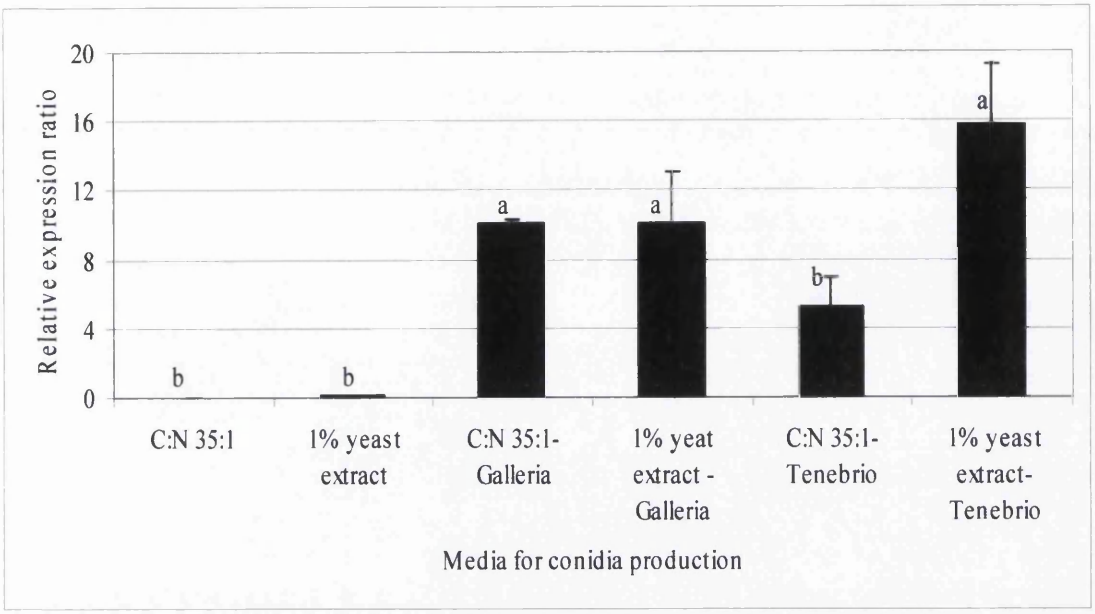


Fig 3.4. Relative expression of try *1* in un-germinated conidia of *M. anisopliae* V245. Sources of conidia include, conidia produced on intermediate CN (35:1), conidia produced on 1% yeast extract media (1% yeast extract), conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (% yeast extract -*Tenebrio*). Bars labelled with same letter differ non-significantly ($P < 0.05$, Tukey test).

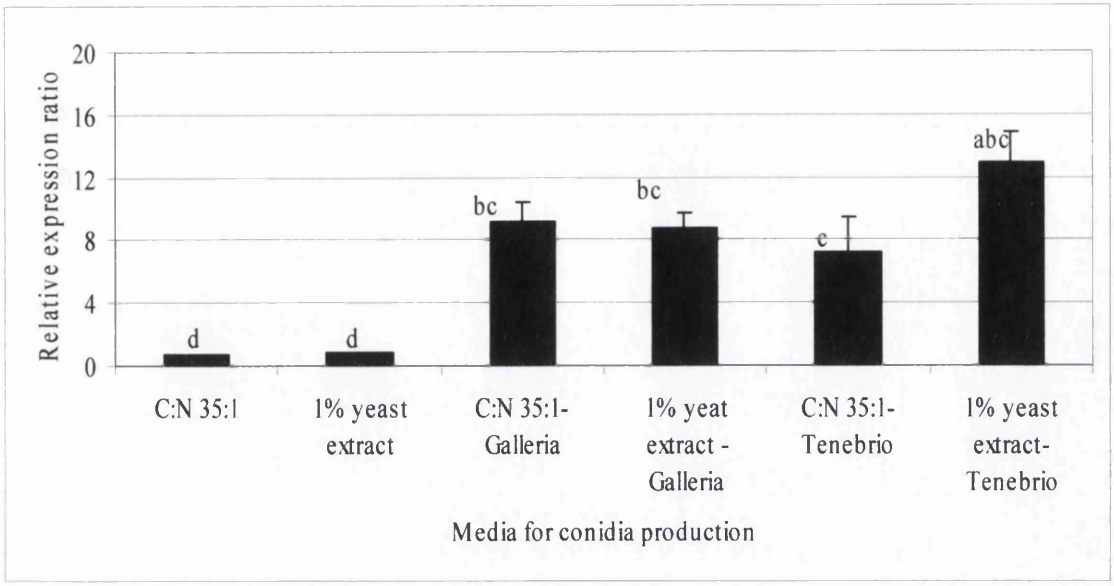


Fig 3.5. Relative expression of *chy 1* in un-germinated conidia of *M. anisopliae* V245. Sources of conidia include, conidia produced on intermediate CN (35:1), conidia produced on 1% yeast extract media (1% yeast extract), conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (% yeast extract -*Tenebrio*). Bars labelled with same letter differ non-significantly ($P < 0.05$, Tukey test).

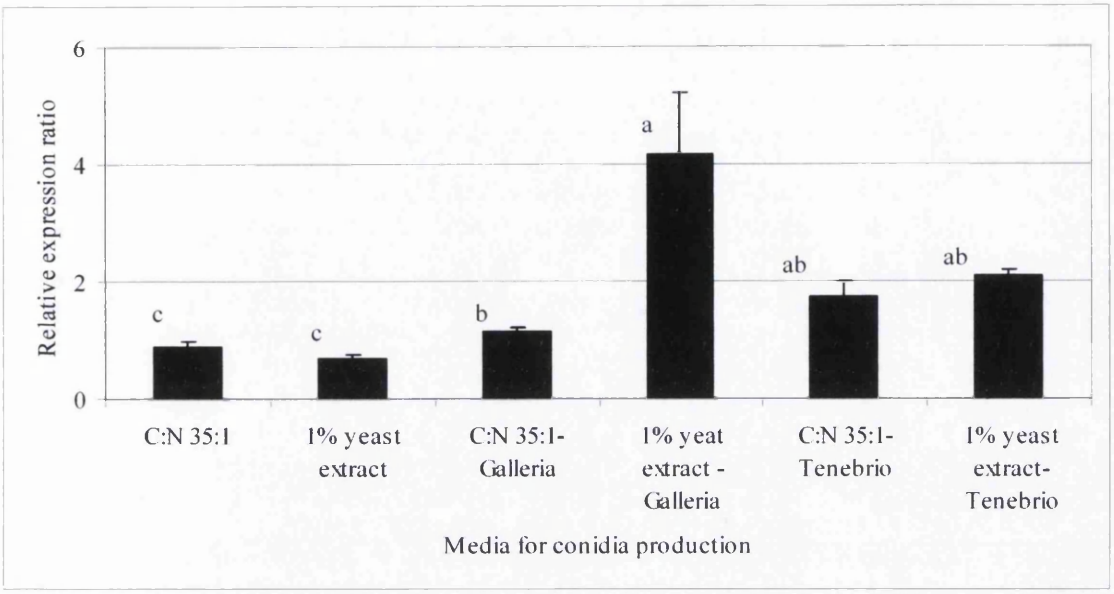


Fig 3.6. Relative expression of *ste 1* in un-germinated conidia of *M. anisopliae* V245. Sources of conidia include, conidia produced on intermediate CN (35:1), conidia produced on 1% yeast extract media (1% yeast extract), conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (% yeast extract -*Tenebrio*). Bars labelled with same letter differ non-significantly ($P < 0.05$, Tukey test).

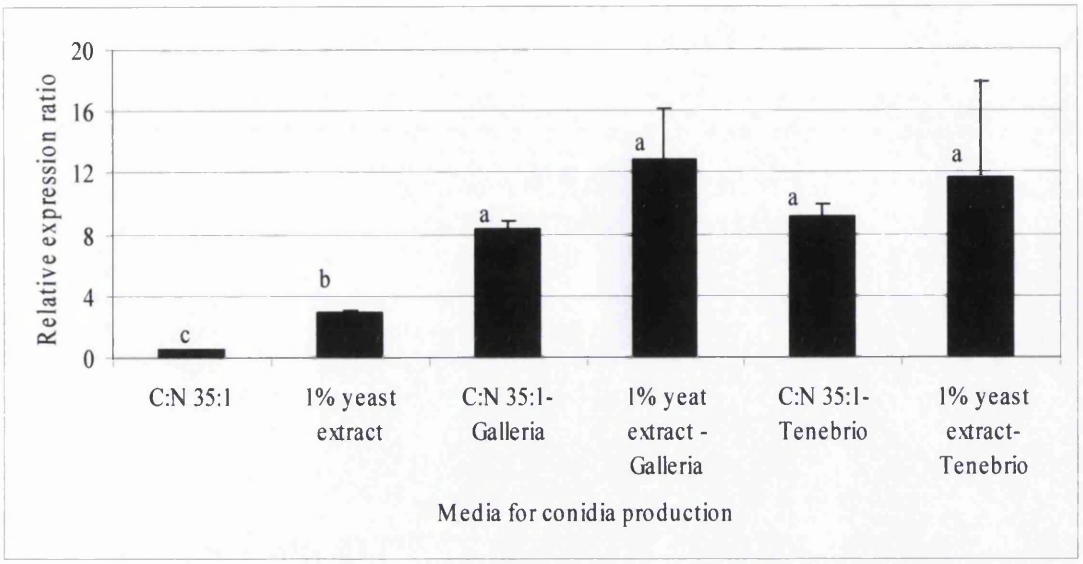


Fig 3.7. Relative expression of *pr1 A* in un-germinated conidia of *M. anisopliae* V275. Sources of conidia include, conidia produced on intermediate CN (35:1), conidia produced on 1% yeast extract media (1% yeast extract), conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (% yeast extract -*Tenebrio*). Bars labelled with same letter differ non-significantly ($P < 0.05$, Tukey test).

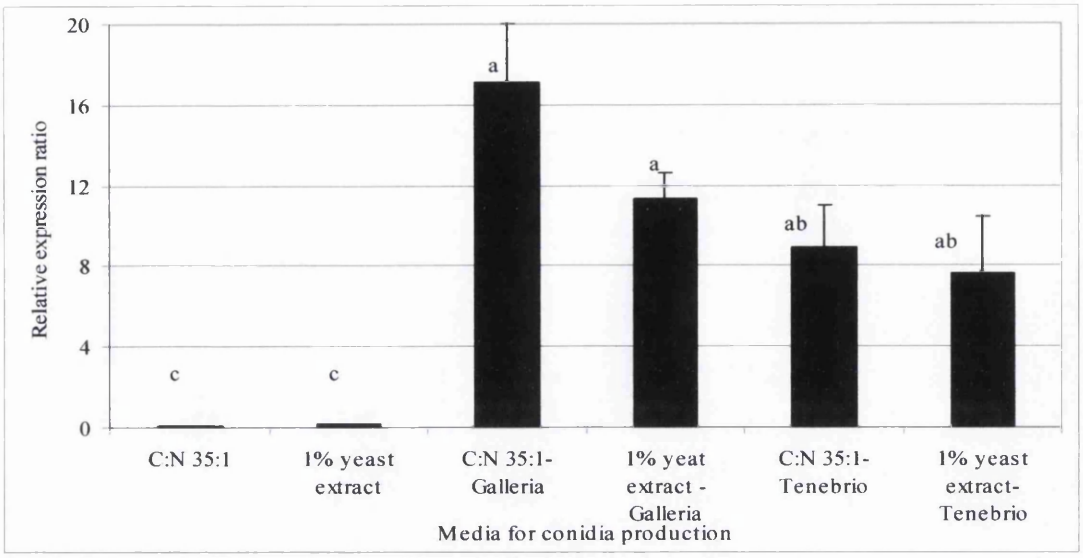


Fig 3.8. Relative expression of *try 1* in un-germinated conidia of *M. anisopliae* V275. Sources of conidia include, conidia produced on intermediate CN (35:1), conidia produced on 1% yeast extract media (1% yeast extract), conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (% yeast extract -*Tenebrio*). Bars labelled with same letter differ non-significantly ($P < 0.05$, Tukey test).

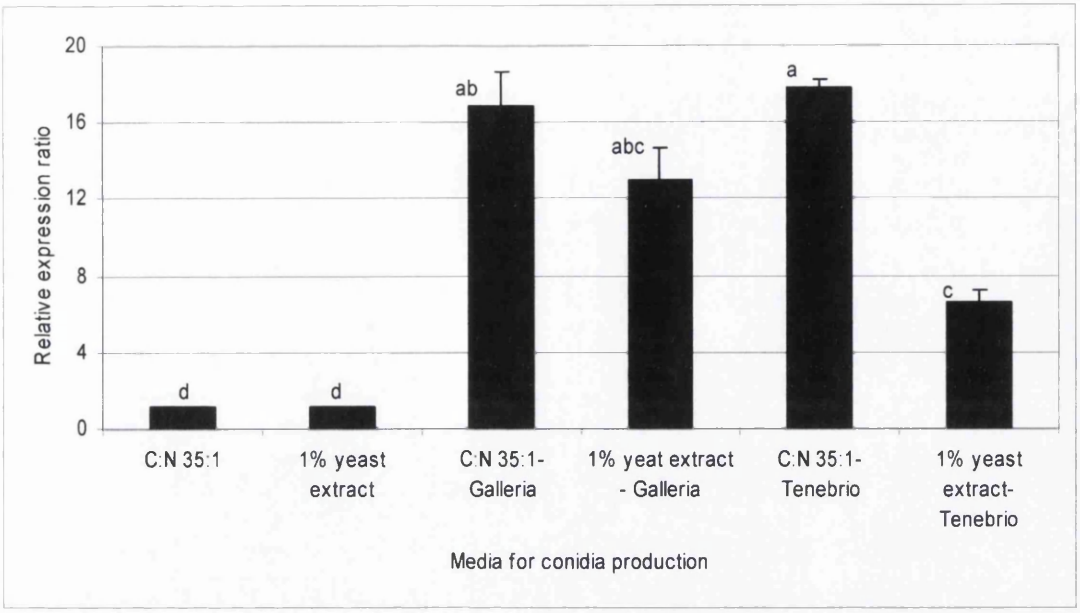


Fig 3.9. Relative expression of *chy 1* in un-germinated conidia of *M. anisopliae* V275. Sources of conidia include, conidia produced on intermediate CN (35:1), conidia produced on 1% yeast extract media (1% yeast extract), conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (% yeast extract -*Tenebrio*). Bars labelled with same letter differ non-significantly ($P < 0.05$, Tukey test).

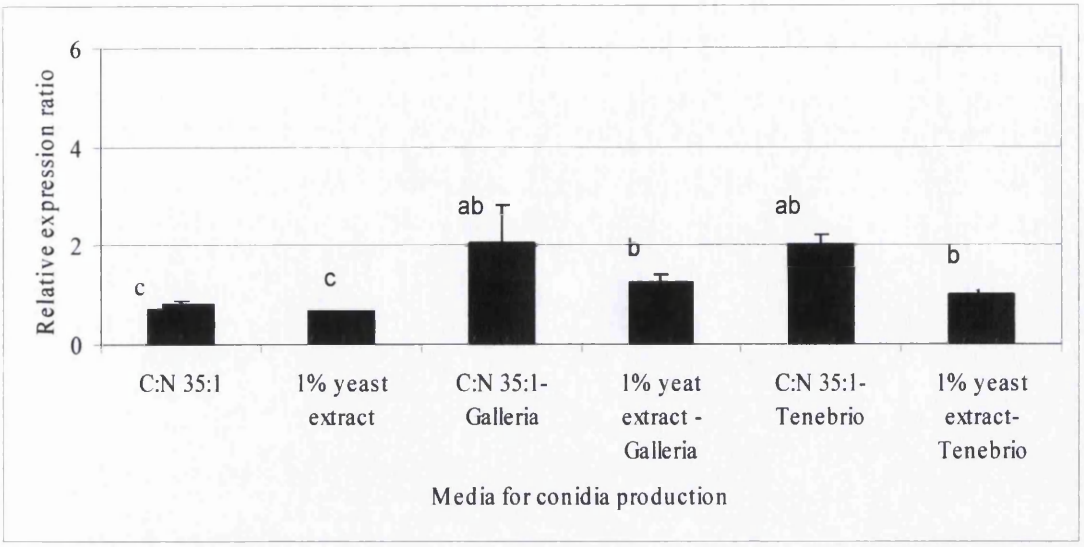


Fig 3.10. Relative expression of *ste 1* in un-germinated conidia of *M. anisopliae* V275. Sources of conidia include, conidia produced on intermediate CN (35:1), conidia produced on 1% yeast extract media (1% yeast extract), conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (% yeast extract -*Tenebrio*). Bars labelled with same letter differ non-significantly ($P < 0.05$, Tukey test).

3.4 Discussion

Conidial yield of *M. anisopliae* is dependent upon the fungal strain and nutrition and does not appear to be linked with radial growth. Earlier studies also reported that optimum conidia production for *Beauveria bassiana* and *M. anisopliae* was dependent upon the isolate and the medium type (Kamp and Bidochka 2002). Conidial yields of V275 and V245 were optimal in the medium with a CN ratio of 35:1 (similar to SDA) whereas studies on other isolates reported highest yields in PDA (Kamp and Bidochka 2002). Optimal spore yield of the mycoherbicide, *Colletotrichum* was also achieved in media with a CN ratio of 30:1 and not at higher or lower CN ratios (Jackson and Schisler, 1992). In contrast, spore production of *M. anisopliae*, *B. bassiana* and *Paecilomyces fumosoroseus* was highest in a broth medium with CN ratio of 10:1 (Vega *et al.*, 2003).

Nutrition impacted not only on spore production but also spore quality. Media with a CN ratio of 35:1 and PDA resulted in poor germination and virulence for both V245 and V275 but exactly why germination should be better at CN ratios higher and lower than 35:1 is unclear. Conidia of *C. truncatum* also germinated more rapidly and were more virulent if produced in CN 10:1 media (Jackson and Schisler 1992). The rapid germination was linked to the relatively high protein content of this inoculum (Jackson and Schisler 1992). In our study, we did not discriminate between the different endogenous reserves but noted that virulent conidia of *M. anisopliae* had relatively low CN ratios and high germination rates. It is possible that the high nitrogen levels in *M. anisopliae* correspond to storage and structural (e.g. cytoskeletal, membrane) proteins as well as enzymes that facilitate rapid germination. Conidia produced in OSM were an exception; their rapid germination may be due to other reasons. For example, *M. anisopliae* conidia produced under osmotic stress have been observed to accumulate higher amount of polyols and consequently germinate faster (Hallsworth and Magan 1994; 1995).

The most aggressive inocula of *M. anisopliae* V245 and V275 were produced on insect hosts, OSM, 2% peptone and 1% yeast extract. These disparate substrates

probably require different pathways for the utilisation of nutrients and subsequently regulation of sporulation and virulence genes. Indeed, earlier studies show that nitrogen compounds that support growth were less favourable for spore germination and, since different amino acids stimulated particular stages of growth and sporulation, a complex nitrogen source was required to optimise these processes (Li and Holdom 1995). Presumably, yeast extract, peptone and the insects used in our studies possess nutritional components not present in simple, traditional, inexpensive mycological media. We postulate that starvation conditions whether *in vivo* or *in vitro* results in de-repression of Pr1 and that elevated levels of this enzyme enhances fungal virulence. Pr1 is without doubt an important virulence determinant. Mutants lacking the *pr1* gene are less pathogenic and those over-expressing Pr1 are hypervirulent (Wang *et al.*, 2002; St. Leger *et al.*, 1996). Nutrient deprivation triggers increased Pr1 transcription and rapid secretion of this enzyme (St. Leger *et al.*, 1991, 1992). Furthermore, Pr1 production far exceeds synthesis of other proteins when *M. anisopliae* differentiates infection structures (St. Leger *et al.*, 1989). It is tempting to speculate that conidia with low levels of endogenous reserves would rapidly exhaust these reserves and starvation would induce Pr1 and other virulence determinants. This may explain why conidia from mycosed insects were aggressive since they had a low carbon content (ca. 30-50% lower than conidia from artificial media) and subsequently fewer endogenous reserves (e.g. glycogen, lipid).

Detailed studies of the virulent conidia produced on mycosed insects and 1% yeast extract showed that they shared several attributes including relatively high levels of spore bound Pr1 activity (>2μM/min/min), CN ratios <5.2:1 and high germination rates. Conidium from mycosed cadavers, i.e. passaged inoculum, was marginally more virulent than inoculum produced *in vitro* and clearly had higher germination rates and significantly more *pr1 A* mRNA. Conidia produced on yeast extract had comparatively more *pr1 A* transcripts and spore bound Pr1 than the least virulent conidia produced on CN 35:1 media. Besides *pr1 A*, virulent conidia, especially passaged inoculum, had significantly higher levels of transcripts of other pathogenicity related genes compared with less virulent conidia. High levels of transcripts of the cuticle degrading enzymes would accelerate production of these

enzymes and result in faster germination and infection. The cuticle-degrading enzymes Pr1 and Pr2 have already been shown to release peptides that induce more Pr1 (Paterson *et al.*, 1994). Presence of cuticle-degrading enzymes in the conidial cell wall suggest that enzymes were secreted during conidiation and the level of activity appeared to be correlated with the amount of transcripts in the cell. Higher levels of enzyme activity were detected on conidia from infected *Manduca sexta* larvae than those from SDA suggesting that environmental conditions in which conidia develop pre-adapts them for the pathogenic life style (St. Leger *et al.*, 1991). Our study shows for the first time that the passaged inoculum is virulent irrespective of the original culture medium or insect host. The insect host clearly provides the nutrition and development cues for production of virulent conidia. This is quite important since the pathogen may only have a narrow window of opportunity where host density and environmental conditions are favourable for induction of epizootics in pest populations. Since it is uneconomical to produce conidia using insects, we have identified several parameters that could help monitor the virulence of *M. anisopliae* in large-scale fermentation systems. These parameters could also help in the design of new, inexpensive production media.

Virulent conidia were also consistently produced on OSM but growth and sporulation were poor. The relatively high level of C and N in the conidia suggests that osmotic stress did not prevent nutrient uptake. Ibrahim *et al.* (2002) also noted that conidia of *M. anisopliae* produced in nutrient poor minimal media or OSM were more aggressive than those produced on nutrient rich media (Ibrahim *et al.*, 2002). Since stress (osmotic, starvation) is a phenomenon independent of the strain, it suggests a shared pathway. The endogenous reserves of fungal spores include protein, glycogen, polyols, and lipids (Carlilie and Watkinson, 1996) but so far no worker has been able to establish a link between specific reserves and virulence. We have shown that CN ratios below 5.2:1 are a good indicator of virulence, particularly if used with other parameters such as spore bound Pr1 and high germination rates. The virulent conidia produced on OSM were an anomaly since these had CN ratios similar to those of the less virulent conidia produced on CN 35:1. The high salt levels did influence *M. anisopliae* physiology since it resulted in conidia that were paler

and less hydrophobic than those produced on the other culture media and infected insects (Ibrahim *et al.*, 2002). Again this phenomenon was independent of strain. By comparing CN ratios we do not discriminate between the endogenous reserves since these may vary depending on the source of nutrients and fungal species or strain. For example, the polyol and trehalose content of conidia of *B. bassiana*, *M. anisopliae* and *P. farinosus* were influenced by the carbohydrate type and concentration in solid agar media (Hallsworth and Magan 1994).

Bio-manufacturers of *M. anisopliae* or any other fungal biocontrol agent must take into account several factors when developing inexpensive media for the mass production of these agents. Culture media must not only maximise spore yield but also enhance qualities such as desiccation tolerance, stability as a dry preparation and virulence. Much attention has focused on manipulating nutritional conditions during growth and sporulation towards accumulation of appropriate endogenous reserves so that the newly formed conidia possessed the above qualities (Wraight *et al.*, 2001). Our study has helped identify specific quality control parameters that could help in the development of inexpensive media for the mass production of virulent inoculum. However, further work is needed to determine the relationship between endogenous reserves and the desired attributes of virulence and prolonged shelf life

Chapter 4: Development of assays to quantify adhesion forces of *M. anisopliae* conidia and their application to study the effect of nutrition on the adhesion properties of *M. anisopliae* conidia

4.1 Introduction

For all pathogenic fungi (human, plant, invertebrate), adhesion of propagules (conidia, blastospores or hyphae) to the host surface constitutes the first and probably most important step in the infection process. Failure to adhere is one of the attributes of attenuated or weak pathogens (Alter *et al.*, 1999). Adhesion is a two-step process; (1) the initial, passive event usually involves preformed factors and (2) the active, consolidation phase usually involves secretion of mucilage and enzymes (Fargues, 1984; St. Leger *et al.*, 1991b).

Conidia of many species of entomopathogenic fungi are thought to initially attach nonspecifically (Boucias and Pendland 1991). The specific mechanisms involved in spore attachment to host cuticle is not known, however, certain attributes of conidia are linked with their attachment to insect cuticle. These include electrostatic charges, lectins, surface carbohydrates and hydrophobins. (Fargues, 1984, Boucias *et al.*, 1988; Jeffes *et al.*, 1999; Ibrahim *et al* 2002). The hydrophobicity and electrostatic forces of fungal spore and bacteria have been shown to vary in different isolates of same species and physiological state of the spore or bacteria (Grasso *et al* 1996; Giradin *et al* 1999; Pascual *et al.* 2000). Recently, Ibrahim *et al* (2002) reported that nutrition during conidia production of the *M. anisopliae* influence their adhesion properties by altering surface carbohydrates. However, very little is known about the role of nutrition on the hydrophobicity, electrostatic forces and in particular the adhesion strength of the fungal conidia.

A major reason for this lack of information could be the unavailability of tools/methods to quantify adhesion forces. Most previous studies on adhesion forces have focussed on qualitative differences in adhesion with no information on the quantification of these forces. In the past, Radial Flow Chamber (RFC) assays have been used to quantify the adhesion of bacterial cells to different surfaces of varying physical and chemical properties (Tegoulia *et. al.*, 2002). However, the assays require optimisation for the each test organism due to the variation in physio-chemical properties of each organism. The most important consideration is to select the surface chemistry simulating the natural adhesion site of the test organism. The insect cuticular lipids were observed to play an important role in *M. anisopliae* adhesion (Sosa-Gomez *et al.*, 1997). The extraction and immobilisation of insect cuticular lipids on to the glass surface of the RFC could, therefore, provide an opportunity to quantify the adhesion of *M. anisopliae* conidia. Secondly, each organism varies in its morphology thus offering variable surface area which comes in contact with flow of liquid in the RFC. The same flow rate applied to different surface area would result in shear strength proportional to the surface area. Therefore, it is important to determine the flow rate proportional to the size of the test organism.

The present study was designed to address two important issues, firstly to optimise RFC assays for the measurement of adhesion forces of *M. anisopliae* conidia, together with measurement of electrostatic and hydrophobic forces contributing toward adhesion. Secondly, to see how the nutrition during the conidia production influences adhesion forces. The study provided optimised methods for the quantification of *M. anisopliae* adhesion forces and the role of nutrition in altering these forces has been discussed.

4.2 Materials and Methods

4.2.1 Background of Radial Flow Chamber (RFC)

The Radial Flow chamber (RFC) provides a method of assessing adhesion by generating a shear force gradient across the collector surface in question (Tegoulia *et al.* 2002). The geometry of RFC provides a well-defined laminar flow field. The RFC consists of two parallel discs (stainless steel disc and glass disc) which are separated at narrow space (1mm) to ensure high flow pressure.

The use of RFC in conjunction with a travelling microscope allows the observation of attachment in real time at various distances. The hydrodynamics in the RFC are well defined and shear rate varies inversely with radial distance in the chamber (Tegoulia *et al.* 2002). The shear rate at the surface is defined as

$$S = 3Q/\pi rh^2 \qquad \text{Eq. 4.1}$$

Where S is the shear rate at the surface of the disc, Q is the volumetric flow rate, r the radial distance and h the gap width.

4.2.2 The design of radial flow chamber

The RFC used in this study was originally designed to assess biofilm formation (Fowler and Mackay, 1980). The chamber consisted of a central inlet pipe of internal diameter of 5mm. The manifold outside radius of the chamber was 85mm, and inside radius of the collector disc was 50mm. The collector disc itself was 60mm radius. Three spacers separated the parallel plates by 1mm at 120° intervals. The outlet pipes were also equally spaced around the circumference of the chamber. Each outlet pipe had an external diameter of 10mm. The initial design is shown below in Figures 4.1 and 4.2.

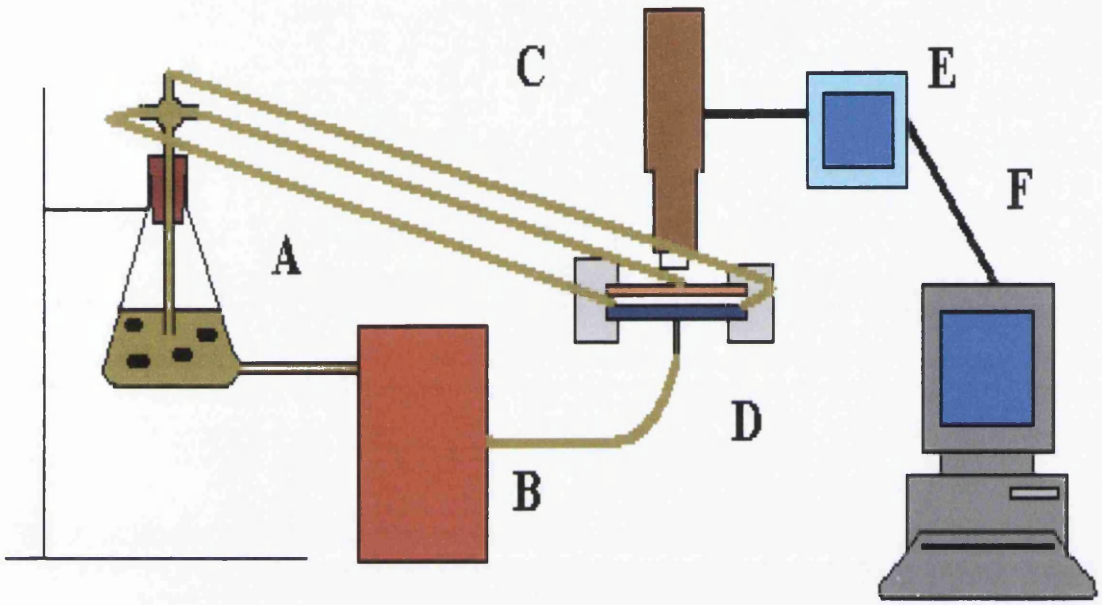


Fig. 4.1 A diagrammatical representation of the Radial Flow Chamber and supporting apparatus, A. reservoir, B. Pump, C. Camera attached to microscope, D. RFC surfaces, E. TV monitor, F. Computer for image analysis

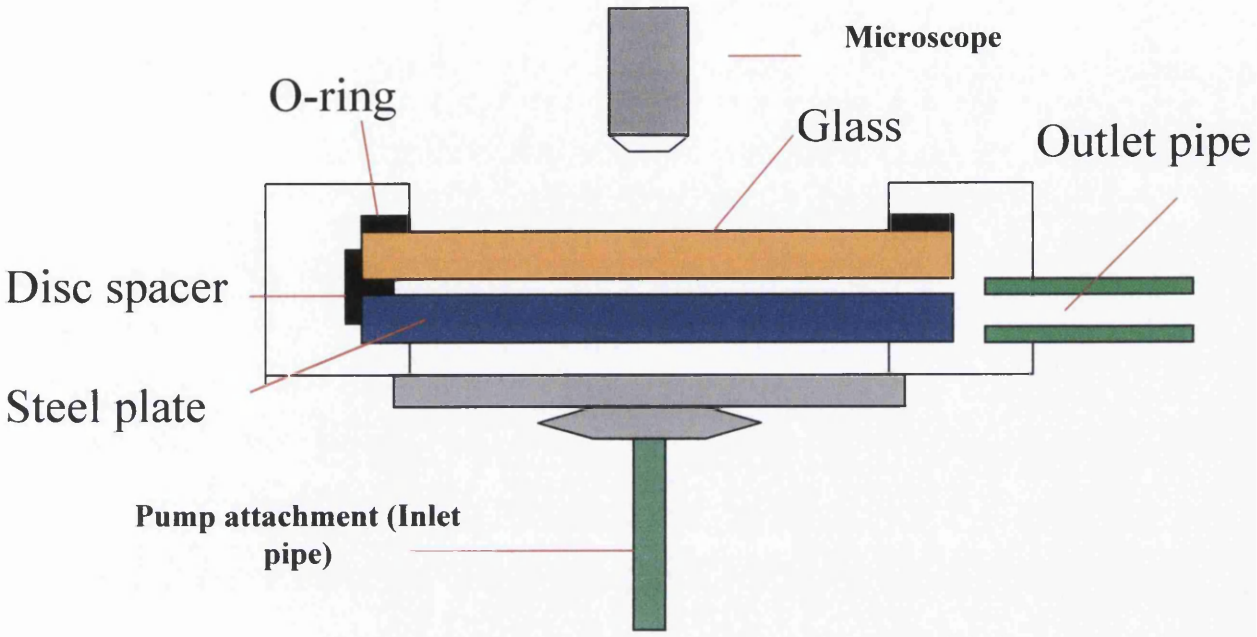


Fig. 4.2 Schematic representations of Radial Flow Chamber's collector plates, Conidial attachment was assessed simultaneously on both glass and steel

4.2.2.1 Pump

The pump (model 5003U) used in conjunction with the RFC was supplied by Michael Smith Engineers (Surrey, U.K.). The maximum flow rate generated by the pump was 10 l/min. The flow rate was adjusted to 0.2 l/min. by using a clamp to restrict the flow and was kept constant throughout experiments. The pump was linked to the chamber by silicone tubing as shown in figure 4.2. The spore suspension was pumped through the chamber and returned to the pump through three outlet pipes via a reservoir. The reservoir consisted of a 250 ml Pyrex flask with a side arm at its base. The reservoir performed two functions. Firstly it enabled easy handling of the suspension within the apparatus. The second function was that the volume of the suspension ensured that concentration would not vary should large level of adhesion occur.

4.2.2.2 Collector Surface

Both glass and stainless steel were used in conjunction with the RFC. This allowed the comparison of results generated from two different surfaces i.e. non-specific stainless steel surface and cuticular lipid coated glass surface. The benefit of the RFC was that it enabled the adhesion to glass and steel to be assessed simultaneously in real time.

4.2.2.3 Glass surface

The glass disc used in the original design was 8mm thick. This proved to be far too thick for microscopic inspection of both surfaces due to the limited focal length of the microscopes objectives. The apparatus therefore required adjustment to minimise the thickness of glass to 2mm.

The stainless steel surface was used as provided by Swansea Precision Engineering. No alterations were required, except polishing with abrasive.

4.2.3 Experimental procedure to study the adhesion of *M. anisoplaie* using RFC

4.2.3.1 Adjustment of flow rate

As the RFC is mostly used to study the adhesion of spherical shaped bacterial organisms, the same flow rate described in earlier studies proved to be too high for rod shaped and relatively bigger *M. anisopliae* conidia. A range of flow rates (from 0.2 to 1.4 l/min) were evaluated to determine an optimum flow rate, which could allow reproducible assays with *M. anisopliae* conidia.

4.2.3.2 Extraction of cuticular lipids and coating of the glass surface with lipid extracts

To mimic the adhesion surface with that of insect cuticle, glass surfaces were coated with cuticular lipid extracts from *T. molitor* larvae. Cuticular lipids were extracted as described by Sosa-Gomez *et al.*, (1997), briefly, the cuticles of 4-5th instar *T. molitor* larvae were flushed with Hexane (Sigma) at room temperature for 5 minutes. The hexane extract was then concentrated under a N_2 stream. Paste like lipid extract was then dissolved in hexane at a concentration of 10 mg/ml and stored at –20 °C until required. To coat the glass surface, a Langmuir – Blodgett Film technique was used (Peterson, 1990). Briefly, One ml of lipid extract was applied at the surface of water in a tank. Glass surface was already embedded in this system at an angle of 45 degrees. The glass surface was then slowly pulled out from the system, resulting in uniform distribution of hydrophobic lipid onto the glass surface. This method of coating has been assessed using atomic force microscopy to confirm complete coverage of the disc (Wright C.J., unpublished observations).

4.2.3.3 Video observations of adhesion

The RFC was mounted on the motorized stage of a Leitz wetzlar (Germany) travelling microscope. The microscope and accompanying video camera ((Panasonic (UK) TV camera, WV-1550/B), colour monitor (JVC (UK) video monitor, TM-123) comprised an automated video microscopy. Video microscopy allowed direct observation of the attachment of individual cells to the surface in real time under a

600x magnification. Attachment of individual cells was observed at ten different radii (0-35 mm from centre) and ten different positions within each radius.

4.2.3.4 *M. anisopliae* injection into RFC

M. anisopliae conidia obtained from different media were suspended in 0.03% aq. Tween 80 (Fisher Scientific, U.K.) to the final concentration of $1x10^9$ conidia/ml as described in section 2.2.3. The reservoir of the RFC contained 95 ml of deionised water. Conidial suspension (5 ml per treatment) was then introduced into the reservoir. The suspension was allowed to run at a flow rate of 0.2 l/min into the RFC for 15 minutes prior to adhesion observations. These conditions were kept constant for the study of all treatments.

4.2.4 Measurement of zeta potential (surface charge)

Surface charge of the conidia produced on different media was determined as described in section 2.2.6

4.2.5 Measurement of surface hydrophobicity

The relative hydrophobicity of the conidia produced on different media was assessed as described in section 2.2.7.

4.3 Results

4.3.1 Effect of flow rate on the adhesion of *M. anisopliae*

Adhesion of *M. anisopliae* conidia was inversely proportional to the flow rate. At higher flow rates i.e. 1.4 l/min. to 0.6l/min., no adhesion was observed either on stainless steel or cuticular lipid coated glass surface (Fig. 4.3). At slow flow rates of 0.3 to 0.5 l/min, adhesion of *M. anisopliae* conidia gradually increased but adhesion was inconsistent among the replicate experiments (Fig. 4.3). Consistent and

considerable higher adhesion was observed on 0.2 l/min flow rate, which was then used for all other experiments (Fig. 4.3).

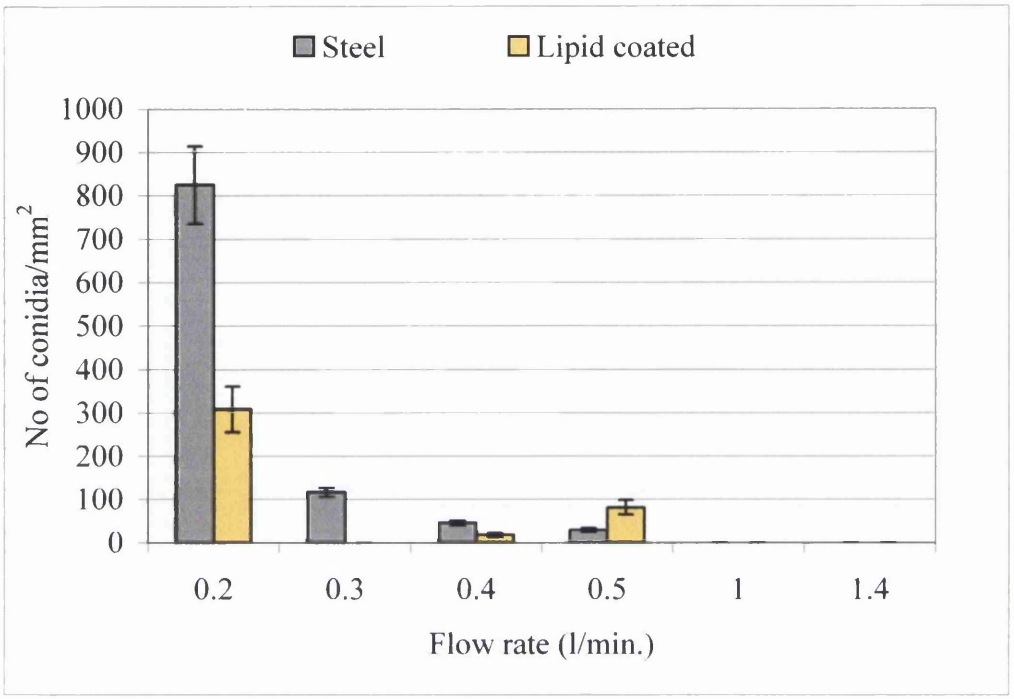


Fig. 4.3 Effect of flow rate on the adhesion on *M. anisopliae* V275 conidia produced on CN 35: 1 media to the stain less steel and cuticular lipid coated glass surface.

4.3.2 Effect of cuticular lipid coating on the adhesion of *M. anisopliae* on to glass surface

Immobilisation of cuticular lipids on the glass surface significantly increased *M. anisopliae* adhesion. In the absence of cuticular lipid coating, very low adhesion was observed on the glass surface even at a very slow flow rate of 0.2 l/min. After lipid immobilization, adhesion of *M. anisopliae* conidia increased 10 fold on the glass surface (fig. 4.4). Adhesion to glass surfaces was independent of adhesion to steel surface i.e. whether glass surface had low or high adhesion; it did not influenced adhesion to steel surface (Fig. 4.4).

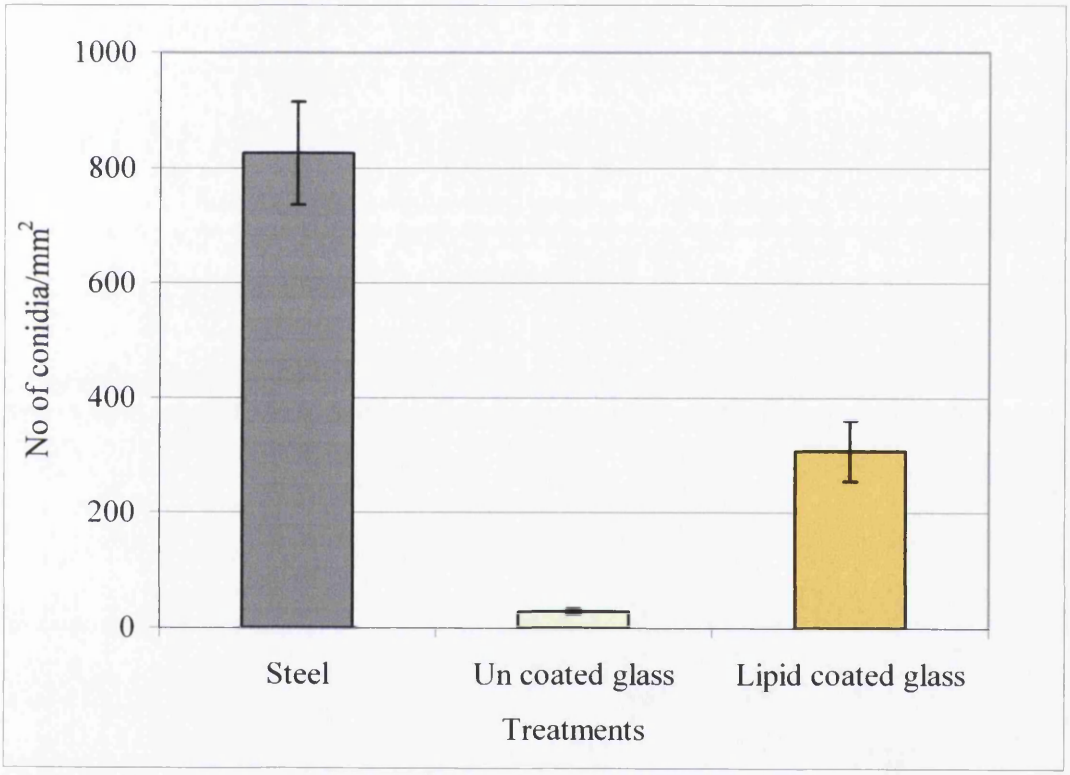


Fig. 4.4 Effect of cuticular lipid coating on glass surface on the adhesion on *M. anisopliae* V275 conidia produced on CN 35: 1 media conidia under the flow rate of 0.2 l/min

4.3.3 Adhesion of *M. anisopliae* conidia produced on different media

Adhesion of *M. anisopliae* conidia varied significantly among the treatments as well as between the two surfaces. Irrespective of the treatment, adhesion was relatively higher on stainless steel surface than that of cuticular lipid coated glass surface. On the stainless steel surface, V245 conidia grown on 1% yeast extract and 2% peptone media were most adhesive. Remaining treatments had no major differences in their adhesion to stainless steel surface (Fig.4.5). On the cuticular lipid coated glass surface, conidia of V245, conidia grown on 1% yeast extract and 2 % peptone media were most adhesive while the conidia produced on KCl, CN 35:1 media and PDA were least adhesive (Fig. 4.5).

On Stainless steel surface adhesion of V275 conidia produced on 1% yeast extract, CN of 10:1 and 75:1 were significantly higher than the conidia produced on the rest of the media (Fig. 4.6). Conidia produced on KCl were the least adhesive while the rest had intermediate adhesion on stainless steel surface. On cuticular lipid coated glass surface, no major differences in adhesion were observed between the V275 conidia produced on CN 35:1 media, PDA, 1% yeast extract and CN 75:1 media. However, adhesion was significantly lower for the conidia produced on KCl, 2% peptone and CN 10:1 media than the conidia produced on media described above (Fig. 4.6).

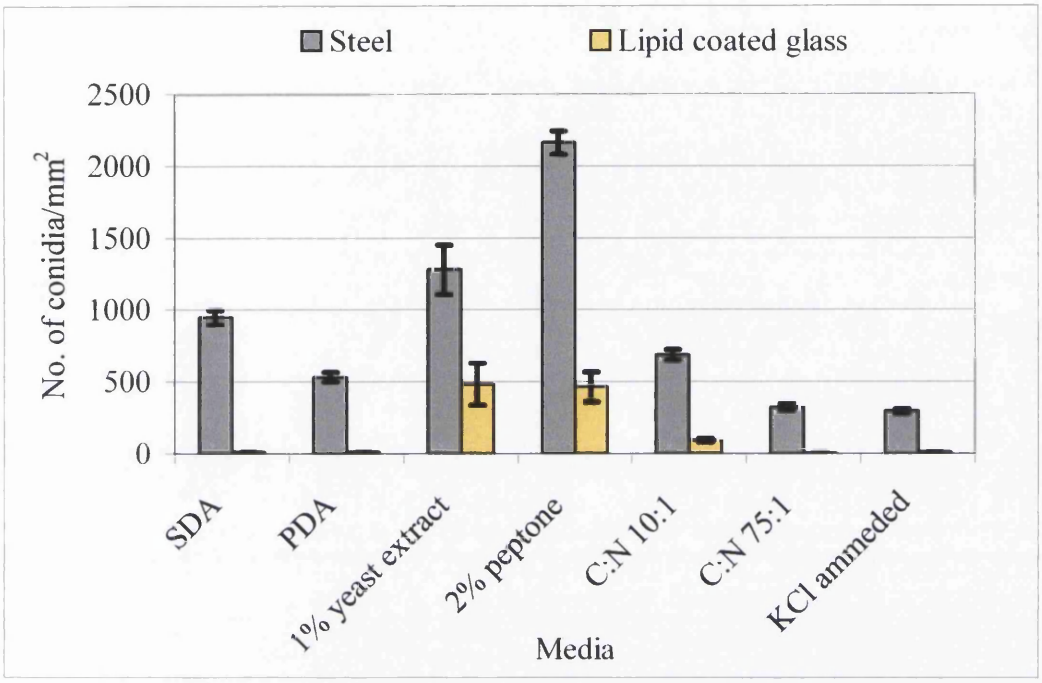


Fig. 4.5 Influence of nutrition on the adhesion of *M. anisopliae* V245 conidia to stainless steel and cuticular lipid coated glass surface under a flow rate of 0.2l/min.

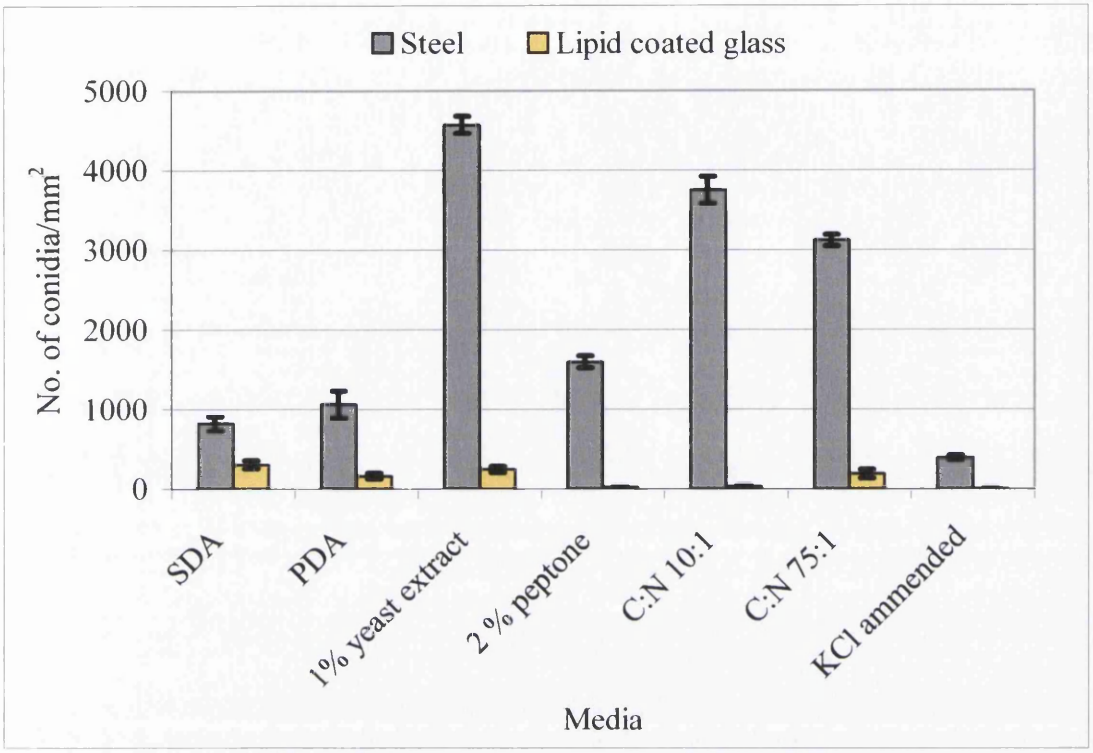


Fig. 4.6 Influence of nutrition on the adhesion of *M. anisopliae* V275 conidia to stainless steel and cuticular lipid coated glass surface under a flow rate of 0.2l/min.

4.3.4 Influence of nutrition on the zeta potential of the *M. anisopliae* conidia

Zeta potential varied significantly ($P < 0.05$, Tukey Test) among the different treatments. The conidia of V245 produced on intermediate and low CN media had the least zeta potential than rest of the treatments (Table 4.1). These two treatments varied significantly ($P < 0.05$, Tukey Test) with rest of the treatments. Highest Zeta potential was observed for the conidia produced on 2% peptone or 1% yeast extract media.

In the case of V275, the conidia produced on 1% yeast extract and 2% peptone had the least zeta potential. The conidia from different CN media varied significantly ($P < 0.05$, Tukey Test) and an increase in CN of the media resulted in a gradual increase in zeta potential of the conidia produced on these media. The conidia produced on PDA and KCl had the highest zeta potential than all other treatments except for the conidia produced on high CN media (Table 4.1).

Table 4.1 Influence of nutrition on the zeta potential of *M. anisopliae* conidia. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated three times. Values in parenthesis represent standard errors.

Media	Zeta potential (mV^2)	
	V245	**V275**
PDA	-31.72^{b} (0.48)	-34.62^{f} (0.32)
1% YE	-36.74^{e} (0.27)	-23.84^{a} (0.11)
2% Pep	-37.01^{e} (0.16)	-27.41^{b} (0.21)
CN 10:1	-28.01^{a} (0.19)	-29.41^{c} (0.19)
CN 35:1	-28.01^{a} (0.19)	-31.67^{d} (0.40)
CN 75:1	-35.30^{d} (0.21)	-36.77^{g} (0.31)
KCl	-34.0^{c} (0.20)	-33.26^{e} (0.26)

4.3.5 Influence of nutrition on the relative hydrophobicity of *M. anisopliae* conidia

Relative hydrophobicity of the conidia produced on different media also varied significantly among the treatments (Table 4.2). In the case of V245, inoculum produced on either low or high CN media were most hydrophobic. These conidia varied non-significantly ($P < 0.05$, Tukey Test) with the conidia produced on CN 35:1 media or PDA but varied significantly with the least hydrophobic conidia produced on either KCl or 2 % peptone media (Table 4.2).

In the case of V275 highly hydrophobic conidia were produced on 1% yeast extract, CN 75:1, 2% Peptone and CN 35:1 (Table 4.2). Conidia produced on either PDA, KCl or low CN of 10:1 were significantly ($P < 0.05$, Tukey Test) less hydrophobic.

Table 4.2 Influence of nutrition on the relative hydrophobicity of *M. anisopliae* conidia. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated three times. Values in parenthesis represent standard errors.

Media	Relative hydrophobicity (%)	
	V245	**V275**
PDA	89.42^{ab} (0.44)	74.99^{b} (1.60)
1% Yeast extract	80.17^{bc} (2.12)	86.18^{a} (0.99)
2% peptone	75.63^{c} (4.21)	84.26^{a} (0.46)
CN 10:1	91.35^{a} (0.94)	75.98^{b} (1.78)
CN 35:1	83.48^{abc} (1.12)	81.32^{ab} (1.49)
CN 75:1	92.23^{a} (1.44)	83.77^{a} (1.01)
KCl	78.74^{c} (1.96)	74.90^{b} (2.30)

4.4 Discussion

The Radial Flow Chamber Assay proved to be an effective method for quantifying adhesive properties of *M. anisopliae* conidia. It also facilitated comparative study of adhesion to the cuticular lipid coated and inert surfaces simultaneously. Earlier uses of RFC had focussed on bacterial adhesion (Tegoulia *et al.*, 2002); consequently the

existing protocols did not work for *M. anisopliae*. In the RFC, the shear force is generated by the flow rate, which is a function of its speed as well the surface with which it strikes. The rod shaped *M. anisopliae* conidia provided too large a surface area, thereby, the minimum flow rate used for bacterial cells proved to be far too high for *M. anisopliae* conidia. Similarly, the smooth and hydrophilic surface of glass did not provide any physical or chemical cues for *M. anisopliae* adhesion; however, its immobilization with cuticular lipids not only simulated the real adhesion environment (biochemical cues) but also provided specific hydrophobic surface for adhesion.

The adhesion of fungal spores to a surface is a function of both the surface in question and the spores. Our study indicated that *M. anisopliae* could attach to both specific and non-specific surfaces. Similar studies on fungal adhesion but using static conditions, (Sosa-Gomez *et al.*, 1997: Hajek and Eastburn 2003) demonstrated that both surface topography and chemistry influence the attachment of *M. anisopliae* and *Entomophaga maimaiga* conidia to the surface in question.

Spore adhesion to the host cuticle is thought to be an important indicator of virulence. In our study the more aggressive strain V275 showed higher adhesion than V245. The adhesion profile suggested that though each isolate responds differently to the same nutritional environment, however, certain similarities were consistently observed. For example nutrient poor media e.g. 1% yeast extract appeared to positively effect the adhesion of both isolates, while an excess of carbon and salt negatively affected the overall adhesion. Nutrition has also been shown to affect the surface carbohydrates of *M. anisopliae* conidia, which in turn could alter the adhesion properties (Jeffs *et al.* 1999, Ibrahim *et al.* 2002.).

The aerial conidia of *M. anisopliae* exhibit a distinct rodlet layer, which is highly hydrophobic (Bociaus *et al.*, 1988). This rod like layers imparts hydrophobicity to the

conidia. The hydrophobicity of conidia of other fungi has been shown to vary due growth media, cell starvation and growth phases (Doyle 2000 and references therein). According to Wessels (1997), the hydrophobins are deposited on the walls of aerial structures like conidia. The nature and number of hydrophobins deposited at the surface of conidia could therefore, depend on the nutritional level and/or composition of nutrition. Relatively high hydrophobicity observed in complex media like yeast extract and peptone based media suggest the deposition of more hydrophobic amino acids on the conidia surface.

The dry conidia of *M. anisopliae* are generally hydrophobic and possess a net negative charge on its surface (Boucias and Pendland, 1991). The zeta potential assays helped to determine the electrostatic force of *M. anisopliae* conidia. This is the first time that electrostatic forces of any entomopathogenic fungi have been quantified. Previous studies only describe the qualitative difference in the electrostatic forces but here it was shown that even the conidia produced under different growth condition could vary significantly for their electrostatic surface charge. On the basis of this study, it is not possible to describe the mechanism involved in altering the surface charges of conidia. However, it may be suggested that changes in surface properties of conidia on different type of nutrients (Jeff *et al.*, 1999; Ibrahim *et al.*, 2002) may have some role in this phenomenon.

Apart from hydrophobins, other molecules e.g. glycoproteins, lipids are also present on conidial surface (Cole *et al.*, 1979; Latge *et al.*, 1988; Tronchin *et al.*, 1997). Different biological molecules e.g. lipids, proteins, carbohydrates exhibit varying levels of surface charge. Differences in nutrition during conidia production may alter the composition and or nature of these compounds, thereby, in directly influencing the surface charge. Both strains were observed to vary in their surface charge on complex media based on yeast extract and peptone suggesting a strain specific response to complex nutrition. Such

strains specific response may have some role in altering the conidial surface, however further studies are needed to understand this mechanism.

Surface of the conidia exhibit a net negative charge but our results indicate that the growth medium on which conidia are produced influence this charge quantitatively. A higher negative charge on the surface of conidia would mean it would be repelled rather than attracted toward the negatively charged insect cuticle. On the other hand conidia with less negative value would be overridden by attractive forces. These forces come into play when conidia and cuticle are not in physical contact but are in the region of an electric field where they either repel or attract each other. Results of our studies confirmed this hypothesis, as relatively less negatively charged conidia e.g. conidia produced on 1% yeast extract media were more adhesive. However, in other cases, this effect was not very clear suggesting that electrostatic forces alone cannot exert a decisive role on adhesion but facilitates the adhesion process.

The adhesion mechanism appeared to be quite complex and involves numbers of factors e.g. hydrophobic forces, electrostatic forces, surface carbohydrates etc. The results of this study do suggest the nutritional conditions could be optimized to manipulate the adhesion properties of conidia. Such manipulations could prove very important in situations where pest is likely to have low exposure to *M. anisopliae* application, thereby, efficacy would rely mainly on good adhesion as mortaility is dose related (Butt *et al.* 2002)

Chapter 5: Influence of nutrition on the production and physiology of sectors produced by *M. anisopliae*

5.1 Introduction

Formation of distinct morphological variants or sectors are often observed in fungal cultures maintained on artificial media (Jennings and Lysek, 1996; Vannacci and Cristani, 1998). The type and frequency of sectoring varies among different fungal species and strains and has often been attributed to mutation, transposons, double-stranded RNA mycoviruses and genomic rearrangements (Becker *et al.*, 2003; Firon *et al.*, 2002; Chu *et al.*, 2002, Fowler and Mitton, 2000; Pontecorvo and Gemmell, 1944). Some studies suggest that sectors arise as a result of cultural degeneration caused either by the age of culture, method of propagation, or nature of the culture medium (Booth, 1971; 1975). Sectors can differ from the parent culture in a range of morphological and physiological characteristics including a decline in the production of spores and certain metabolites (Chu *et al.*, 2002; Guzman-de-pena and Jose Ruiz, 1997). Some plant pathogenic fungi, especially those containing mycoviruses, can decline in virulence (Chu *et al.*, 2002; Chen *et al.*, 1996).

There is much interest in the development of insect-pathogenic fungi as environmentally friendly alternatives to chemical insecticides for pest control (Butt, 2002; Inglis *et al.*, 2001). Formation of sectors in cultures of these biocontrol agents (BCAs) could have serious commercial implications. For example, avirulent inoculum would dilute the efficacy of the harvested inoculum making the product less efficacious. Sterile sectors would reduce spore yield thus raising production costs and making the BCA less cost-effective compared with competing agents. Knowledge of the factors triggering sector formation could help in developing strategies to prevent them forming. This study focuses on identifying the cultural conditions favouring sector formation in the insect pathogen *Metarhizium anisopliae* and the analysis of the physiological attributes of the

sectors in particular the production of Pr1 and destruxins, two important pathogenicity determinants (St. Leger *et al* 1994; Kershaw *et al.* 1999; Amiri *et al*, 2000; Wang *et al.*, 2002).

5.2 Materials and Methods

5.2.1 Influence of culture conditions on sector formation

Two isolates of *Metarhizium anisopliae* V245 and V275 from Finnish soil and *Cydia pomonella,* respectively were passaged through *Galleria melonella* larvae and isolated on Oatmeal dodine agar. Single spore colonies were transferred to Sabouraud Dextrose Agar (SDA) and these cultures were used in subsequent studies.

The effect of nutrition on sector formation was investigated using seven different media as described in section 3.2.2 and shown in table 5.1. Each treatment was replicated 10 times and the whole experiment was repeated four times. Each plate was inoculated with 2mm diameter mycelial plugs taken from the edge of the colony. Cultures were maintained at 25°C and after 15 days of growth, the frequency and morphology of sectors were recorded. Each sector was subsequently subcultured by transferring a 2mm plug as described above to fresh SDA. After 15 days incubation at 25°C, sector morphology was recorded and those sectors that did not revert back to the parent phenotype were kept at 4°C until required. Twenty-two sectors (from >150) representing the different morphological groups, media and isolates were selected for further studies (Table 5.2).

5.2.2 Production of Pr1 and other enzymes by sectors

Parents and sectors (Table 5.2) were grown in Sabouraud dextrose broth for 3 days and washed with sterilized distilled water then 1 g wet weight of mycelium was used to inoculate 100 ml inductive medium (1% oven-dried cockroach *Blaberus discoidalis*

homogenate in minimum medium) which is known to induce Pr1 and several other cuticle-degrading enzymes (Wang *et al.*, 2002). Cultures were incubated in a GallenKamp orbital incubator (Sanyo) at 24°C and 120 rpm. Culture filtrates were collected 3 days post inoculation and assayed spectrophotometrically for Pr1, Pr2, exochitinase and non –specific protease activity according to St. Leger *et al.*, (1987) and Segers *et al.*, (1995), respectively.

For selected sectors, an additional set of nineteen different enzymes was investigated using the semi-quantitative API- ZYM test (BioMerieux Vitek Inc., France). Briefly, 65 µl of culture filtrate was applied to each well and incubated at 37 °C for 4 hr and the reactions scored as advised by the manufacturer.

5.2.3 RT-PCR analysis of cuticle-degrading enzymes in sector mycelium

Quantitative Real Time PCR was used to compare the expression of Pr1 and three other genes (esterases, trypsin, chymotrypsin) linked to the infection process in the mycelium harvested from the above cultures. Total RNA was extracted from the mycelium of sectors and parent cultures using the Qiagen RNAeasy kit (Valencia, CA). Rest of the procedure was same as described in section 3.2.11.

5.2.4 Production of destruxins

Three-day old mycelium (2.5g wet weight) of parent cultures and selected sectors produced in Sabouraud dextrose broth was used to inoculate 250ml Czapek dox broth (Sucrose, 3%, sodium nitrate, 0.2%, K_2HPO_4, 0.1%, $MgSO_4$, 0.05% KCl 0.05% and $FeSO_4$, 0.001%) supplemented with 0.5% peptone in 500 ml conical flasks. The flasks were incubated in an orbital shaker (120 rpm) at 24 °C for 7 days. Destruxins were extracted from culture filtrates using dichloromethane: ethyle acetate (1:1) and analysed by HPLC as described in section 2.2.12.

5.2.5 Statistical analysis

The whole study was repeated twice with each treatment replicated three times unless stated otherwise. Data was subjected to one-way ANOVA followed by Duncan test for determining significant differences. For all statistical analysis SPSS 11 software was used.

5.3 Results

5.3.1 Influence of nutrition on sector production

The sectoring frequency of *M. anisopliae* varied significantly between the isolates V245 and V275 ($P < 0.05$, Duncan test) with the former producing more sectors on all the media investigated (Table 1). Nutrition did influence sector formation, with the least sectors being produced on media with either low nutrients or low CN ratios (Table 5.1). Media containing >4% glucose yielded significantly ($P < 0.05$, Duncan test) more sectors for both isolates (Table 5.1). Sectors were either sterile or sporulated poorly. Rarely did sectors sporulate as profusely as the original parent cultures. Conidia, if present, were usually paler than those of the parent (Table 2). The sectors varied in shape and size (Figs. 5.1-5.6). Most sectors were a variation of a V-shape but occasionally other shapes were noted such as a square (Fig. 5.1). Whereas some sectors arose close to the culture periphery others appeared to arise close to the point of inoculation (i.e. near centre).

Table 5.1 Frequency of sector formation by V245 and V275 on different media. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Duncan Test). Each treatment was replicated ten times. Values in parenthesis represent standard errors.

Culture Media	**% Frequency of sector formation**	
	V245	V275
PDA	60^{a} (9.1)	22^{abcde} (4.7)
1 % Yeast extract	30^{bc} (4.0)	10^{cde} (4.0)
CN 8:1 (2 % Peptone)	37^{b} (4.7)	05^{e} (2.8)
CN 10: 1 (0.6% glucose, 1% peptone)	32^{bc} (6.2)	07^{de} (4.7)
CN 35:1 (4 % glucose, 1% peptone, corresponds to SDA)	65^{a} (11.9)	12^{bcde} (2.5)
CN 75: 1 (9.1 % glucose, 1% peptone)	62^{a} (9.4)	27^{abcd} (4.7)
KCl-amended medium (8% glucose, 2% peptone, 5.5% KCl, 5.5% agar).	60^{a} (9.1)	15^{bcde} (2.8)

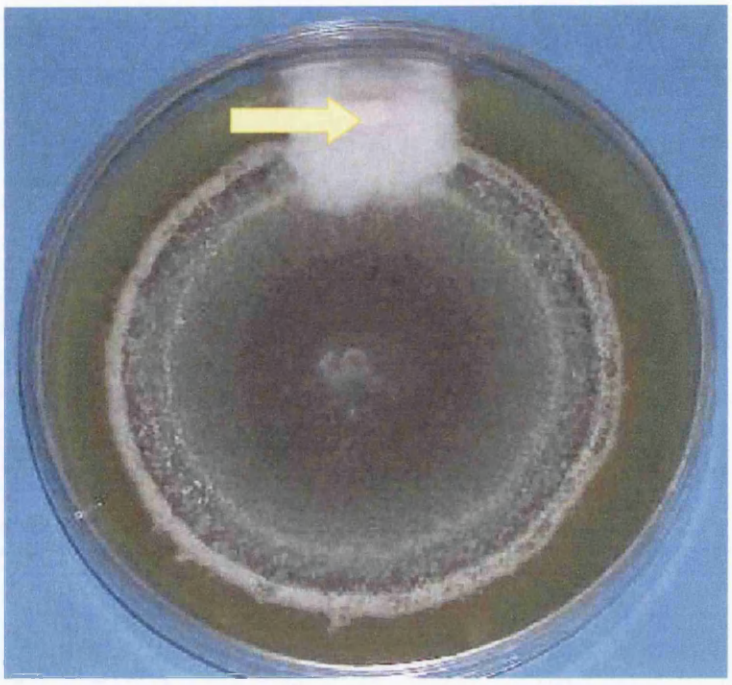


Fig. 5.1 Culture of *M. anisopliae* V 245 on culture medium with CN 75:1. Note square-shaped sector consisting of fluffy, sterile mycelium (arrow). Sector initially arises as typical V shaped but can form any shape later depending on their growth rate.

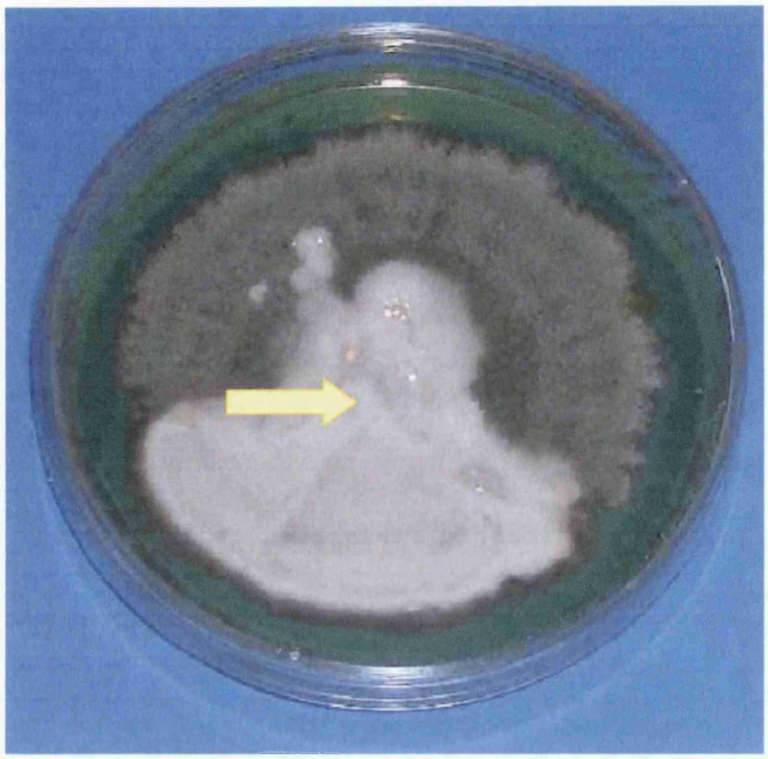


Fig. 5.2. Culture of *M. anisopliae* V 245 on culture medium with CN 35:1. Note sterile mat like mycelium growth of sector growing faster than parent colony.

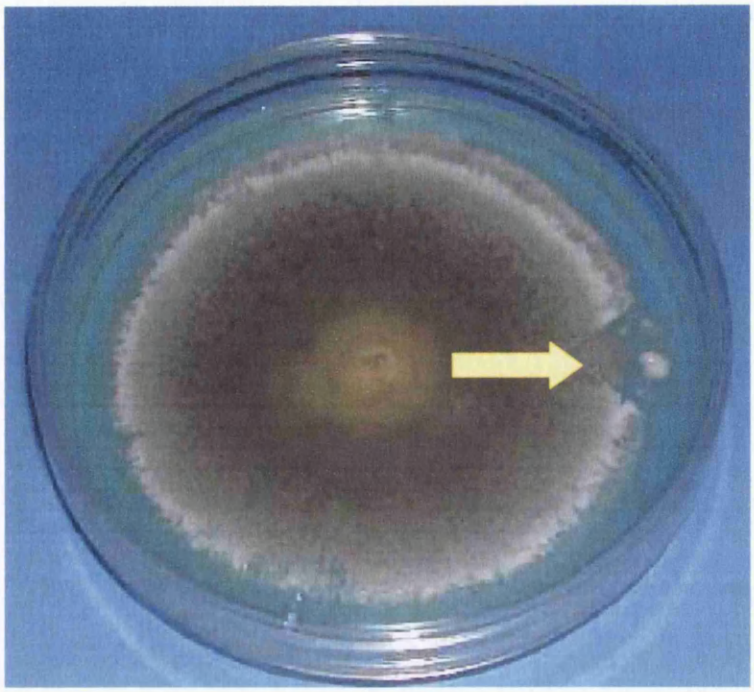


Fig. 5.3. Culture of *M. anisopliae* V245 maintained on culture media containing 1% yeast extract as sole source of nutrition. Note "V-shaped" sector formed at the cultures periphery (arrow).

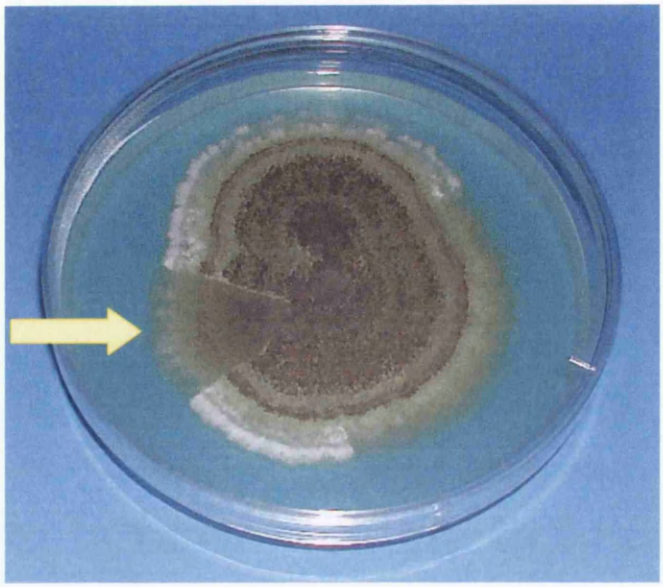


Fig. 5.4. Culture of *M. anisopliae* V 275 on PDA. Note typical V shaped sector with less dense sporulation (arrow) as compared to parent culture.

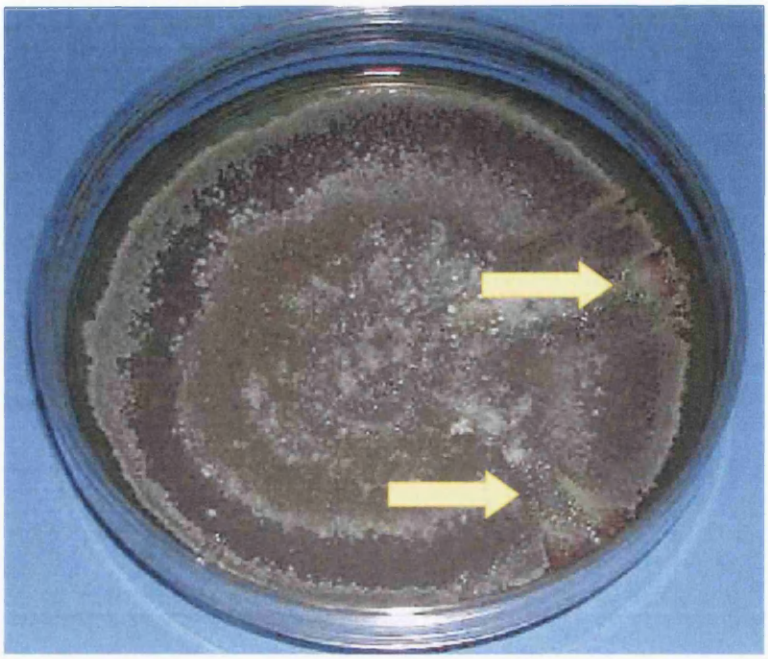


Fig. 5.5. Two sectors formed in a culture of *M. anisopliae* V 275 maintained on 2% peptone. Note the sectors are less pigmented than parent culture.

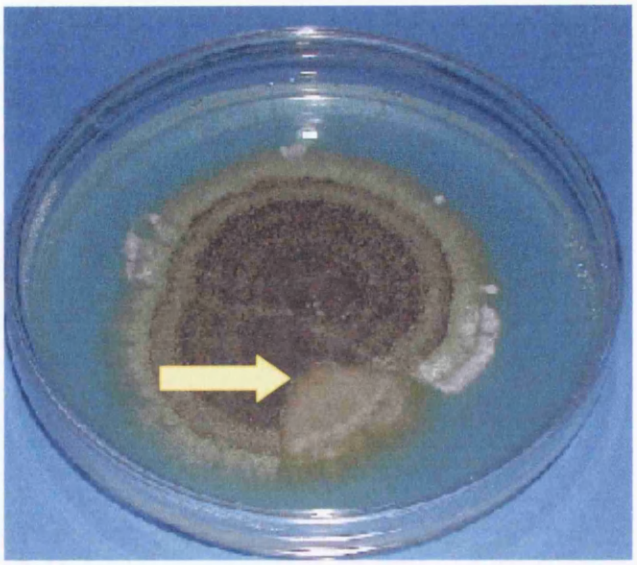


Fig. 5.6. Culture of *M. anisopliae* V 275 on PDA. Note typical V shaped sector having sparse growth and sporulation (arrow) as compared to parent culture.

5.3.2 Production of Pr1 and other enzymes by sectors

Sectors varied significantly ($P < 0.05$, Duncan Test) in the production of Pr1 and other cuticle degrading enzymes in inductive media (Table 5.2). Non-specific protease activity also varied between the two isolates and sectors. Pr1 production was highest in the parent strains of *M. anisopliae* and in sectors 12-22 and lowest in sectors 1-5 (Table 5.2). The remaining sectors had intermediate Pr1 production (Table 5.2). In case of Pr2 and NAG'sae production most of the sectors either had higher Pr2 and NAG'ase production or varied non-significantly ($P < 0.05$, Duncan test) with parental strains (Table 5.2). There appeared to be no obvious link between the production of Pr1, Pr2, NAG'ase and non-specific protease activity, and the isolate, media and sector phenotype (Table 5.2).

The API–ZYM test revealed distinct physiological profiles for the parents and sectors (Table 5.3). Both parent strains had similar enzyme profiles except that V275 generally produced higher quantities than those of V245 in the inductive media. There were some exceptions; V275 produced comparatively less N-acetyl-β-glucosaminidase and no β-galactosidase. No obvious pattern was observed among the sectors. Sectors varied between each other and with the parent strains. However, it was higher α-chymotrypsin activity was noted in sectors S1 and S3 and higher N-acetyl-β-glucosaminidase activity in S7 (V275). Neither parents nor sectors exhibited β-glucuronidase, α-glucosidase and α-fucosidase activity (Table 5.3).

Table 5.2 CDEs profile of sectors and parent cultures. Means within a column followed by the same letter are not significantly different ($P < 0.05$, Duncan Test). Sector ID codes are based on the Pr1 activity with sector 1 showing the least Pr1 activity. Each treatment was replicated two times. Values in parenthesis represent standard errors.

Sector ID code	**Sector Phenotype**	**Parent Isolate**	**Culture Medium**	**Non specific protease activity** (U/ml)	**Pr1 activity** (μ mol/ml/min)	**Pr2 activity** (μ mol/ml/min)	**NAGase activity** (μ mol/ml/hr)
V245	Good sporulation, grey-green conidia	V245	C:N 35:1	0.83^{cdef} (0.0)	2.79^{ab} (0.12)	1.15^{hij} (0.04)	0.19^{mn} (0.0)
V275	Good sporulation, grey-green conidia	V275	C:N 35:1	1.54^{a} (0.05)	2.74^{ab} (0.10)	1.18^{ghij} (0.11)	0.29^{ijk} (0.0)
S 1	Sterile, white mycelium	V245	C:N 35:1	0.26^{h} (0.02)	0.53^{k} (0.11)	1.04^{j} (0.07)	0.35^{fghi} (0.01)
S 2	Sterile, white mycelium	V245	C: N10:1	0.53^{fgh} (0.19)	0.58^{k} (0.07)	1.20^{ghi} (0.07)	0.39^{efg} (0.11)
S 3	Sterile, white mycelium	V275	C:N 35:1	0.98^{bcde} (0.11)	0.77^{ijk} (0.0)	0.66^{l} (0.10)	0.32^{ghij} (0.01)
S 4	Sterile, white mycelium	V245	C:N75:1	0.73^{efg} (0.04)	0.89^{ijk} (0.28)	1.29^{cdefgh} (0.08)	0.38^{efgh} (0.02)
S 5	Mycelial mat, poor sporulation, light	V245	C:N 35:1	0.36^{h}	0.92^{hijk}	1.32^{bcdefg} (0.02)	0.37^{fgh} (0.01)

	green conidia			(0.10)	(0.30)		
S 6	Poor sporulation, light green conidia	V275	PDA	0.93^{bcde} (0.01)	$1.34g^{hij}$ (0.38)	1.38^{bcdef} (0.02)	0.29^{ijk} (0.0)
S 7	Mycelial mat, poor sporulation, light grey-green conidia	V275	PDA	0.78^{defg} (0.09)	1.38^{fghij} (0.55)	1.46^{ab} (0.10)	0.46^{de} (0.01)
S 8	Mycelial mat, poor sporulation, light grey-green conidia	V275	KCl	0.80^{defg} (0.01)	1.51^{efghi} (0.09)	0.80^{k} (0.06)	0.14^{n} (0.0)
S 9	Fluffy mycelial over growth on light green conidia	V245	KCl	1.08^{bcd} (0.02)	1.65^{efgh} (0.47)	1.39^{bcdef} (0.05)	0.56^{bc} (0.02)
S 10	Poor sporulation, light grey-green conidia	V275	PDA	1.10^{bcd} (0.37)	1.91^{defg} (0.15)	1.30^{cdefg} (0.07)	0.61^{ab} (0.04)
S 11	Poor sporulation, light grey-green conidia plus fluffy mycelium	V245	PDA	0.99^{bcde} (0.02)	1.94^{cdefg} (0.23)	1.42^{bcd} (0.08)	0.62^{ab} (0.03)
S 12	Sparse conidia plus sterile mycelium	V245	C:N10:1	1.14^{bc} (0.01)	2.10^{bcdef} (0.28)	1.28^{defghi} (0.03)	0.66^{a} (0.02)
S 13	Poor sporulation, light green conidia	V275	C:N 35:1	0.99^{bcde} (0.02)	2.11^{bcdef} (0.48)	1.27^{efghi} (0.09)	0.23^{klm} (0.01)
S 14	Distinct sporulating sector similar in appearance to parent	V275	C:N75:1	1.07^{bcd} (0.03)	2.15^{bcde} (0.16)	1.24^{fghi} (0.07)	0.31^{hij} (0.02)
S 15	Sterile white mycelium	V245	C:N 35:1	1.03^{bcde} (0.01)	2.17^{bcde} (0.07)	1.17^{ghij} (0.06)	0.61^{ab} (0.02)
S 16	Sterile white mycelium	V275	C:N 35:1	1.00^{bcd}	2.19^{bcde}	1.28^{defghi} (0.03)	0.30^{hijk} (0.01)

				(0.03)	(0.21)		
S 17	Sparse conidia on mat like mycelium plus certain areas of fluffy mycelium	V275	C:N 35:1	1.17^{b} (0.02)	2.22^{abc} (0.20)	1.13^{ij} (0.02)	0.35^{fghi} (0.01)
S 18	Sterile white mycelium	V245	C:N 35:1	1.08^{bcd} (0.02)	2.56^{abcd} (0.08)	1.26^{efghi} (0.06)	0.46^{de} (0.05)
S 19	Sparse conidia similar in colour to parent	V275	KCl	0.95^{bcde} (0.06)	2.61^{abcd} (0.23)	1.28^{defghi} (0.08)	0.33^{ghij} (0.05)
S 20	Fluffy mycelial over growth on light green conidia	V275	C:N 35:1	0.98^{bcde} (0.01)	2.71^{ab} (0.05)	1.41^{bcde} (0.18)	0.49^{cd} (0.12)
S 21	Distinct sporulating sector similar in appearance to parent	V275	C:N 35:1	1.05^{bcd} (0.06)	2.74^{ab} (0.11)	1.43^{bc} (0.05)	0.43^{def} (0.05)
S 22	Distinct sporulating sector similar in appearance to parent	V245	C: N75:1	0.98^{bcde} (0.01)	2.94^{a} (0.02)	1.57^{a} (0.06)	0.61^{ab} (0.02)

Table 5.3. Enzyme activities of parent and sectors as determined by API- ZYM test. Each treatment was replicated two times. Values in parenthesis represent standard errors.

Enzyme assayed	V245	S1	S4	S5	S9	V275	S3	S7	S 13	S 16
Alkaline phosphatase	1	2.5	2.5	1.5	4.5	4	0	3	1	0.5
Esterase (C 4)	2	2	1	2.5	2	3	3	2	2	1.5
Esterase Lipase (C 8)	1	2	2	3.5	2.5	4	3	3	4	3
Lipase (C 14)	1	1	1	1	1	1	1	2	1	0
Leucine arylamidase	4	5	5	5	5	5	5	5	5	5
Valine arylamidase	4	5	5	5	5	5	5	5	5	5
Cystine arylamidase	3	3	2.5	2.5	3	3	4	4	3	4
Trypsin	5	3	5	2.5	5	5	5	5	5	3
α-chymotrypsin	1	3	1	1	1.5	1	5	2	1	5
Acid phosphatase	5	5	5	5	5	5	5	5	5	5
Naphthol -AS-BI-phosphohydrolase	4	5	5	5	5	5	5	5	4	4
α- galactosidase	2	1	1.5	1	1.5	3	0	0	0	0
β - galactosidase	1	1	1.5	0.5	0	0	0	0	0	0
β - glucuronidase	0	0	0	0	0	0	0	0	0	0
α- glucosidase	0	0	0	0	0	0	0	0	0	0
β - glucosidase	3	2.5	3	2.5	2	4	3	2	1	1
N- acetyl – β glucosaminidase	5	3	3.5	3	5	3	2	5	2	1
α- mannosidase	4	0	1.5	0	0.5	3	0	1	0	0
α- fucosidase	0	0	0	0	0	0	0	0	0	0

5.3.3 RT-PCR analysis of cuticle-degrading enzymes in sectors

Quantitative Real Time PCR showed that parent strains expressed more *prl A* than their corresponding sectors (Fig. 5.7). Sectors produced 6 to 100 times less *prl A* than the parent strains (Fig. 5.7). Only S9 (V245) and S13 (V275) varied non-significantly from the parent strains. The esterases (*ste 1*) expression ratio also showed the same pattern as that of *pr 1A*, however, two sectors S9 and S13 that varied non-significantly with their parents for *pr 1A* had significantly ($P < 0.05$, Duncan test) lower *ste 1* expression. In

general 1-2.5 fold decrease in *ste 1*expression was observed among the sectors compared to their parent strains (Fig. 5.8).

Expression of the trypsin (*try 1*) and chymotrypsin (*chy 1*) genes differed in both parent strains and sectors (Figs 5.9-5.10). Up-regulation of *try 1* usually resulted in a decline in *chy 1* and *vice versa* in parent strains and sectors, with the exception of S9 and S13 where the expression of both *try 1* and *chy 1* was high (Figs. 5.9-5.10). There was a tendency for V245 and all its sectors to be inclined towards expression of *chy 1* whereas V275 was biased towards *try 1.* S7 and S13, both derived from V275 expressed more *chy 1* than any other treatment. All V245 derived sectors with the exception of S9 produced up to 15-fold less *chy 1* (Fig. 5.10). Similarly all the sectors produced by V275 with the exception of S13 produced 6 fold less *try 1* (Fig.5.9). Interestingly, S13 expressed more *try 1* than the parent culture.

In general, the parent strains had higher expression for *pr1 A*, *ste 1* and either of *try 1* (V275) or *chy 1* (V245). Sector S1 had least expression for all the genes. Sectors S4, and 5 had intermediate expression for all the genes while sectors S7, S16 and S3 had variable expression for each gene but ranged from intermediate to higher expression. Sectors S9 and S13 except for the *ste 1* expression generally varied non-significantly with parent cultures (Figs 5.7–5.10). There appeared to be no obvious relationship between the expression of any specific enzyme(s) and sector phenotype or culture medium.

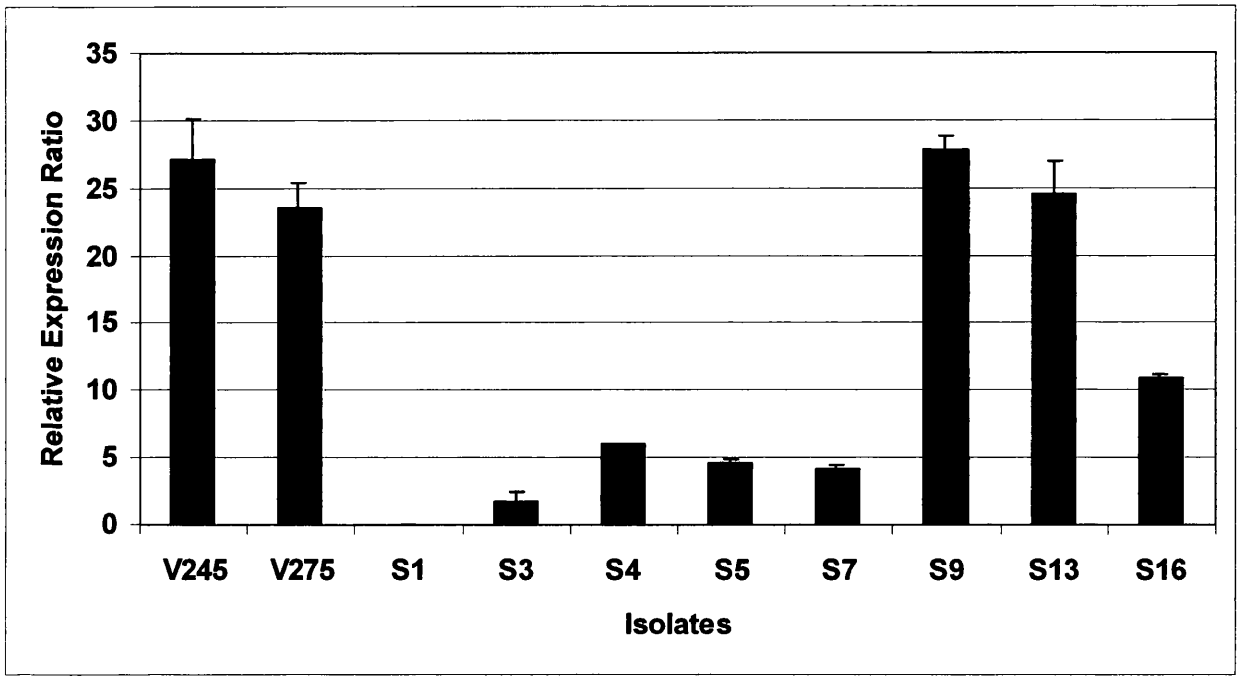


Fig. 5.7 Relative expression of *pr1A* by selected sectors and parent cultures.

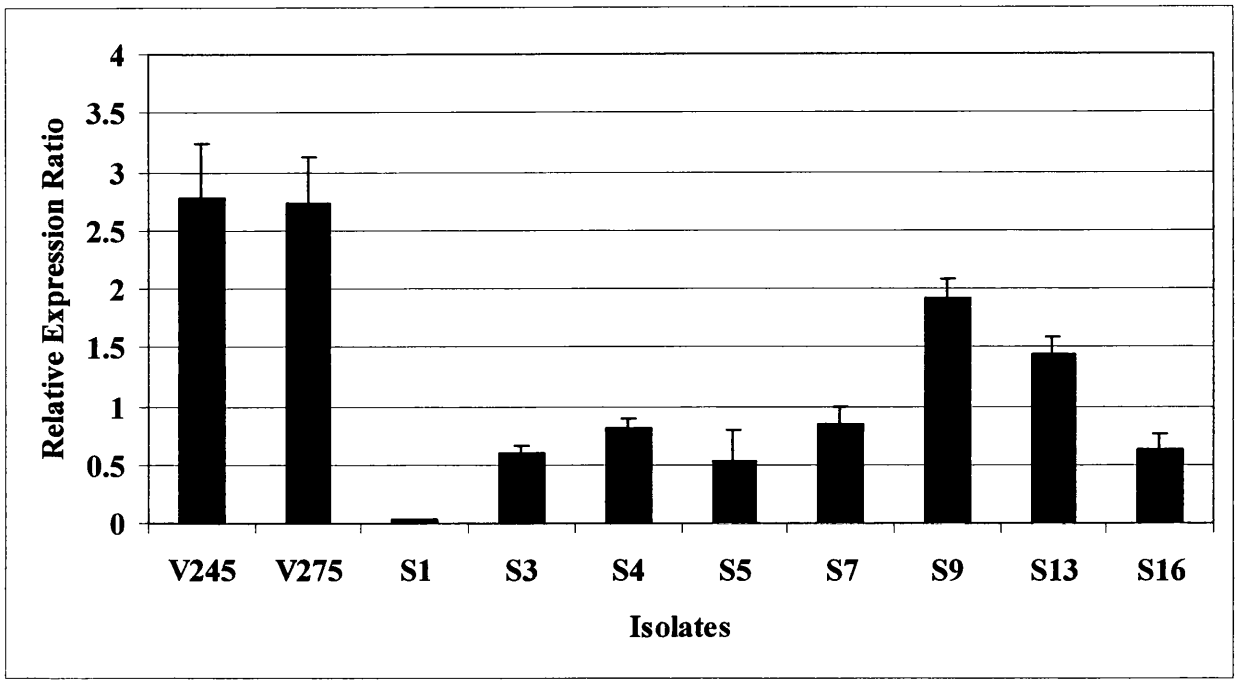


Fig. 5.8 Relative expression of esterases (*ste 1*) by selected sectors and parent cultures

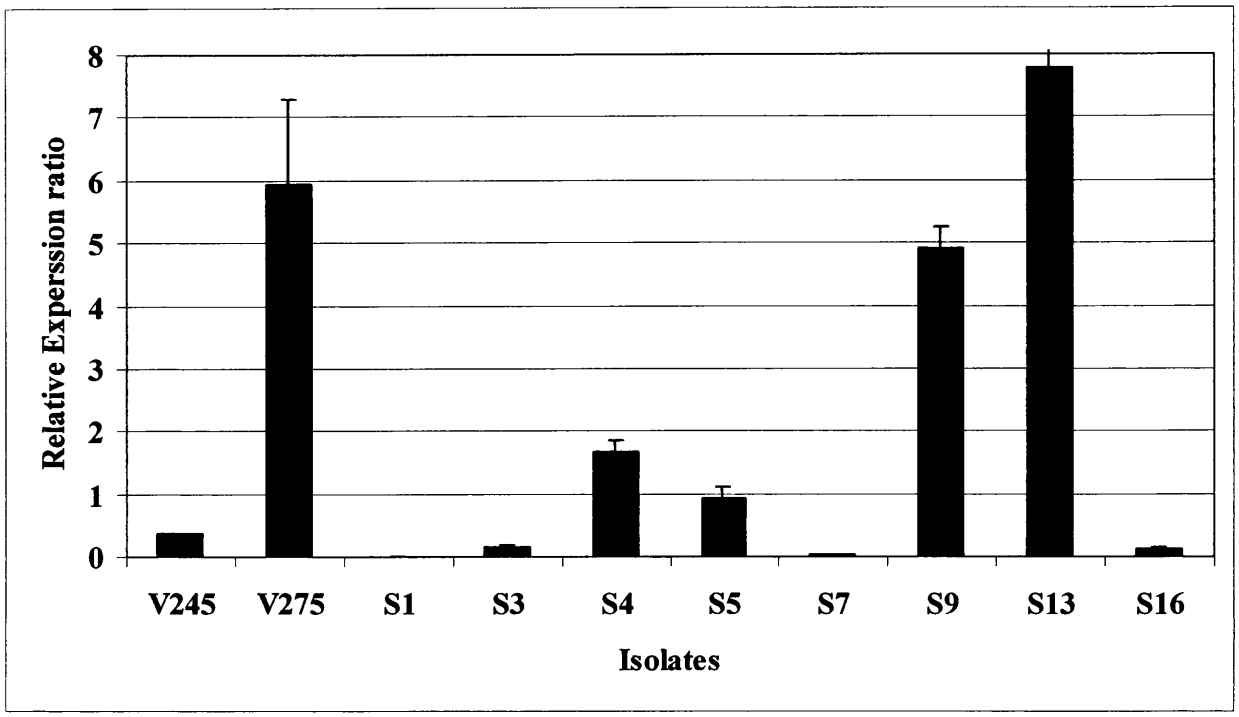


Fig. 5.9 Relative expression of trypsin (*try 1*) by selected sectors and parent cultures.

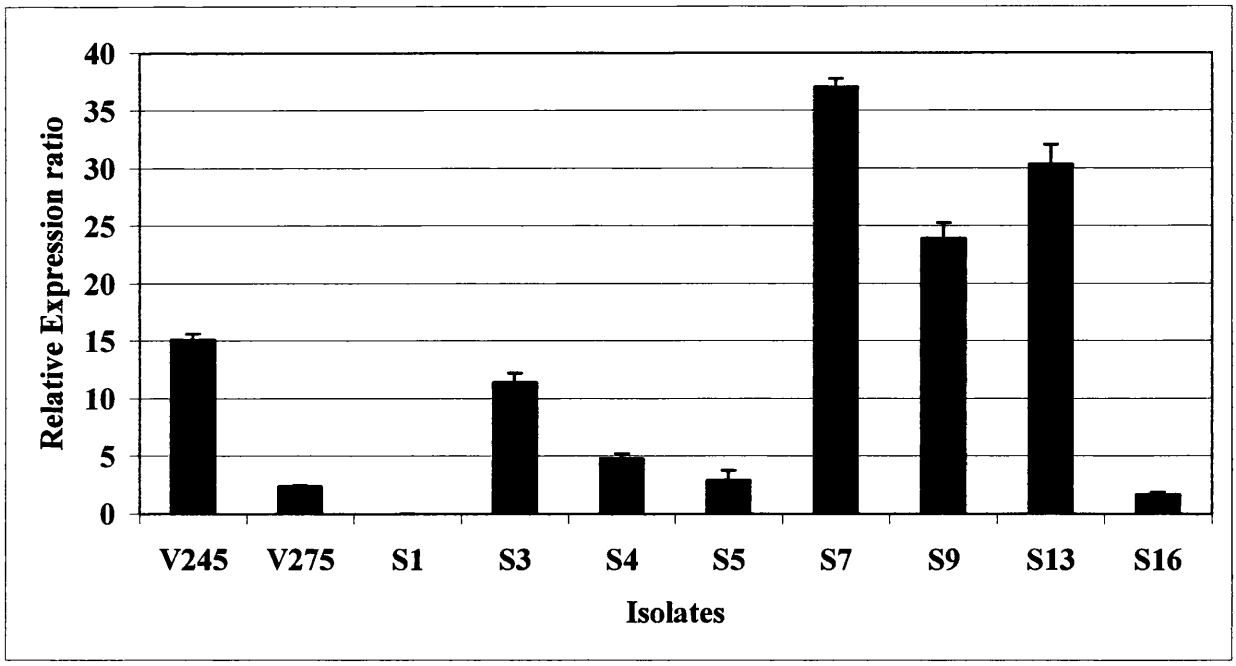


Fig. 5.10 Relative expression of chymotrypsin (*chy 1*) by selected sectors and parent cultures.

5.3.4 Destruxins production

Sectors showed a significant reduction in destruxin (dtx) production compared to the parent strains, usually declining more than half that observed in parent cultures (Table 5.4). Both parent strains produced significantly ($P < 0.05$) higher quantities of dtx A, and E than the sectors. Dtx B production varied significantly both between parent strains and among the sectors (Table 5.4), but was significantly ($P < 0.05$) higher in V275 than all other treatments.

The ratio of dtx A, B and E also varied among the parents and sectors (Table 5.4). More dtx A, B and E was produced by the sectors than the parental strains. In both V245 and V275 parent cultures, the ratio of dtx A, B and E was such that dtx E predominated. Sectors produced more dtx A and B than dtx E (Table 5.4).

Table 5.4 Crude toxin extract and destruxins (dtx) profile of selected sectors and their parent cultures. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Duncan Test). Each treatment was replicated two times. Values in parenthesis represent standard errors.

Sector's ID	Crude toxin (mg/l)	dtx A (mg/l)	dtx B (mg/l)	dtx E (mg/l)	Ratio of dtxs A, B, E
V245	136.80^{a} (5.60)	25.94^{a} (0.02)	5.45^{b} (0.05)	29.94^{a} (0.02)	4.75:1: 5.48
V275	157.80^{a} (18.20)	28.16^{a} (4.42)	14.70^{a} (2.20)	31.72^{a} (5.09)	1.93:1: 2.15
S 1	22.20^{e} (5.40)	0.56^{c} (0.17)	0.76^{cd} (0.24)	ND*^{c}	0.74:1: 0
S3	24.8^{e} (0.80)	0.24^{c} (0.00)	ND^{d}	ND^{c}	0.24:0:0
S 4	42.80^{cde} (8.80)	0.12^{c} (0.03)	0.10^{d} (0.02)	ND^{c}	1.15:1: 0
S 5	38.00^{cde} (12.4)	4.41^{bc} (1.33)	1.89^{cd} (0.61)	1.15^{c} (1.15)	2.34:1: 0.61
S 7	58.40^{cd} (6.80)	0.15^{c} (0.00)	ND^{d}	ND^{c}	0.15:0:0
S 9	24.00^{e} (0.80)	0.03^{c} (0.00)	0.18^{d} (0.00)	ND^{c}	0.17:1:0
S13	54.80^{cd} (4.40)	2.20^{c} (0.16)	2.84^{c} (0.23)	0.59^{c} (0.59)	0.78:1: 0.20
S16	97.10^{b} (1.70)	ND^{c}	ND^{d}	ND^{c}	ND

* Not detected

5.4 Discussion

This study shows that sector formation is influenced by the fungal strain and nutritional conditions. Clearly, *M. anisopliae* V245 was less stable than V275 since it produced more sectors under a wide range of cultural conditions. The sectors could be divided into poorly sporulating or sterile colonies that arose almost anywhere in the parent colony, a phenomenon also observed in other fungal species (Jennings and Lysek, 1996; Vannacci and Cristani, 1998). Sector frequency appears to increase with increasing levels of glucose; presumably the sectors are physiologically adapted to exploit such conditions. Furthermore, by investing energy in growth as opposed to sporulation they avoid being smothered by the parent culture. These observations support one of the proposed models in which the differential growth rate was described as a selective advantage to sectors over the parent strain (Pontecorvo and Gemmel 1944). The varying sector size may reflect not just differential growth but the involvement of different numbers of hyphae. Since the sectors arose almost anywhere in the parent culture suggests that this was a spontaneous event. Exactly what triggers sector formation remains unclear, but is probably dependent on multiple cues, one of which would be differences between hyphae in their ability to utilize certain nutrients. In nutrient rich conditions, sectors may assimilate nutrients at a faster rate than the parent mycelium, hence the high frequency of sectors under such conditions. Even in the nutrient rich osmotic stress medium the frequency was comparatively high. In contrast, the low sector frequency on nutrient poor media was probably due to the sectors being unable to compete with the parent culture and establish their identity.

Whatever the cause of sector formation, we show for the first time that sectors do not respond to inductive media since they produce less Pr1 and other pathogenicity-related enzymes. RT-PCR confirmed fewer gene transcripts for pathogenicity related genes (*pr1 A, chy 1, try 1, ste 1*) in sectors than parent cultures. It is tempting to speculate that sectors have faulty transcription control mechanism(s) and/or receptors that do not respond to cuticular cues due to alterations in the receptor structure or composition (type

and number). The importance of the signal transduction machinery in fungal development and pathogenesis is well documented (Bolker 1998). Recent studies have shown that disruption of the *cpg-1* gene encoding for the Gα subunit, negatively affected the growth, reproduction and virulence of the plant pathogen *Cryphonectria parasitica* (Segers and Nuss 2003).

API-ZYM helped investigate the production of an additional set of enzymes in inductive media. It showed that the activity of some enzymes increased whilst that of others decreased or was lost altogether. Whether these enzymes are regulated by glucose has still to be substantiated but glucose is known to repress fungal genes used to metabolise alternate carbon sources (Ronne, 1995). Pr1 is known to be under carbon catabolite repression control and its production is repressed by excess glucose (Screen *et al.*, 1997). Sectors of the *Metarhizium* strains V275 and V245 differed in the type and number of enzymes affected. In a previous study, the production of ten enzymes by sectors produced by a single strain of *M. anisopliae* was examined and authors found that one sector had lost activity of three enzymes relative to the original profile (Ryan *et al.*, 2002). In our study, RT-PCR showed that increased levels of *try 1* transcripts often was accompanied by depressed levels of *chy* 1 transcripts and *vice versa* in parent strains and sectors, with the exception of S9 and S13 where the expression of both *try 1* and *chy 1* was high. These observations confirm that different strains of *Metarhizium* differ in their physiology and stability and that the sector enzyme profile may differ from that of the parents and also between sectors.

Successive subculturing of *M anisopliae* V275 may also result in whole cultures degenerating starting with silencing of the *pr1* gene followed by the loss of the small chromosome carrying the *pr1* gene (Wang *et al.*, 2003). Similarly, chromosome loss has been reported to be associated with the "fluffy" sectoring in *Agaricus bisporus* (Horgen *et al.*, 1996). However, our studies show that sectors exhibit Pr1 activity, therefore, are unlikely to have lost the small, conditional dispensable chromosome.

Destruxins constitute one of the major secondary metabolites produced by *Metarhizium anisopliae* and are also considered to be an important pathogenicity determinant (Amiri *et al.*, 2000; Kershaw *et al.*, 1999; Wang *et al.*, 2003). This study shows for the first time that *M. anisopliae* sectors produce significantly less destruxin A, B and E than the parent cultures. This is a more widespread phenomenon, since the production of fungal secondary metabolite production is frequently associated with developmental processes such as sporulation and pigmentation (Calvo *et al.*, 2002). A loss or reduction in the production of secondary metabolites is often associated with phenotypic changes in particular reduced sporulation and pigmentation (Adams and Hu 1998; Gao and Nuss, 1996 Kale *et al.*, 1994). We show that if *Metarhizium* sectors produce conidia then these are sparse and always paler than those of the parent and that this is independent of the strain. Even whole cultures of a mutant strain of V275, which does not produce destruxins produced few pale conidia (Wang *et al.*, 2003).

Production of sectors by fungal biocontrol agents like *M. anisopliae* has to be taken seriously since it could raise production costs and by diluting the virulent inoculum with less aggressive inoculum it could result in reduced efficacy and ultimately reduce sales of the product. By careful selection of stable strains and manipulation of cultural conditions it may be possible to minimise sector production. However, more work is needed to elucidate the underlying mechanisms for the localised instability. The fact that so many attributes of the sectors produced by *M. anisopliae* have been described in other fungi suggests that similar mechanisms must be involved.

Chapter 6: Influence of repeated subculturing on the virulence of single spore and parent cultures of *M. anisopliae*

6.1 Introduction

Cultural stability in terms of morphology and virulence is of paramount importance to ensure consistency and reliability of fungal BCAs e.g. *M. anisopliae*. Degenerate cultures i.e. those that differ from parent are generally discarded and not used in any production systems (Jenkins *et al.*, 1998). However cultures similar to parental phenotype may also differ in virulence. Single spore isolates of *B. bassiana* were observed to have enhanced virulence compared to parent cultures (Samsinikova and Kalalova, 1983). Single spore colonies have also been used to identify mutants but these studies are limited to first generation of the culture. The aim of this study was to determine the influence of successive subculturing on the variation within the parent culture. The study will help determine the stability of *M. anisopliae* upon successive subculturing.

Attributes of phenotypically degenerate cultures are described in chapter 5 and therefore, the present study was focussed on the cultures identical to parent phenotype. The result revealed that single spore colonies vary in virulence and Pr1 production with their parents. Variation in the single spore colonies increased with successive subculturing suggesting a shift in the relative proportion of virulent and avirulent individuals in the culture.

6.2 Materials and Methods

6.2.1 Fungal cultures

M. anisopliae V245 and V275 were passaged through *G. mellonella* and then repeatedly subcultured onto SDA media up to 10^{th} subculture as described in Chapter 2.

6.2.2 Preparation of single spore colonies

Single spore colonies were produced from the selected multi spore subcultures (1^{st}, 5^{th} and 9^{th}) of both strains. Single spore colonies were prepared by inoculating 100µl of 1x 10^2 conidia/ml of each treatment on SDA plates. After inoculation, Petri dishes were incubated at 25°C for 48hours. Thirty single spore colonies of each treatment were then transferred to individual SDA plates. After 14 days of incubation at 25°C, all the single spore colonies of each subculture were then examined for morphological difference and divided into 3 main groups. These group included A. (parent type colonies), B. (sector forming colonies), and C. (colonies exhibiting differential morphology than parent culture). In order to determine whether morphologically similar colonies vary in virulence, five single spore colonies with identical morphology as that of parent culture were selected from each multi spore subculture as described in fig 6.1.

6.2.3 Comparison of virulence between the single spore and parent

Virulence of selected single spore colonies and multi spore colonies were evaluated against *T. molitor* larvae according to the procedures described in section 2.2.3. Mother cultures of V245 and V275 were used as a positive control.

6.2.4 Comparison of CDEs production by the single spore and parent cultures

Production of CDEs and their activities were determined as described in section 2.2.9.

6.2.5 Statistical analysis

The whole study was repeated twice, with thirty single spore colonies observed from each subculture for phenotypic grouping and 5 for other studies. Each treatment was replicated three times unless stated otherwise. Data was subjected to one-way ANOVA followed by Tukey test for determining significant differences. For all statistical analysis SPSS 11 software was used.

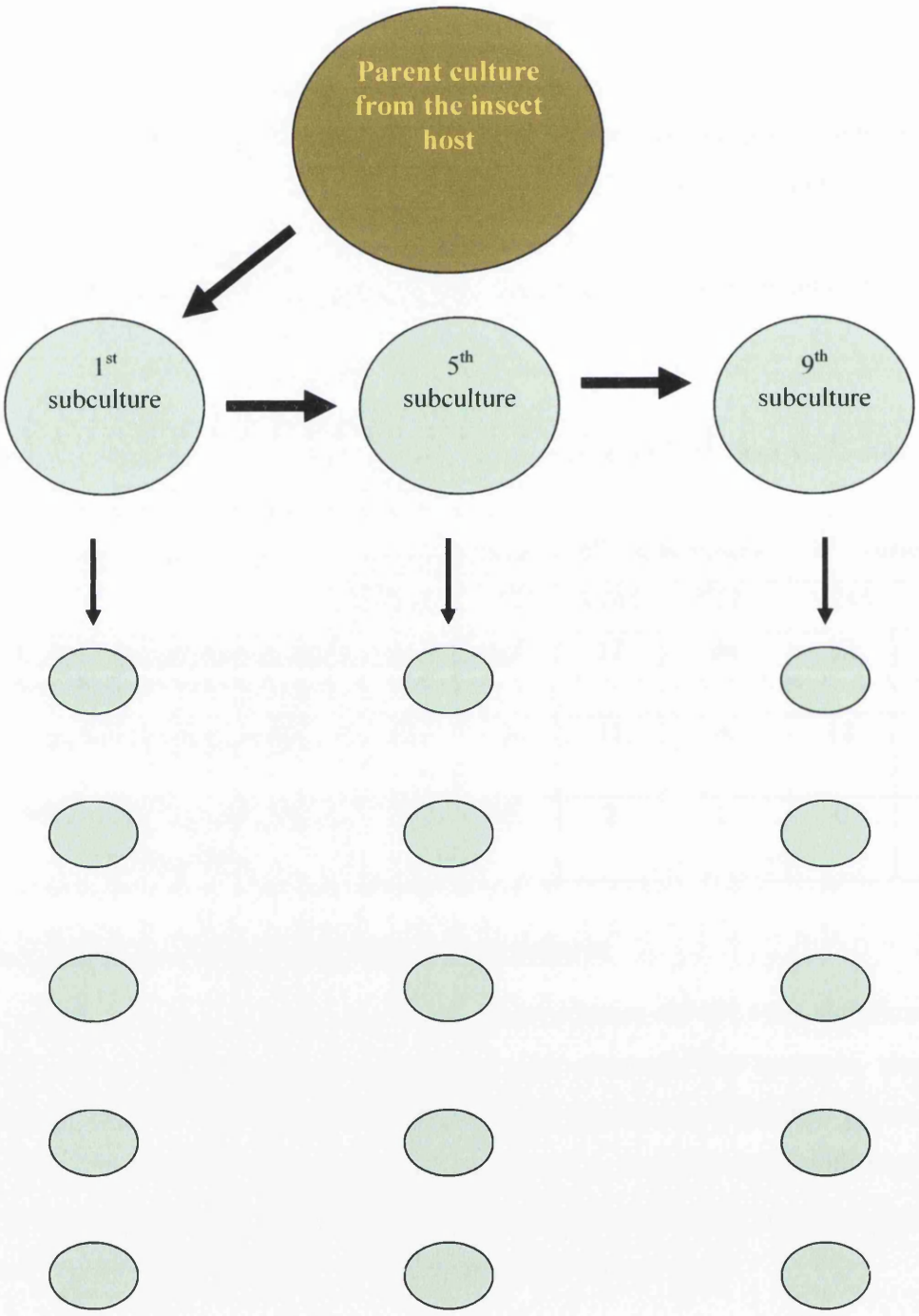


Fig 6.1 Selection of single spore colonies from different subcultures to compare their virulence and CDEs production with that of parent cultures.

6.3 Results

6.3.1 Phenotypic grouping of single spore colonies

M. anisopliae V245 was relatively unstable as its first subculture and subsequent generations produced a large number of sectors or colonies, which differed phenotypically with parent cultures (Table 6.1). V275 was more stable with only few sectors/phenotypes being produced (Table 6.1). In both strains, an increase in sectors and/ or different phenotype colonies was observed on successive subculturing (Table 6.1).

Table 6.1 Phenotypic grouping of single spore colonies of V245 and V275 subcultures

Phenotypic group	**1st subculture**		**5th subculture**		**9th subculture**	
	V245	V275	V245	V275	V245	V275
Parent type (greyish green conidia, uniform colony)	15	25	17	24	12	15
Sector forming colony	15	5	11	6	18	10
Phenotype varying from parent culture	0	0	2	0	0	5

6.3.2 Virulence of single spore and parent cultures

Generally virulence of single spore and parent cultures did not vary significantly ($P < 0.05$, Tukey Test) but certain exceptions were observed. For example, single spore colonies of the 5th and the 9th subcultures varied significantly with their parent cultures. These colonies were more aggressive and varied non-significantly with those of first subculture (Table 6.2, 6.3). In the case of V245, one single spore colony from 1st subculture had significantly low virulence than its parent culture.

In general, single spore and parent cultures of both strains declined in virulence upon successive subculturing (Table 6.2, 6.3). The LT_{50} ranged between 3.87 to 8.10 dpi and 3.31 to 4.48 dpi for V245 and V275 cultures respectively (Table 6.2, 6.3).

6.3.3 CDEs production by single spore and parent cultures

6.3.3.1 Total protein production

The total protein and CDEs production varied significantly ($P < 0.05$, Tukey Test) among the different treatments (Tables 6.2, 6.3). The single spore colonies varied with each other and with parent cultures (Tables 6.2, 6.3).

In case of V245, highest protein concentration was observed in the multi spore colony of 1st subculture, while the least was observed in one of its single spore colonies. Least total protein was also for all the single spore and parent cultures of 5th and 9th subcultures (Table 6.2). In the case of V275, Generally non-significant differences in the total protein production were observed (Table 6.3).

6.3.3.2 Non-specific protease activity

Non-specific protease activity varied non-significantly ($P < 0.05$, Tukey Test) among the most single spore and parent cultures of both strains (Table 6.2, 6.3).

6.3.3.4 Pr1 activity

The Pr1 activity varied significantly ($P < 0.05$, Tukey Test) among the treatments. Highest activities were observed in single spore and parent cultures of 1st and 5th subcultures of both strains (Table 6.2, 6.3). Least Pr1 activities were observed in parent and single spore colonies of 9th subcultures. Occasionally single spore colonies had either higher or lower Pr1 activity than the parent cultures (Tables 6.2, 6.3).

6.3.3.4 Pr2 activity

The trypsin like protease Pr2 varied non-significantly ($P < 0.05$, Tukey Test) in most of the single spore colonies and parent cultures of both strains (Table 6.2, 6.3).

Table 6.2 CDEs production and virulence of selected single spore and parent cultures of V245. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated two times except for bioassays which were replicted three times. Values in parenthesis represent standard errors.

Colony ID	Total protein µg/ml	Non specific proteases U/ml	Pr1 activity µmol/ml/min	Pr2 Activity µmol/ml/min	Virulence LT_{50} (DPI)
1st MS	332.5^{a} (13.1)	1.37^{cd} (0.11)	2.40^{a} (0.02)	0.64^{abc} (0.02)	3.87^{a} (0.02)
1A245	252.5^{abc} (7.50)	1.43^{cd} (0.02)	2.44^{a} (0.02)	0.73^{abc} (0.05)	4.11^{abcd} (0.01)
1B	190.0^{abcd} (23.8)	1.65^{bcd} (0.08)	2.56^{a} (0.03)	0.57^{abc} (0.02)	4.04^{abc} (0.04)
1C	305.0^{ab} (78.0)	1.52^{cd} (0.0)	2.38^{a} (0.01)	0.69^{abc} (0.07)	4.12^{abcd} (0.06)
1D	07.5^{f} (4.7)	2.04^{bcd} (0.13)	2.48^{a} (0.08)	1.20^{a} (0.07)	4.31^{cd} (0.04)
1E	167.5^{bcde} (44.9)	1.50^{cd} (0.11)	2.41^{a} (0.04)	0.64^{abc} (0.01)	3.97^{ab} (0.02)
5th MS	057.5^{def} (19.7)	1.31^{cd} (0.07)	2.28^{a} (0.1)	1.12^{ab} (0.02)	4.31^{cd} (0.04)
5A	057.5^{def} (13.1)	2.01^{bcd} (0.24)	2.42^{a} (0.01)	1.20^{a} (0.07)	4.79^{e} (0.09)
5B	132.5^{cdef} (25.6)	1.61^{bcd} (0.03)	0.81^{b} (0.02)	0.82^{abc} (0.34)	5.42^{f} (0.08)
5C	70.0^{def} (10.8)	1.65^{bcd} (0.18)	2.44^{a} (0.05)	0.50^{bc} (0.24)	4.05^{abcd} (0.02)
5D	100.0^{def} (21.2)	1.94^{bcd} (0.25)	2.44^{a} (0.04)	0.38^{c} (0.12)	4.23^{bcd} (0.05)

5E	70.0^{def} (7.0)	3.56^{a} (0.02)	2.37^{a} (0.03)	1.10^{ab} (0.03)	4.07^{abcd} (0.04)
9^{th} MS	95.0^{def} (55.4)	2.18^{bc} (0.43)	0.60^{b} (0.03)	0.99^{abc} (0.08)	4.88^{e} (0.09)
9A	37.5^{ef} (11.0)	1.92^{bcd} (0.14)	0.84^{b} (0.12)	1.13^{ab} (0.03)	4.32^{d} (0.02)
9B	22.5^{f} (4.7)	1.60^{bcd} (0.17)	0.56^{b} (0.03)	0.63^{abc} (0.0)	4.23^{bcd} (0.04)
9 C	40.0^{ef} (4.0)	2.42^{b} (0.14)	0.90^{b} (0.17)	0.97^{abc} (0.04)	8.10^{g} (0.02)
9D	55.0^{def} (5.0)	1.49^{cd} (0.17)	2.63^{a} (0.11)	0.39^{c} (0.12)	4.08^{abcd} (0.04)
9E	40.0^{def} (8.1)	1.21^{d} (0.12)	0.55^{b} (0.03)	0.54^{bc} (0.15)	5.61^{f} (0.02)

Table 6.3 CDEs production and virulence of selected single spore and parent cultures of V275. All the means within a column followed by the same letter are not significantly different ($P < 0.05$, Tukey Test). Each treatment was replicated two times except for bioassays which were replicted three times. Values in parenthesis represent standard errors.

Colony ID	Total protein µg/ml	Non specific proteases U/ml	Pr1 activity µmol/ml/min	Pr2 Activity µmol/ml/min	Virulence LT_{50} (DPI)
1st MS	212.5^{bc} (18.8)	1.55^{bc} (0.28)	2.52^{a} (0.12)	0.98^{a} (0.07)	3.67^{abcd} (0.04)
1A275	47.5^{c} (17.5)	1.83^{b} (0.02)	2.21^{ab} (0.12)	0.86^{a} (0.01)	3.58^{abc} (0.02)
1B	077.5^{c} (21.3)	2.85^{a} (0.49)	2.16^{ab} (0.13)	0.91^{a} (0.03)	3.49^{ab} (0.1)
1C	210.0^{bc} (4.0)	1.46^{bcd} (0.14)	2.38^{ab} (0.15)	0.97^{a} (0.03)	3.47^{ab} (0.04)
1D	215.0^{bc} (6.4)	1.29^{bcde} (0.02)	2.55^{a} (0.04)	0.91^{a} (0.03)	3.94^{cdef} (0.05)
1E	215.0^{bc} (11.9)	0.95^{bcde} (0.05)	2.51^{a} (0.04)	0.95^{a} (0.05)	3.31^{a} (0.02)
5th MS	157.5^{bc} (68.2)	0.78^{cde} (0.01)	2.01^{b} (0.20)	0.87^{a} (0.06)	4.21^{g} (0.13)
5A	152.5^{bc} (43.8)	0.96^{bcde} (0.08)	1.20^{c} (0.05)	0.63^{b} (0.04)	4.25^{g} (0.12)
5B	222.5^{bc} (16.5)	0.66^{cde} (0.16)	2.41^{ab} (0.08)	0.96^{a} (0.03)	3.95^{cdef} (0.11)
5C	225.0^{bc} (13.2)	1.03^{bcde} (0.05)	2.35^{ab} (0.03)	0.96^{a} (0.07)	4.21^{defg} (0.13)
5D	292.5^{b} (69.2)	1.10^{bcde} (0.36)	2.21^{ab} (0.05)	0.97^{a} (0.06)	3.61^{abc} (0.05)
5E	142.5^{bc} (10.3)	0.77^{cde} (0.11)	2.15^{ab} (0.08)	0.91^{a} (0.01)	3.88^{bcdef} (0.05)
9th MS	485.0^{a} (30.1)	0.53^{e} (0.03)	0.48^{d} (0.04)	0.38^{c} (0.04)	4.48^{g} (0.10)
9A	170.0^{bc} (37.6)	0.69^{cde} (0.08)	2.24^{ab} (0.02)	0.87^{a} (0.06)	3.82^{bcde} (0.02)

9B	210.0^{bc} (57.8)	0.66^{cde} (0.00)	0.70^{d} (0.03)	0.29^{c} (0.00)	4.04^{def} (0.09)
9 C	137.5^{bc} (13.7)	0.62^{de} (0.03)	2.42^{ab} (0.03)	0.94^{a} (0.04)	3.85^{bcdef} (0.09)
9D	122.5^{bc} (44.7)	0.75^{cde} (0.02)	2.23^{ab} (0.06)	1.00^{a} (0.04)	3.99^{cdef} (0.07)
9E	180.0^{bc} (18.2)	0.56^{e} (0.13)	2.24^{ab} (0.06)	0.89^{a} (0.01)	3.36^{a} (0.04)

6.4 Discussion

Parental cultures of *M. anisopliae* were shown to consist of individuals, which differ in their virulence even though they had parental phenotype. *M. anisopliae* V275 yielded more single spore colonies similar to parent in appearance and virulence than V245. This supports earlier findings that V275 is more stable than V245. There are about 10^9 to 10^{11} air dried conidia /g of *M. anisopliae*. Each conidium is a potential infective unit and can establish a new culture. Under natural conditions, mutants or less virulent isolates in a mixed population would be removed due to selection pressure, however, under artificial conditions, these less virulent individuals would increase in number at the same rate (if not at greater rate) than those of virulent individuals, thereby out numbering the virulent individuals after several generations. The overall effect of such a situation would result in reduced efficacy over time. The result of this study clearly shows an increase in variability of the culture with increasing number of subcultures.

The changes in virulence among the single spore colonies and their parents further suggest that virulence is the net effect of its individual spores. Earlier studies conducted by Samsinkova and Kalalova, (1983) describe the variation in virulence of single spore colonies from the same parent culture. However, they did not look at the possibility that this variation in virulence among the single spore colonies may have some role in attenuation of virulence. Secondly, their studies were only based on bioassays, whereas the present study showed that these single spore colonies also vary in their physiological state particularly in the production of Pr1.

Similar observations were also made in other organisms e.g. Ferea *et al.*, (1999) observed that under nutrient limited conditions; yeast clones of higher fitness successively replace one another over time. Such shifts had been observed to occur on the order of once every 50 generations. They demonstrated that evolved strains differed in their genome wide gene expression compared to the parental strain. Such adaptations may also be taking place in *M. anisopliae* resulting in the survival and selection of physiologically or genetically fitter individuals after host passage and an increase in the weak/avirulent individuals during successive growth under nutrient rich conditions.

In another report, Mostowy *et al.*, (2003) demonstrated that the *in vitro* cultivation of BCG vaccine have undergone relatively much higher genetic loss than those observed in *M. tuberculosis* pathogen itself. They concluded that bacterial survival in the laboratory requires a different set of genetic tools than are needed to cause clinical tuberculosis. These authors explained their results in the light of *M. tuberculosis* culturing practice. *M. tuberculosis* grows as clumps and individuals were neither observed nor selected. Therefore each bi weekly transfer of BCG to fresh media involves million and more likely billions of individual bacteria, this serial passaging may then create a competitive environment within the culture, under which selectively advantageous mutants may out compete the wild type and thereby dominate the culture. A similar situation in fungal culture e.g. *M. anisopliae* is also very likely, where each laboratory subculture involves millions of individual conidia.

The relative virulence or its loss therefore, then partly depends on the population composition. A higher number of virulent individuals would result in virulent cultures but their loss or mixture with individuals of average or lower virulence than that of the parental population would result in more rapid loss of virulence.

7.1 General Discussion

The stability and virulence of the entomogenous fungus *M. anisopliae* was influenced by nutrition and dependent on the strain. Nutrient rich conditions favoured good vegetative growth and high conidial yields but was also likely to result in attenuation and spontaneous production of sectors. Successive subculturing resulted in further degeneration of the cultures with increasing tendency to produce sectors or pale sterile mycelium. In contrast, nutrient poor or osmotic stress media gave rise to fewer but more aggressive conidia. These findings suggest that on exposure to stress conditions the priority of the *M. anisopliae* changes towards survival in adverse conditions, which may limit its growth but make it more aggressive. Nutrient starvation is therefore likely to be a key environmental signal for the switch from a saprophytic to a pathogenic mode of nutrition (Clarkson and Charnley, 1996). This study shows that nutrient poor media e.g. 1% yeast extract media provides a compromising balance between conidial yield and virulence. Though osmotic stress media also yielded highly virulent conidia, conidial yield was too low to be considered for cost effective mass production of the inoculum. Both strains varied in their virulence when produced on similar media but overall trend of high virulence under nutrient stress was strain independent.

Nutrition also influenced fungal stability, which was reflected in the sector frequency. Nutrient rich conditions yielded significantly higher number of sectors. Since this phenomenon was strain independent, these nutrient rich media could be used to determine fungal stability of other strains of *M. anisopliae*. Relatively fewer sectors formed by V275 on all media indicate its stability under different nutritional conditions. Studies on single spore cultures also confirmed that V275 is more stable as it yielded more single spore colonies identical to parental phenotype and virulence.

Prl, particularly spore bound Prl, appeared to be very good indicator of virulence. Levels of spore bound Prl were consistently higher in all the virulent conidia, which

could be used as a potential quality control marker to monitor the changes in virulence. Earlier studies on null or over expressing *pr1* mutants also indicate its role in *M. anisopliae* virulence (St. Leger *et al.*, 1996; Wang *et al.*, 2002). Significantly higher levels of transcripts for *pr1* from virulent conidia observed in this study confirms its role in virulence. Low levels of transcripts for *pr1* and a decline in spore bound Pr1 activity upon successive subculturing suggests a role for nutrition in predetermining the pathogenic or saprophytic mode of *M. anisopliae*. The levels of inductive Pr1 and other cuticle degrading enzymes were inconsistent in different studies suggesting the limited application of these enzymes to monitor changes in virulence.

Conidia CN ratios and speed of germination were also good indicators of virulence. Nutritional conditions appeared to influence conidial CN ratios as nutrient rich media consistently had higher CN ratios. Except for osmotic stress media, conidia with high CN ratio, i.e. above 5.2:1, germinated slowly, had low spore bound Pr1 activity and virulence. In contrast, virulent conidia from insect hosts or nutrient poor media had low CN ratios, germinated faster and had higher spore bound Pr1 activity. These findings further support the role of nutrition in virulence and highlights the coordinated events leading to changes in *M. anisopliae* virulence.

Nutrition also influenced several other pathogenicity determinants such as the adhesive properties of conidia and destruxins production; however results were inconsistent in different set of studies or strains. For example, overall adhesion, surface hydrophobicity and electrostatic forces of the conidia declined upon successive subculturing and showed a good relationship with virulence. In contrast, conidia produced on different media did vary in these attributes but the link with virulence was tenuous. It is possible that when grown on different media, other factors like surface carbohydrates; mucilage production may be affected, thereby influencing overall adhesion. Ibrahim *et al.* (2002) showed that nutrition influenced surface carbohydrates of *M. anisopliae* but its role in virulence

remained unclear, therefore, further studies are required to determine the effect of nutrition on various factors contributing to the adhesion of *M. anisopliae* conidia.

Several factors appeared to contribute in the attenuation of virulence. Successive subculturing on nutrient rich media influenced important virulence determinants such as adhesion, Pr1 and destruxins. A decline in the expression and production of these attributes attenuated *M. anisopliae* virulence. Beside important virulence determinants, other attributes of *M. anisopliae* such as conidial yield, sectors formation or appearance of variant single spore colonies were also influenced by successive susbculturing or nutrition. Phenotypic changes particularly the loss of sporulation was observed to be linked with decline in the production of important virulence determinants suggesting a link between phenotypic stability and attenuation of virulence. This study also showed for the first time that cultures identical to the parental morphological phenotype may differ in virulence and this variability increases with successive subculturing. The relative ratio of virulent and avirulent single spore colonies is strain dependent; however, successive subculturing on nutrient rich media appeared to favour an increase in the population of less virulent individuals within the culture. Similar reports in other organisms suggest that this phenomenon may also have role in the long-term attenuation of virulence (Ferea *et al.*, 1999, Mostowy *et al.*, 2003).

Another important feature of this study is the optimisation and application of novel tools to monitor various attributes of *M. anisopliae*. The only notable study to date on the adhesive properties of entomogenous fungal spores is that of Boucias *et al.*, (1988) which mentions various adhesion forces but provides no detailed measurement of the forces. Disparate adhesion assays developed in this project (e.g. Radial Flow Chamber assays, electrophoretic mobility, hydrophobicity assays) have helped to determine the contribution of the different adhesion mechanisms of spores during the initial, passive attachment phase of the infection process. Protocols developed for the Radial Flow

Chamber assay could be adapted for more in depth analysis of adhesion to different substrates (e.g. cuticles from different hosts or regions of the same arthropod).

Similarly, this study is the first to quantify electrostatic forces, conidial CN ratios and attributes of insect derived conidia. This study showed for the first time that irrespective of strain or culture media, *M. anisopliae* regains its virulence upon host passage. Identification of several pathogenicity and or virulence determinants and the influence of nutrition on their expression would facilitate further studies on the underlying mechanisms of virulence of fungal pathogens.

In conclusion, this study has helped elucidate some of the underlying mechanisms of attenuation and virulence in *M. anisopliae*. Certain strain independent parameters such as conidial CN, spore bound Pr1 and the speed of germination were shown to be useful markers to monitor fungal virulence. Nutritional studies showed that there was much scope for developing inexpensive media for the mass production of stable, virulent inoculum.

It is suggested that future studies should be directed to understand how a particular nutrient or medium influences upsteam events e.g. signalling responses, gene expression and gene silencing of particular strain or set of strains. Similarly further studies on the genetic variability among single spore colonies could explain the role of culture stability in virulence. At present very little is known about the molecular mechanisms involved in sector formation therefore further studies on the genetics of sectors e.g. number of chromosomes, DNA sequencing, could be used to identify causes of sector formation. Further studies on attenuation of virulence should also take in account whether attenuation of virulence influence specifity of the strain or not. If specificity is lost or reduced upon attenuation, then it would be interesting to note which genes are involved in the attenuation and/or specificity of the *M. anisopliae*.

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Appendix 1

Table 1 List of the commercially available fungal BCAs products which are used against several insect pests (Butt and Copping, 2000).

Product	Fungus	Target	Producer
Mycotal	*Verticillium lecanii*	Whitefly and thrips	Koppert, The Netherlands
Vertalec	*Verticillium lecaniii*	Aphids	Koppert, The Netherlands
Biogreen	*Metarhizium anisopliae*	Scarab larvae on posture	Bio-care Technology, Australia
Metaquino	*Metarhizium anisopliae*	Spittle bugs	Brazil
Bio-Path	*Metarhizium anisopliae*	Cockroaches	Eco-Science, USA
Bio-Blast	*Metarhizium anisopliae*	Termites	Eco-Science, USA
Cobican	*Metarhizium anisopliae*	Sugarcane spittle bug	Probioagro, Venezeula
Conidia	*Beauveria bassiana*	Coffee berry borer	Live Systems Technology, Colombia
Ostrinil	*Beauveria bassiana*	Corn borer	Natural Plant Protection (NPP), France
CornGuard	*Beauveria bassiana*	European corn borer	Mycotech, USA (now Emerald Bioagriculture)
Mycotrol GH	*Beauveria bassiana*	Grasshoppers, locusts	Mycotech, USA
Mycotrol WP & Botanigard	*Beauveria bassiana*	Whitefly, aphids, thrips	Mycotech, USA
Naturalis-L	*Beauveria bassiana*	Cotton pests e.g. bollworms	Troy Biosciences, USA
Proecol	*Beauveria bassiana*	Army worm	Probioagro, Venezuela
Boverol	*Beauveria bassiana*	Colorado bettle	Czechoslovakia
Boverosil	*Beauveria bassians*	Colorado bettle	Czechoslovakia
Engerlingspilz	*Beauveria brongniartii*	Cockchafer	Andermatt, Switzerland
Schweizer Beauveria	*Beauveria brongniartii*	Cockchafers	Eric Schweizer, Switzerland
Melocont	*Beauveria brongniartii*	Cockchafers	Kwizda, Austria
Green Muscle	*Metarhizium anisopliae var. acrididae*	Locusts, grasshoppers	CABI- BioScience, UK
PFR-97	*Paecilomyces fumosoroseus*	Whitefly	ECO-tek, USA
Pae-Sin	*Paecilomyces fumosoroseus*	Whitefly	Agrobionsa, Mexico
Laginex	*Lagenidium giganteum*	Mosquito larvae	AgraQuest, USA

Appendix 2

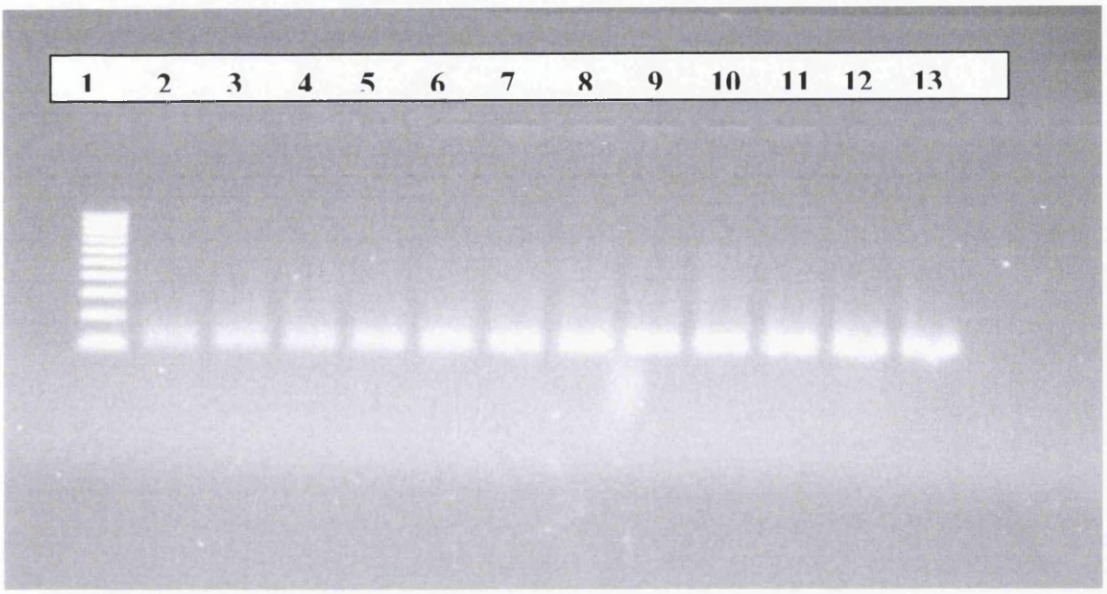

Fig. 1 Real Time PCR product (110 bp expected size) of the ribosomal rDNA (5.8S) gene from the *M. anisopliae* conidia. Lane 1 (100bp Ladder), lane 2 (V245 conidia produced on intermediate CN (35:1), lane 3 V245 conidia produced on 1% yeast extract media (1% yeast extract), lane 4, V245 conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), lane 5, V245 conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), lane 6, V245 conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), lane 7, V245 conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (1% yeast extract –*Tenebrio*. lane 8 (V275 conidia produced on intermediate CN (35:1), lane 9 V275 conidia produced on 1% yeast extract media (1% yeast extract), lane 10, V275 conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), lane 11, V275 conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), lane 12, V275 conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), lane 13, V275 conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (1% yeast extract -*Tenebrio*

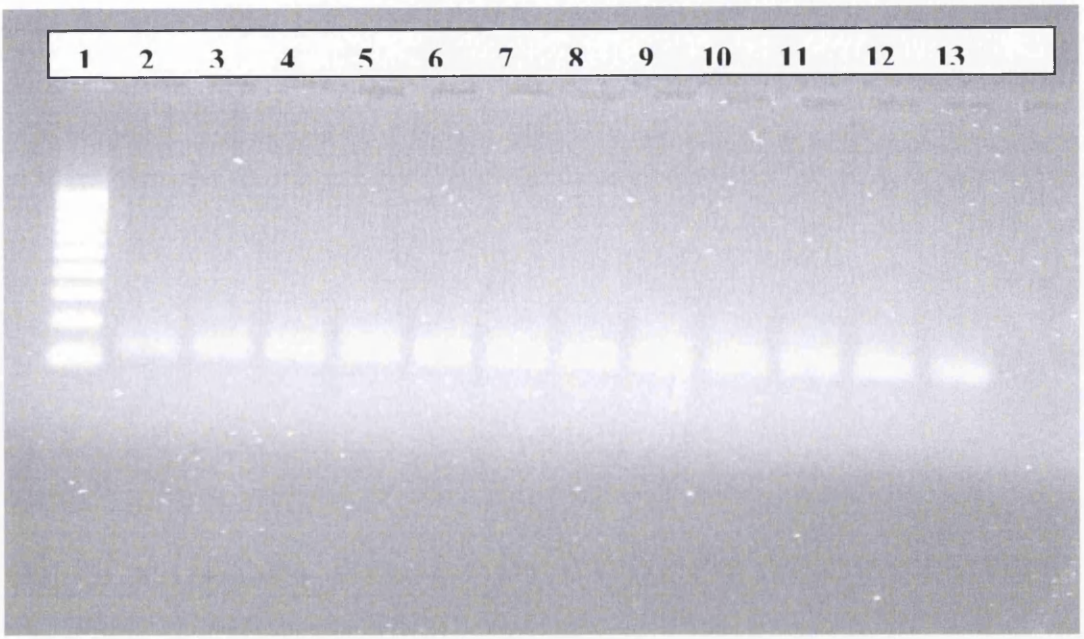

Fig. 2 Real Time PCR product (180 bp expected size) of the *pr1A* gene from the *M. anisopliae* conidia. Lane 1 (100bp Ladder), lane 2 (V245 conidia produced on intermediate CN (35:1), lane 3 V245 conidia produced on 1% yeast extract media (1% yeast extract), lane 4, V245 conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), lane 5, V245 conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), lane 6, V245 conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), lane 7, V245 conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (1% yeast extract –*Tenebrio*. lane 8 (V275 conidia produced on intermediate CN (35:1), lane 9 V275 conidia produced on 1% yeast extract media (1% yeast extract), lane 10, V275 conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), lane 11, V275 conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), lane 12, V275 conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), lane 13, V275 conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (1% yeast extract –*Tenebrio*

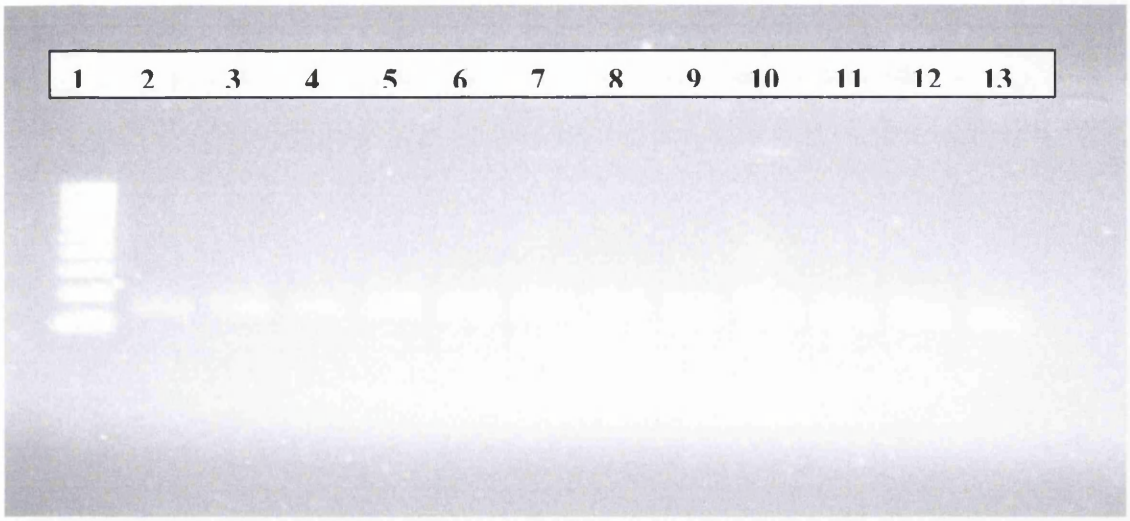

Fig. 3 Real Time PCR product (138 bp expected size) of the *ste1* gene from the *M. anisopliae* conidia. Lane 1 (100bp Ladder), lane 2 (V245 conidia produced on intermediate CN (35:1), lane 3 V245 conidia produced on 1% yeast extract media (1% yeast extract), lane 4, V245 conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), lane 5, V245 conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), lane 6, V245 conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), lane 7, V245 conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (1% yeast extract –*Tenebrio*. Lane 8 (V275 conidia produced on intermediate CN (35:1), lane 9 V275 conidia produced on 1% yeast extract media (1% yeast extract), lane 10, V275 conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), lane 11, V275 conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), lane 12, V275 conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), lane 13, V275 conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (1% yeast extract –*Tenebrio*

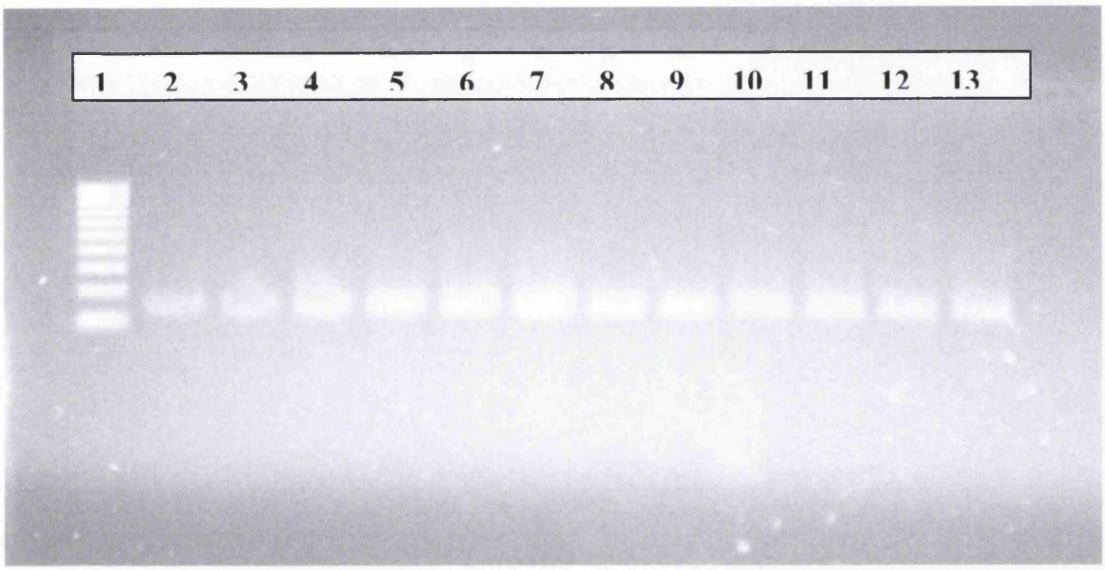

Fig. 4 Real Time PCR product (147 bp expected size) of the *chy1* gene from the *M. anisopliae* conidia. Lane 1 (100bp Ladder), lane 2 (V245 conidia produced on intermediate CN (35:1), lane 3 V245 conidia produced on 1% yeast extract media (1% yeast extract), lane 4, V245 conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), lane 5, V245 conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), lane 6, V245 conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), lane 7, V245 conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (1% yeast extract –*Tenebrio*. lane 8 (V275 conidia produced on intermediate CN (35:1), lane 9 V275 conidia produced on 1% yeast extract media (1% yeast extract), lane 10, V275 conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), lane 11, V275 conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), lane 12, V275 conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), lane 13, V275 conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (1% yeast extract -*Tenebrio*

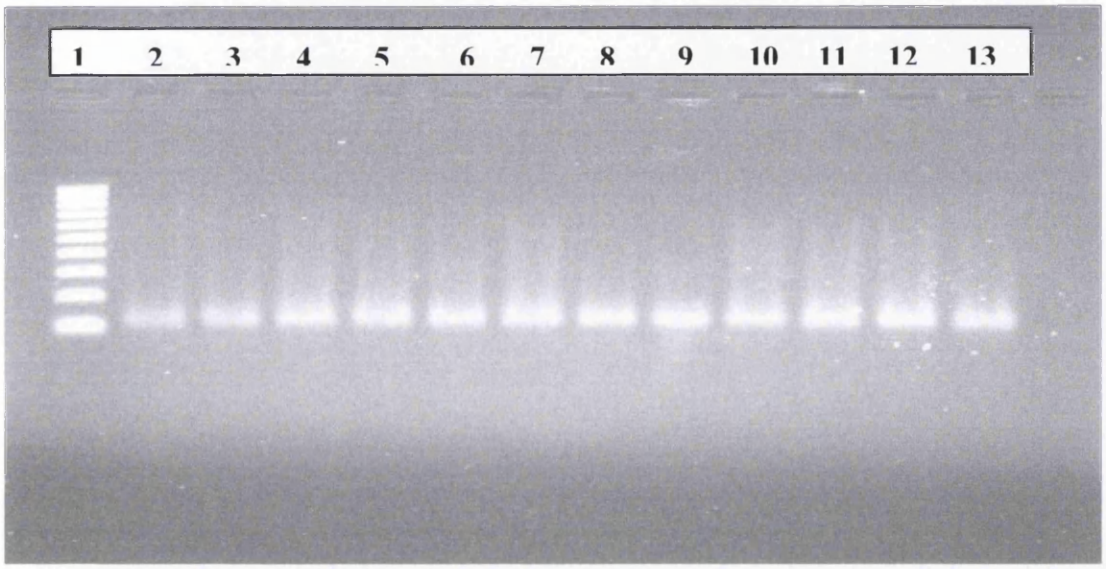

Fig. 5 Real Time PCR product (120 bp expected size) of the *try 1* gene from the *M. anisopliae* conidia. Lane 1 (100bp Ladder), lane 2 (V245 conidia produced on intermediate CN (35:1), lane 3 V245 conidia produced on 1% yeast extract media (1% yeast extract), lane 4, V245 conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), lane 5, V245 conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), lane 6, V245 conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), lane 7, V245 conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (1% yeast extract –*Tenebrio.* lane 8 (V275 conidia produced on intermediate CN (35:1), lane 9 V275 conidia produced on 1% yeast extract media (1% yeast extract), lane 10, V275 conidia initially produced on intermediate CN media but passaged through *Galleria mellonella* larvae (CN 35:1-*Galleria*), lane 11, V275 conidia initially produced on 1% yeast extract media but passaged through *G. mellonella* larvae (1% yeast extract-*Galleria*), lane 12, V275 conidia initially produced on intermediate CN media but passaged through *Tenebrio molitor* larvae (CN 35:1-*Tenebrio*), lane 13, V275 conidia initially produced on 1% yeast extract media but passaged through *T. molitor* larvae (1% yeast extract -*Tenebrio*

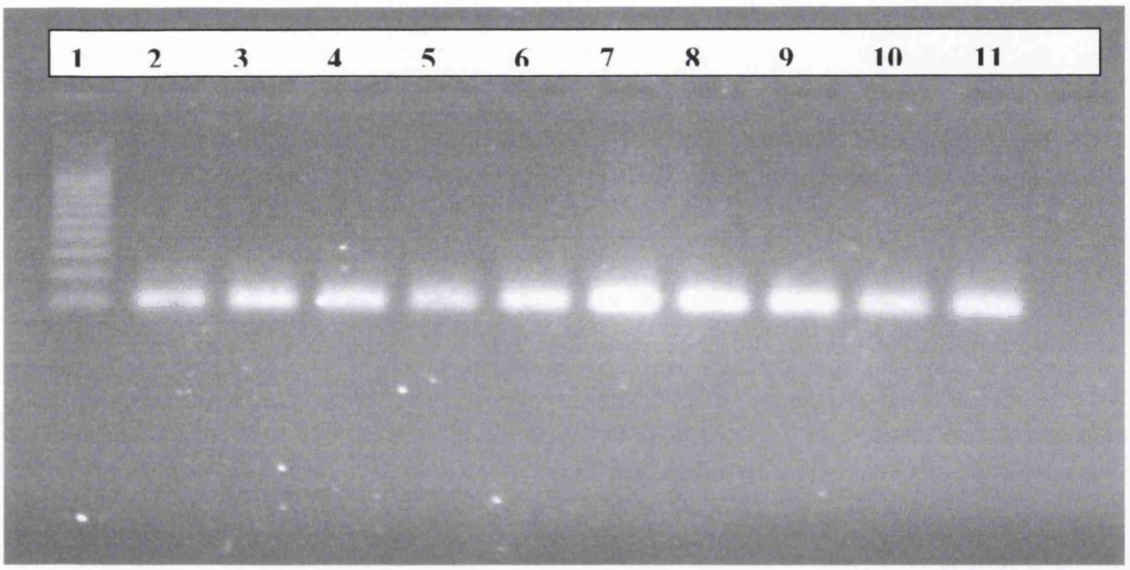

Fig.6 Real Time PCR product (110 bp expected size) of the ribosomal rDNA (5.8S) gene from the *M. anisopliae* sector and parent cultures. Lane 1 (100bp Ladder), Lane lane 2, (S1), Lane 1 (100bp Ladder), lane 2, (S1), lane 3, (S4), lane 4 (S5), lane 5 (S7), lane 6, (S9), lane 7 (S13), lane 8, (S16), lane 9, (S 21), lane 10 (V245), lane 11 (V275)

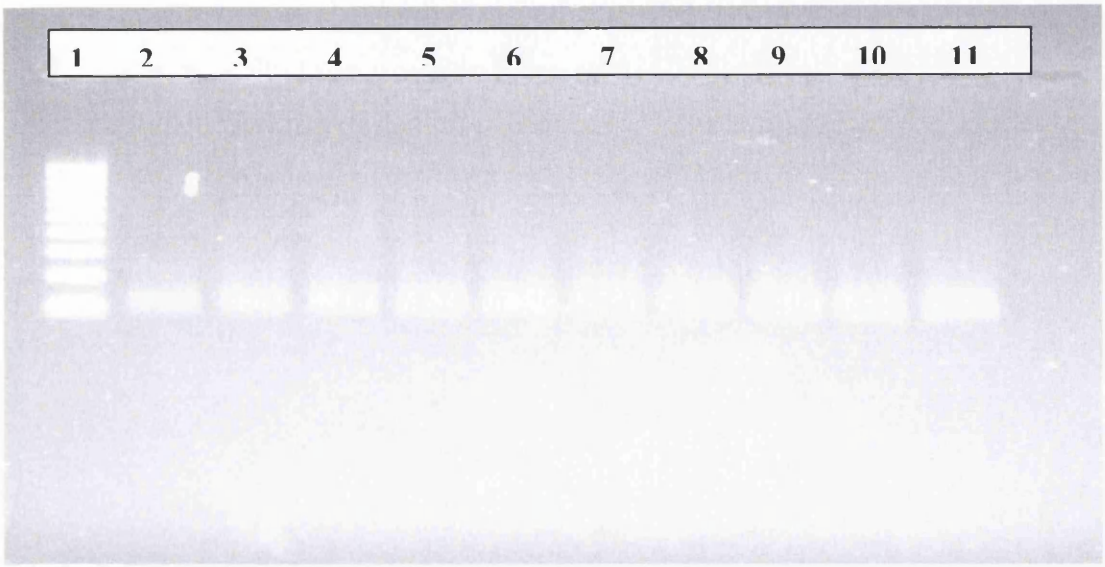

Fig.7 Real Time PCR product (180 bp expected size) of the *pr1 A* gene from the *M. anisopliae* sector and parent cultures. Lane 1 (100bp Ladder), lane 2, (S1), lane 3, (S4), lane 4 (S5), lane 5 (S7), lane 6, (S9), lane 7 (S13), lane 8, (S16), lane 9, (S 21), lane 10 (V245), lane 11 (V275)

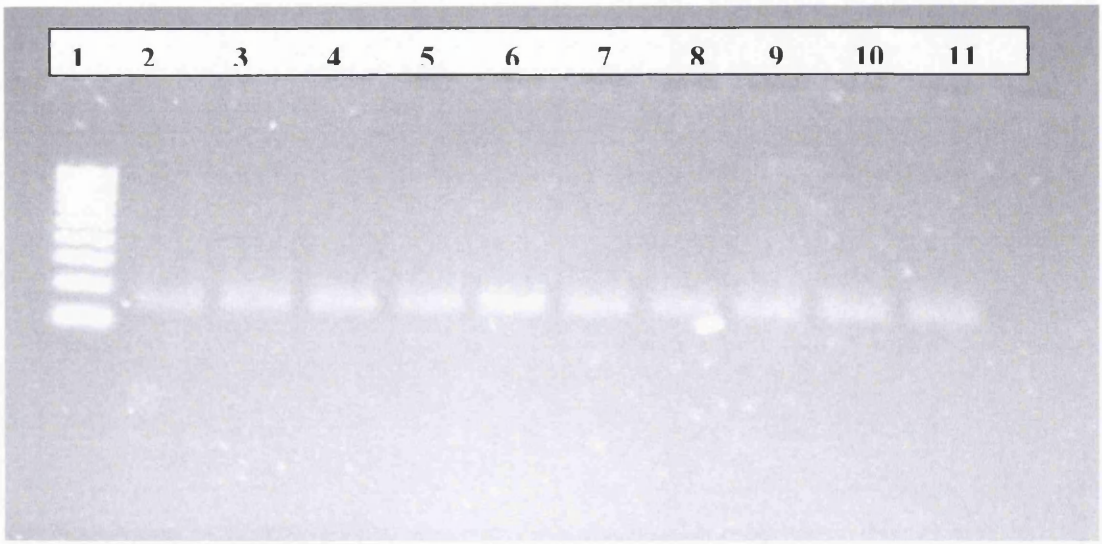

Fig.8 Real Time PCR product (138 bp expected size) of the *ste1* gene from the *M. anisopliae* sector and parent cultures Lane 1 (100bp Ladder), lane 2, (S1), lane 3, (S4), lane 4 (S5), lane 5 (S7), lane 6, (S9), lane 7 (S13), lane 8, (S16), lane 9, (S 21), lane 10 (V245), lane 11 (V275)

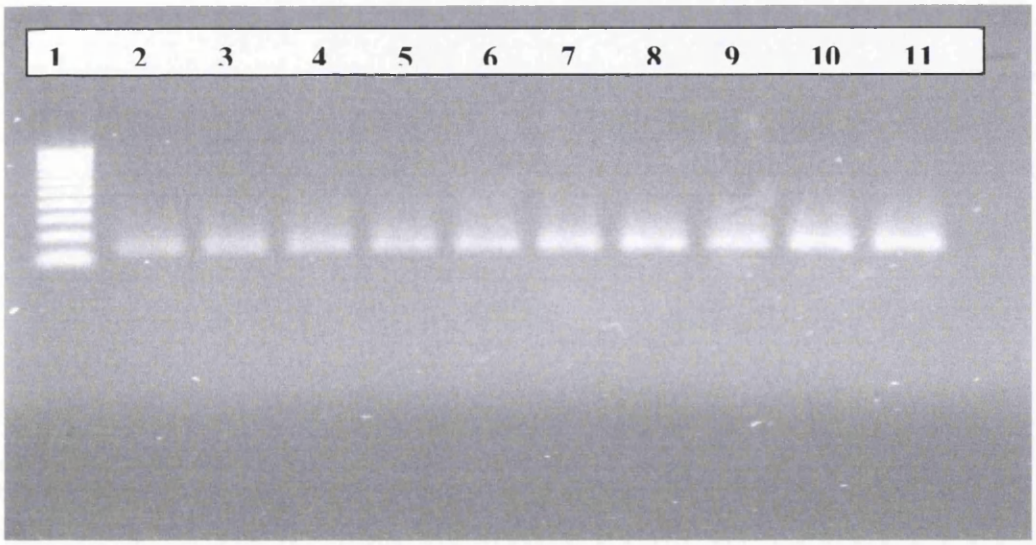

Fig.9 Real Time PCR product (147 bp expected size) of the *chy 1* gene from the *M. anisopliae* sector and parent cultures. Lane 1 (100bp Ladder), lane 2, (S1), lane 3, (S4), lane 4 (S5), lane 5 (S7), lane 6, (S9), lane 7 (S13), lane 8, (S16), lane 9, (S 21), lane 10 (V245), lane 11 (V275)

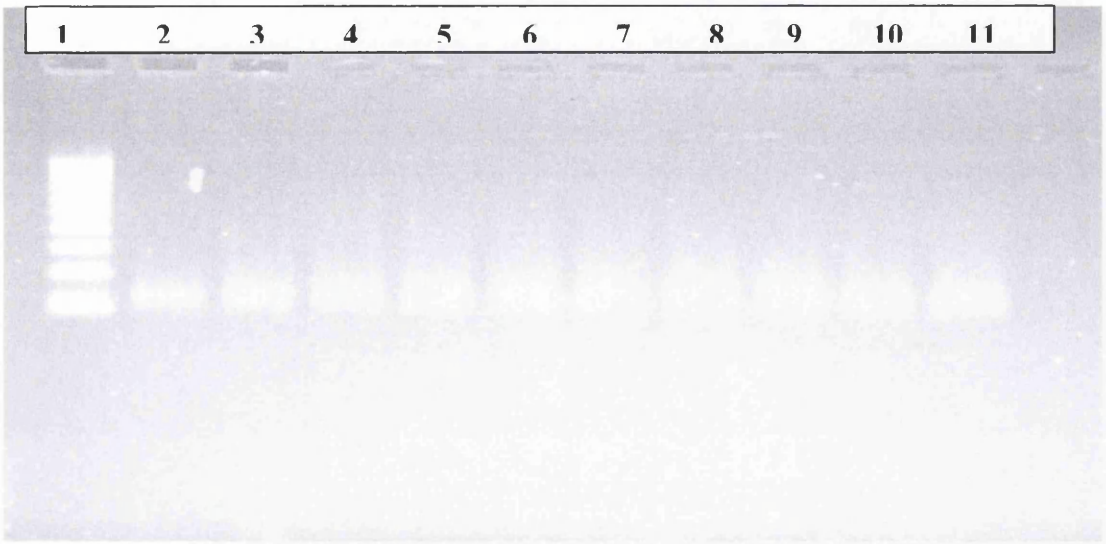

Fig.10 Real Time PCR product (120 bp expected size) of the *try 1* gene from the *M. anisopliae* sector and parent cultures. Lane 1 (100bp Ladder), lane 2, (S1), lane 3, (S4), lane 4 (S5), lane 5 (S7), lane 6, (S9), lane 7 (S13), lane 8, (S16), lane 9, (S 21), lane 10 (V245), lane 11 (V275)