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Evaluation of exotic and South African isolates of *Beauveria bassiana* as potential mycoacaricides of *Tetranychus urticae* Koch

Nainisha Morar Bhana

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science.

Johannesburg, 2007

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____

NAINISHA MORAR BHANA

_____ day of _____, 2007.

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ABSTRACT

Economic losses caused by the two spotted spider mite (*Tetranychus urticae* Koch) in the agricultural industry prompted the initiation of this project to evaluate the potential of native and exotic *Beauveria bassiana* isolates as biocontrol agents against this pest. Before the selection of a potential mycopesticide for use at a commercial scale, several crucial criteria must be taken into consideration. The application of some of these criteria have been the focus of this study and include: production of virulent *B. bassiana* inoculum, an evaluation of the virulence of the fungus against *T. urticae*, monitoring of the fungal infection cycle in *T. urticae* and establishing a phylogenetic evaluation of *B. bassiana* isolates using rDNA sequence analysis.

A two-stage *in vitro* diphasic fermentation process produced *B. bassiana* inoculum. In the first submerged phase the impact of nutrient treatments (carbon and nitrogen) at different concentrations (3% and 4%) in a 1:1 ratio was investigated for mycelium dry mass production and spore yield. The 4% nutrient concentration yielded a higher mycelium dry mass yield compared to the 3% and was therefore used in the second semi-solid phase to stimulate aerial conidia formation in response to low nutrient stress. The fungal structures produced during the first phase of the diphasic fermentation process were submerged conidia recognized as small, spherical structures with a smooth form. In comparison, inoculum of the second semi-solid phase produced aerial conidia with small, spherical, rough surfaces and a brittle appearance assumed to be related to nutrient deprivation. Nutritional parameters exploited in this study favoured conidia production for use as a potential mycopesticide.

An *in vitro* bioassay compared the infectivity of exotic and native isolates of *B. bassiana* against *T. urticae* adults. All the isolates were pathogenic with mite mortality increasing over time. Differences in the virulence of the *B. bassiana* isolates were demonstrated suggesting host-specificity. With respect to the native isolates *B. bassiana* (PPRI 04305) was more virulent than the *B. bassiana sensu latu* isolates (PPRI 04304 and PPRI 04306). The differences in the virulence of

the native isolates are reflections of genetic differences demonstrated in the phylogenetic analyses in this study. The results of the preliminary bioassay study suggest that *B. bassiana* has the potential as a biocontrol agent of *T. urticae*.

Microscopy was used to morphologically visualise the post infection cycle of a native *B. bassiana* isolate (PPRI 04305) in the two-spotted spider mite. The infection cycle observed in the current study is in agreement with those described in a number of agricultural pests. However, aspects not observed before with *T. urticae* infection included limited hyphal growth on the cuticle surface before penetration, *per os* mode of entry, cuticular melanization, lateral hyphal development under the cuticle and aerial hyphal emergence through the setal annulum on the dorsal surface of the cadaver. These observations will stimulate further research in the development of *B. bassiana* as a mycoacaricide.

rDNA analysis of the ITS1-5.8S-ITS2 regions of different *Beauveria* species was assessed for strain genotyping and population studies. Fitch parsimony and neighbour joining analyses displayed species differentiation and confirmed that *B. bassiana* was not a monophyletic group but a species complex. Distinct clades in the phylogenetic analyses in the current study were matched to four species of *Beauveria*: *B. bassiana, B.* cf. *bassiana, Beauveria brongniartii and Beauveria caledonica* species. Two South African isolates PPRI 04304 and PPRI 04306 morphologically assigned to *B. bassiana*, are assumed to be either *B. caledonica* or a close relative of *B. caledonica* based on the rDNA analysis. However, due to the lack of confirmation of the change of species identification of these native isolates, they are regarded as *B. bassiana sensu latu*. Results from this study demonstrated the importance of rDNA analysis in biocontrol studies for population studies and species differentiation.

The material in this dissertation highlighted some important characteristics relevant for the biocontrol of *T. urticae* by *B. bassiana*. Aerial conidia produced by the cost-effective diphasic fermentation process were virulent against *T. urticae* and demonstrated high percentage mortalities. *B. bassiana* was shown to

be a generalist pathogen with strain-dependent differences in nutrient preferences and virulence against the mite. Differences in the infectivity of the native isolates *B. bassiana* (PPRI 04305) and *B. bassiana sensu latu* (PPRI 04304 and PPRI 04306) were reflections of the genotypic separation of the isolates demonstrated by rDNA analysis. The results obtained from this research project are promising for the ongoing research and development of *Beauveria* isolates as efficient mycoacaricides against *T. urticae* for the South African agricultural market.

Dedicated to my husband

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CHAPTER 1

Literature Review

1.1. INTRODUCTION AND BACKGROUND TO PROJECT

Humans share the planet with as many as 10 million species of organisms and are dominant in various ecosystems relying on other species for food (Hajek, 2004). With an increase in the global human population, humanity is facing challenges in the twenty first century pertaining to food production and pest control (Thacker, 2002).

Man himself has created the pest problem through an increase in the international exchange of infected material (seeds, plants and soil) and also by growing large areas of new and ill-adapted crop varieties (Dent, 1995). Arthropods like insects and mites are regarded as crop pests when they conflict with human welfare, aesthetic value and profits (Gullan and Cranston, 1994).

Challenges in the agricultural sector pertaining to the control of pests have led to the extensive use of chemical pesticides with a global market of $34 billion in 1995 (Hegedus and Khachatourians, 1996). However, continuous application of broad-spectrum chemical pesticides to large scale monocultural crop production systems has resulted in the development of resistance by insect pest populations to these pesticides (Burges and Hussey, 1971). Lacey and Goettel (1995) state that the long term application of pesticides to crops results in the deterioration of natural ecosystem function as contaminating pesticidal residues accumulate in the soils and ground water systems. Some European Union members proposed to decrease chemical control by 50% over a 10 year period (Butt *et al.,* 2001). Pest control programmes have since then dramatically declined pesticide usage to $6 billion (Hegedus and Khachatourains, 1996).

One of the primary objectives in agriculture has been to rely on eco-friendly pest management programmes (Lacey and Brooks, 1997; Aslan *et al.,* 2004). Biological control has been documented as an appropriate alternative approach for plant protection (Shipp *et al.,* 2003) and thus major research efforts with biological control (botanicals and microbes) have been spearheaded (Khetan,

2000). Biological control is termed by Eilenberg *et al.* (2001) as the use of microorganisms to suppress the population of another specific organism, making the specific organism less abundant or less damaging than it would otherwise be (cited in Hajek, 2004).

Biological control using Entomopathogenic Fungi (EPF) has made rapid advances in the plant protection sector worldwide in the 20th century. This has resulted in the development of commercial products that have penetrated the agricultural pest management sector (Khetan, 2000). EPFs have been reported to infect pests with sucking mouthparts such as aphids, whiteflies and spider mites (Khetan, 2000). Spider mites (*Tetranychus urticae* Koch) chosen in this study as the host pest comprise a large group of plant-feeding pests. The spider mites are reportedly the most destructive Acarine pests of over 150 agricultural crops and plants worldwide (Magdalena and Meyer, 1996).

An EPF, *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycota: Hyphomycetes) has one of the longest histories as an experimental mycoinsecticide (Leathers *et al.,* 1993). Agostino Bassi in the 19th century demonstrated that *B. bassiana* caused a white muscardine disease of the silkworm *Bombyx mori* and the fungus was used for medicinal purposes as an antiseptic for wounds and sore throats. Thus, the very first microorganism recognized as a disease-causing agent in insects was a fungus (Thacker, 2002). Since then, the cosmopolitan *B. bassiana* has become a long-standing model as an agent for the world-wide biocontrol of many crop pests such as sweet potato whitefly, colorado potato beetle, european corn borer and migratory grasshoppers (Alexopoulos *et al.,* 1996; Shipp *et al.,* 2003).

B. bassiana, is available on the commercial market as a registered mycoacaricide (Castrillo *et al.,* 1999). A Troy Bioscience Product Naturalis ™ is reported to give excellent control of *T. urticae* on glasshouse roses (Mietkiewski *et al.,* 2000). *B. bassiana* strain GHA has been produced and marketed as Mycotrol® and Botanigard® as a wettable powder and oil-based formulation for control against whiteflies and other leaf feeding insects in the field (Van der Geest *et al.,* 2000).

In South Africa, Biological Control Products sells Bb Plus® to control aphids and the two-spotted spider mite for gardens, tunnels, glasshouses and intensive production units. Due to its economic importance in the South African pest control industry, *B. bassiana* was chosen in this study as a potential mycoacaricide.

In recent biotechnological innovations for the development of a commercial product, a selected EPF isolate must be mass produced cost effectively and display high pathogenicity to the target pest. Molecular techniques must also be attempted to study the taxonomy and population structure of the isolate (Feng *et al.,* 1994; Hegedus and Khachatourians, 1996). These criteria have been the foundation of this study and are reflected in the objectives listed below with some background information thereafter.

1. *To optimise inoculum production in a low-cost diphasic fermentation method for the production of high concentrations of highly virulent infectious conidial structures.*

   Many production techniques have been reported for mass producing EPF such as *B. bassiana* and these are discussed in section 1.6. In this study the diphasic fermentation process was chosen for the production of large quantities of inexpensive *B. bassiana* inoculum. This mass production technology is essential for the development and application of *B. bassiana* to compete with the existing chemical control (Bartlett and Jaronski, 1988).

2. *To determine the basis of virulence and variability of South African B. bassiana strains compared to exotic strains against the two-spotted spider mite under laboratory conditions.*

   Few studies on the infectivity of *B. bassiana* against the two-spotted spider mite have been carried out worldwide (Van der Geest *et al.,* 2000) whilst no studies to our knowledge have been conducted in South Africa. The rationale behind this study was to study the variability in virulence between South African *B. bassiana* strains and exotic strains so as to select for the best

candidate strain as a biocontrol agent against the two-spotted spider mite. Strain evaluation and selection by bioassays is an imperative component in the development of *B. bassiana* for biocontrol.

3. *To observe the infection cycle of the pathogen on and in the host by scanning electron microscopy, light microscopy, confocal scanning laser microscopy and phase contrast microscopy.*

   Understanding the mechanism for the biocontrol of agricultural pests requires visualization of host-pathogen interactions (Connick *et al.,* 1999). The post-infection cycle of a native *B. bassiana* strain (PPRI 04305) was thus monitored using a range of microscopy techniques.

4. *To determine phylogenetic relationships between exotic and South African strains of B. bassiana using PCR amplification and direct sequencing of ribosomal DNA (ITS1, 5.8S gene and ITS2).*

   The advances in biotechnology have led to research and development of molecular fungal detection systems to address questions of EPF taxonomy, identification and genetic variation (Leathers *et al.,* 1993, Hegedus and Khachatourians, 1996). Many methods exist to characterise the DNA of EPF; however, only selected key techniques have been reviewed later in section 1.8. The current study focused on obtaining rDNA sequence information of the representative South African *B. bassiana* strains and to reveal evolutionary relationships of these isolates in relation to exotic isolates from the *Beauveria* genus.

All the above objectives were integral for the present study since fungal strains vary in all respects, thus the most likely candidate is the strain that produces the best results in all the experiments. Laboratory scale research and development of *B. bassiana* will serve as an important step in the studies of the biology, nutrition, genetic diversity and pathogenesis of this fungus against *T. urticae*. This study forms a small part of the programme aimed at developing strains of *B. bassiana* as mycoacaricides against *T. urticae*.

1.2. TWO-SPOTTED SPIDER MITE (*TETRANYCHUS URTICAE* KOCH)

1.2.1. Distribution of the two-spotted spider mite

Mites (Acari) are small arthropods related to spiders and the two-spotted spider mites have been placed in the order Prostigmata. *Tetranychus urticae* Koch (Acari: Tetranychidae) is a plant sucking pest in both greenhouses and the outdoor environment, and is distributed world-wide. It is regarded as one of the most important pest species in the family Tetranychidae (Magdalena and Meyer, 1996). The two-spotted spider mite has been commonly found on wild and cultivated plants, including apple, citrus fruit, papaya, corn, cucumber, aubergine, peach, plum, tomato, cotton, beans, capsicum and strawberries (Schuster and Murphy, 1991; Rott and Ponsonby, 2001; Waite, 2001), and occasionally on grasses (Coates, 1974). In southern Africa, *T. urticae* was first recorded in the Transvaal in 1970 (Annecke and Moran, 1982). Since its introduction into South Africa, *T. urticae* has spread extensively and has displaced other closely related mites (Annecke and Moran, 1982). To date, in South Africa it is considered to be the most abundant mite feeding on higher plants with more than 200 host plants including maize, grain, sorghum, plants in glass houses and plastic tunnels, and chrysanthemums and certain vegetable crops, including cucumbers and tomatoes (Annecke and Moran, 1982; Magdalena and Meyer, 1996).

1.2.2. Damage caused by *T. urticae*

The agricultural system in the 21st century provides an ideal environment with high quality host crops for spider mite population growth. Constant irrigation and fertilization also increases the food quality of crops for the spider mite. Further, continued application of increasing doses of pesticides has reduced the natural enemies of mites discussed briefly in section 1.3.2.5. Worldwide the seriousness of mite pests has become accentuated by its rapid reproduction rate and the development of resistance to acaricides (Meyer-Smith and Craemer, 1999). Recently in South Africa, Korea, Spain and China farming and non-farming populations have been reported to be sensitized to spider mite allergens with the result that many suffer from asthma or bronchitis (Jeebhay *et al.,* 2003). Spider

mites thus rank the highest in importance amongst Acari as agricultural pests' world-wide (Meyer-Smith and Craemer, 1999).

Mites are often associated with the lower surfaces of leaves, buds and flowers. Spider mites have tiny mouthparts (stylets) used for piercing individual plant cells, such as epidermal tissue, to enable them to feed off cells of the palisade layer and the spongy mesophyll (Lacey and Kaya, 2000). Characteristic yellow or white chlorotic speckles and stipplings occur on leaves that become convex during light infestation since the mite initially feeds on the lower surface of the leaf. They are difficult to detect as they spend much of their time on the underside of leaves, within tender buds and even deep within flowers. Spider mites are also considered to be difficult pests due to their small size. As the mite population increases they spread throughout the plant (Fig. 1.1) (Lacey and Kaya, 2000).

Large unicellular glands located in the palpi of mites produce silk webbing (Krantz, 1978). This webbing protects the mites since the mites hide under the webbing canopy. Silk threads also act as a sail to facilitate the migration of the fastidious mites by wind from infested field crops to other crops and wild plants that all serve as pools of new infestations (Wilson and Sadras, 2001). In the greenhouse, clothing and implements serve as dispersal mechanisms (Magdalena and Meyer, 1996). Heavy infestation on foliage lowers the vitality, distorts buds and causes defoliation which reduces photosynthesis and the stress induced on the plants causes the flower petals to darken (Fig. 1.1) (Wilson and Sadras, 2001). After infestation the leaves turn brown, dry out and drop off (Magdalena and Meyer, 1996; Mersino, 2000). In summer the mites reproduce exponentially (Magdalena and Meyer, 1996).

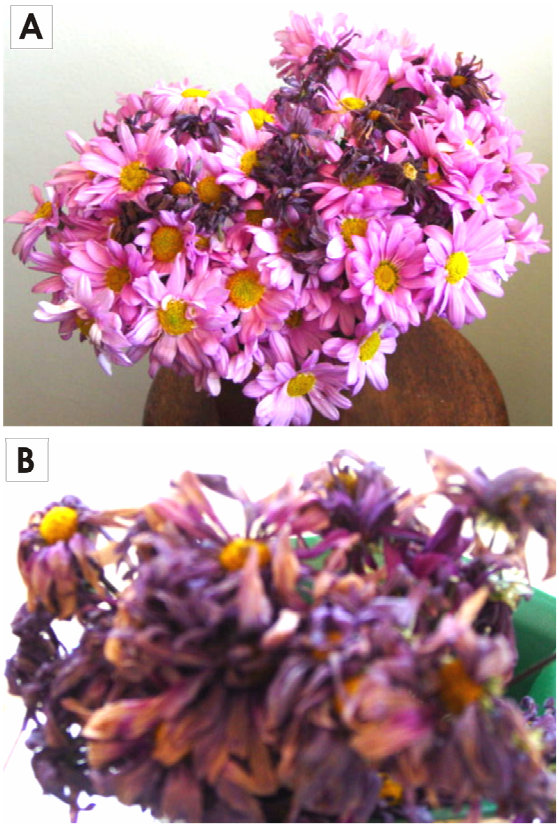

Figure 1.1: A representative view of (A) chrysanthemum flowers (light purple) without spider mites. Destruction of the mite colonized flowers (B) is apparent by darkening of the petals (dark purple-black).

1.2.3. Biology of the two-spotted spider mite

The life cycle of the male is shorter than the female. The male undergoes the same number of developmental stages as the female but the time spent on each stage is slightly shorter. Haploid males develop from unfertilized eggs and diploid females from fertilized eggs (Davidson and Raupp, 1988; Berry, 1998). Sex ratios of the mites vary but are generally 3:1 female to male (Wilson and Sadras, 2001). The male has a thin, straw coloured body ending in a sharp point. Males have longer legs and are more mobile than the female (Coates, 1974). Adult females in summer are oval, 0.5mm long and brownish-red, straw coloured or green with two dark spots on the upper surface, the legs are pale to yellowish (Magdalena and Meyer, 1996).

The colour of the exoskeleton may vary according to their diet and environment. In autumn due to short days and poor host quality the diapause females become orange to brick red (over-wintering) and creep into protected places such as on the underside of leaves or on soil surfaces to hibernate (Annecke and Moran, 1982; Davidson and Raupp, 1988). The mite is non-feeding and non-reproductive at this stage (Wilson and Sadras, 2001).

Virgin and mated females lay eggs (Wilson and Sadras, 2001). In the mite's life cycle, round eggs (Fig. 1.2) are laid near the veins of leaves on fine silk webbing. The colour of the eggs vary from pearly-pink, light red or ivory white (Fig. 1.2) (Magdalena and Meyer, 1996). Females can lay up to 12 eggs daily and 100 in a lifetime, over a period of 3-4 weeks. The eggs turn orange and hatch within 5 days at 25-30ºC and 45-55% relative humidity (Sanderson, 1990). Round bodied larvae (Fig. 1.3) with three pairs of legs are orange then turn green as they feed on chlorophyll. The larvae feed for a few days then seek sheltered areas to rest and molt into the protonymph. The protonymph is also green. The nymphs show variation in colouration, but are normally pale green or yellow with two green spots (Coates, 1974; Davidson and Raupp, 1988). After the quiescence period the protonymph moults into the deuteronymph (Magdalena and Meyer, 1996). The deuteronymph (Fig. 1.2) is initially translucent and after feeding for *ca*: two days it turns green. The deuteronymph also feeds for a few days before molting into the adult female or male. The adult mites begin laying eggs within 1-3 days (Fasulo and Denmark, 1989; Sanderson, 1990; Berry, 1998). The nymph and adult stages have four pairs of legs (Wilson and Sadras, 2001). Temperatures of 26 ˚C to 30 ˚C are optimal for mite development. High temperature and low humidity lead to outbreaks of tetranychid mites whereas low temperature, high humidity and low plant quality induces diapause (Wilson and Sadras, 2001).

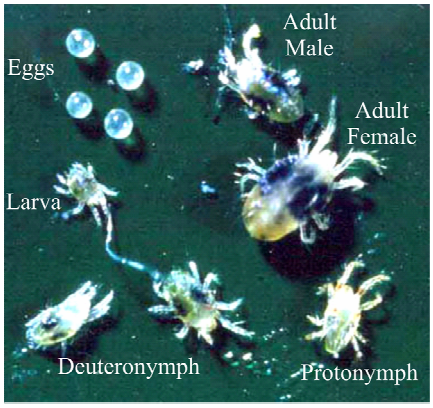

Figure 1.2: General overview of the life cycle of a typical red spider mite. (http://www.agf.gov.bc.ca/cropprot/tfipm/mites.htm)

1.3. CONTROL OF THE TWO-SPOTTED SPIDER MITE

1.3.1. Chemical control

Chemical pesticides are predominantly used in the control of *T. urticae* due to their ready availability, ease of application, effectiveness, low cost and the lack of economically reliable alternative control measures (Sanderson and Zang, 1995). Some examples of chemical control include organophosphates, abamectin, fenpropathrin, dicofol, pyridaben, fenpyroximate and methyl bromate (Motoba *et al.,* 2000; Rauch and Nauen, 2003; Simpson *et al.,* 2004; Recep *et al.,* 2005). Spider mite control with two types of acaricides, contact and systemic acaricides, are currently used to reduce spider mite foliar damage (Lacey and Kaya, 2000). Efficacy of contact acaricides depends on direct physical contact and requires a maximum level of exposure of mites to the chemical agents. Systemic products which are absorbed by the plant are ingested by mites feeding on the plant; however, only young and actively growing plants are in a physiological state to absorb systemic acaricides (Lacey and Kaya, 2000).

Although acaricides (chemical control) play an important role in the control of the two-spotted spider mite, mites still continue to cause a significant yield loss in field and greenhouse crops (Kim and Seo, 2001).

Examples of chemical control problems are listed below:

- Pyrethoids used to control the Southwest corn borer in the U. S. A. has led to outbreaks of spider mites.
- Cross or multiple insecticide resistance often occurs due to the insecticide having specific attack sites so that when a single mutation occurs in the pest genome it has been sufficient to alter or remove the sensitive site.
- During spraying programmes rotation of different acaricides with different chemical compositions are implemented to combat resistance, but this has led to economic losses and major environmental pollution.
- Restriction or elimination of numerous pesticides used for the control of *T. urticae* in the floriculture and greenhouse industry has occurred. Also, the registration of many of the chemical pesticides will not be renewed or the pesticide industry will prefer not to renew registration of the pesticides due to high costs and low profits.

(Davies, 1992; Perfect, 1992; Lacey and Goettel, 1995; Magdalena and Meyer, 1996; Bynum *et al.,* 1997; Aslan *et al.,* 2004)

In the past few years alternative approaches to chemical control have been attributable to various reasons such as a general increase in the public's awareness of the potential adverse hazards of pesticides on the environment with its bioresources (Burges, 1988), concerns for human safety and governmental regulations concerning the contamination of food with harmful pesticide residues (Coates, 1974; Metcalf, 1999; Opit *et al.,* 2004). The current scenario demands an urgency to develop Integrated Pest Management Programmes worldwide (Aslan *et al.,* 2004).

1.3.2. Integrated pest management (IPM)

In the 21st century, agriculture relies heavily on environmentally friendly pest management options (Lacey and Brooks, 1997). IPM has been developed into an alternative approach to the conventional chemical based pest control system, currently used by the agricultural industry. The primary objectives of this approach ensures that levels of pest damage of crops are maintained below

economically damaging threshold levels and that the adverse impact of chemicals in the environment should be reduced to minimal levels so that a medium to long-term sustainable food production system will be maintained (Thacker, 2002).

Integrated control involves a range of pest management practices that combine the use of chemical pesticides with other interventions such as: biological control, plant resistant cultivars, cultural control, genetic manipulation of pests and use of selective chemicals e.g. pheromones and pesticides (Rabbinge, 1976; Thacker, 2002). These methods are discussed briefly below for use against *T. urticae*.

1.3.2.1. Cultural and physical control

Cultural and physical controls are a form of manipulating naturally occurring processes for pest management (Hajek, 2004). Since *T. urticae* outbreaks are unpredictable, regular scouting of the crops to identify the level of infestation and burning of infested plants in the hot dry months minimizes an escalation in crop infestations. Weeds are common mite hosts and are eliminated to prevent the spread of the two-spotted spider mite. Also, separation of infested and newly planted crops reduces the spread of mites (Magdalena and Meyer, 1996).

An increase in plant vigor and reduction of crop stress by irrigation and fertilization has been documented to reduce the susceptibility of plants to *T. urticae* infestation (Walsch *et al.,* 1998). Furthermore, the following strategies can reduce pest establishment: crop cover, mulching, crop rotation and usage of vermicompost (Arancon *et al.,* 2005). Mites can be dislodged from plants by a quarantine method of hot water immersion and spraying affected plants with a steady stream of water (Lester *et al.,* 1997).

1.3.2.2. Plant genetic engineering

Breeding and selection of mite resistant plants remains one of the foundational crop protection strategies. Genes from different *Bacillus thuringiensis* (Bt) strains have been incorporated into some plant varieties that are full or partially resistant against pests. However, commercialization of transgenic products to control the

two-spotted spider mite is a few years away due to a decrease in predacious mites (natural enemies of *T. urticae*) in transgenic plants being documented (Rovenska *et al.,* 2005).

1.3.2.3. Autocidal control

Chemosterilants are generally applied only to pupal, larval or adult stages. Unlike the other control strategies this method focuses more on reducing birth rates than on the death of the pest. This method has been studied in the agrochemical industry against *T. urticae* in great detail. Mating behaviour and dispersal of the pest is required for autocidal control. Sterility induced by chemicals occurs by various mechanisms, such as prevention of mating, failure to produce sperm and/or ova and production of active sperm or ova (Butt and Goettel, 2000).

1.3.2.4. Botanical pesticides

Chilli, garlic and soap extracts are currently been tested against the two-spotted spider mite in Namibia. Neem and Tephrosia have been evaluated against the spider mites in Malawi, Zimbabwe and Kenya (Keizer and Zuurbier, 1998). Recep *et al.* (2005) used insecticidal soaps containing potassium salts and fatty acids against the two-spotted spider mite.

1.3.2.5. Biological control

In recent years biological control has become a major avenue in modern agriculture and has increased in stature (Rabbinge, 1976). Biological control is often defined as “the use of a living organism that suppresses a specific pest organism, making it either less abundant or less damaging then it would be” (Hajek, 2004). Bacteria, fungi, nematodes, protozoa and viruses are entomopathogens regarded as key biocontrol components of IPM (Bidochka *et al.,* 2002; Thacker, 2002) in pest control programmes.

Human intervention by manipulation is performed by enhancing numbers of or introducing new natural enemies of target organisms resulting in safer and environmentally friendly alternatives of pest control (Burges and Hussey, 1971;

Gullan and Cranston, 1994; Hussein, 1999). Complete eradication of the pest is not the aim since it will disturb the balance between the pest and the biological control agents (Schuster and Murphy, 1991). Thus, the success of biological control depends on the activities of these natural enemies in order to facilitate the reduction of density and distribution of the pest and eventually the potential agricultural damage (Gullan and Cranston, 1994).

1.3.2.5.1. Biological control agents

A. Predator

The use of predators for two-spotted spider mite control has received the most attention in the biocontrol industry (Colfer *et al.,* 2004). To date predators including chilean predatory mite (*Phytoseiulus persimilis*), black lady beetles (*Hippodamia convegens*), lacewings (*Chrysoperla carnea*), minute pirate bugs (*Orius insidiosus*) and western predatory mites (*Metasciulus occidentalis*) have been used to control *T. urticae* (Kongchuensin *et al.,* 2001; Jeyaprakash and Hoy, 2004).

Predators are normally susceptible to pesticides such as Carbaryls and Malathion, thus research has been stimulated on pesticide selectivity in the IPM system (Magdalena and Meyer, 1996). Also, although predators are important natural enemies of *T. urticae* they are fragile and the cost of rearing them is higher than pathogens (Hajek, 2004). Another disadvantage is that predacious biocontrol agents are not effective in all crop protection areas (Colfer *et al.,* 2004).

B. Entomopathogens

Entomopathogens include bacteria, fungi, viruses and nematodes used for control of insects and mites. Entomopathogens can be advantageous over predators due to a short generation time, vast amount of propagule production and their application by traditional equipment. Entomopathogens cause diseases in the host by infection, parasitism and/or toxaemia leading to death (Lacey and Brooks, 1997; Van der Geest *et al.*, 2000).

T. urticae has been reported to be susceptible to *Bacillus thuringiensis* (Bt), especially the immature stages. However, spore crystals of this bacterium did not induce mortality of the two-spotted spider mite (Van der Geest *et al.*, 2000). *Wolbachia* are cytoplasmically transmitted bacteria that are obligate symbionts. These bacteria colonize the host by transmission from females to offspring, thus, termed sexual parasites. *Wolbachia* manipulates host reproduction by increasing the number of infected females and by cytoplasmic incompatibility in *T. urticae* (Vala, 2000). A disadvantage in using this bacterium is that if the same bacterial strain present in the male is also present in the fertilized egg, cytoplasmic incompatibility is not induced (Vala *et al.,* 2003). Virus and nematode diseases in *T. urticae* have not been reported but studies on other mites are documented. To date, most studies on *T. urticae* entomopathogens have concentrated on entomopathogenic fungi (Van der Geest *et al.,* 2000).

1.3.3. Entomopathogenic Fungi (EPF)

Work done by Bassi with *Beauveria bassiana* in 1834 proved that fungi cause diseases in silkworm larvae (Wilson and Sadras, 2001). From the 1880s until the present time, epizootics of EPF have led to intensive studies on their potential as pest control agents (Roberts and Hajek, 1992). More recently, EPF have gained considerable attention as potential commercial environmentally friendly agents. About 750 EPF species have been reported to infect insects yet extensive research has only been done on a few species (Sun and Fuxa, 2003).

Although other entomopathogens like bacteria and viruses have potential as biocontrol agents, fungi are of particular interest against arthropod pests since they possess the following useful attributes:

- EPF's use enzymes to breach the integument of the host through direct contact.
- The fungus replicates in the target organism and epizootics have been observed in the pest population.
- There are numerous strains of the same species of the fungus thus advantageous in determining host range of the different isolates.

- The EPF are easily mass produced.
  (Leathers *et al.,* 1993; Sun and Fuxa*,* 2003)

EPF intended for use in biological control are initially obtained from diseased insects or their environment, identified, grown and then evaluated under laboratory conditions or field trials to identify the most promising candidates. A pilot study on economical mass production of the fungus is required. The selected strain should grow rapidly, sporulate abundantly and have a high degree of virulence *in vitro* to target pests. (Burges and Hussey, 1971; Neuenschwander *et al.,* 1993; Butt and Goettel, 2000). EPF are widely distributed and some have been developed or are being developed as promising alternatives to chemical control (Table 1.1) (Wang *et al.,* 2005).

Table 1.1: List of potential mycopesticides and their hosts. Extracted from Roberts and Hajek, (1992).

ENTOMOPATHOGENIC FUNGI				HOST
Subdivision	**Class**	**Order**	**Genus**	
Mastigomycotina	Chytridiomycetes	Blastocladiales	*Coelomomyces*	Mosquitoes
	Oomycetes	Lagenidiales	*Lagenidium*	Mosquitoes
		Saprolegniales	*Leptolegnia*	Mosquitoes
Zygomycotina	Zygomycetes	Entomophthorales	*Entomophaga*	Grasshoppers
				Caterpillars
			Entomophthora	Flies
				Aphids
			Conidiobolus	Aphids
			Zoophthora	Caterpillars
				Weevils
Deuteromycotina	Coelomycetes		*Aschersonia*	Whiteflies
	Hyphomycetes		*Beauveria*	Caterpillars
				Mites
			Culicinomyces	Mosquitoes
			Hirsutella	Planthoppers
				Mites
			Metarhizium	Spittlebugs
				Termites
			Nomuraea	Caterpillars
			Paecilomyces	Nematodes
				Beetles
			Tolypocladium	Mosquitoes
			Verticillium	Aphids
				Mites

The use of entomopathogenic fungi as biocontrol agents against *T. urticae* in the agricultural industry may serve as a solution to the chemical pesticide problems such as the potential environmental contamination and mite resistance. Application of EPF biocontrol agents as an adjunct or as a supplementation of chemical control could improve the pest control economics and increase the sustainability of Acari control (Sun and Fuxa, 2003; Alves *et al.,* 2005).

Reports on fungal infection of phytophagous mites include:

- Infection of the *Tarsonemus spirifex* Marchal, *Botrytis eriophyes* and the mite *Tetranychus spirifex* have been reported by EPF *Hirsutella kirchneri, Eropyes ribes* and *Beauveria bassiana* respectively.
- *Hirsutella thompsonii* has been tested to control *T. urticae* in greenhouses but without success.
- EPF *Neozygites floridana* was reported to induce *T. urticae* mortality. However, culturing of the entomophthoralean fungi *N. floridana* is a drawback.
- *Tolypocladium verticillium* has been registered and is in use in the Peoples Republic of China and Russia as a mycoacaricide.

(Roberts and Hajek, 1992; Mietkiewski *et al.*, 2000; Van der Geest *et al.,* 2000).

1.4. CHARACTERISATION OF EPF

Before the selection and development of particular EPF isolates as potential mycoacaricides, the following criteria have to be considered.

- The strategy to be chosen for the control of the mite pest (section 1.5).
- The ease of production of the EPF isolates in suitable media (section 1.6).
- Pathogenicity of the fungi to the target pest and sporulation on the pest (section 1.7).
- Identification and diversity studies of the EPF isolates (section 1.8).

These characteristics mentioned in the sections above will be discussed below.

1.5. CONTROL STRATEGIES

Control strategies vary based on different types of pests with different attributes in different ecosystems (Hajek and St Leger, 1993). The control strategy chosen is dictated by cost effectiveness, limitations of exposure to non-target organisms, the target hosts and the biology of the natural enemy (Cooks *et al.,* 1996).

Entomopathogenic fungi may be employed under three control strategies:

1. Conservation.
2. Classical biological control or Importation.
3. Augmentation
    a. Inoculation
    b. Inundation

1.5.1. Conservation

This is classified as the oldest form of biological control aimed at modifying or enhancing the natural enemy population already present in the area in order to improve the environmental conditions which have been suppressed due to pesticide application. The conservation strategy can be implemented using EPF by luring, baiting by pheromones or food sources to allow the fungus to contact the host, delivered as spores and released as point-sources of inoculum to spread naturally or be carried by other agents to these sites (Cooks *et al.,* 1996). The current method promotes management practices such as the provision of increased moisture by irrigation, reduction in the usage of pesticides or over wintering sites of alternative hosts (Boucias and Pendland*,* 1998; Shah and Pell, 2003).

This approach has received the least amount of attention compared to the other control strategies because it is passive and directed towards a long-term control programmes and is not suitable for pest control in high value crops (Hajek, 2004).

1.5.2. Classical biological control or importation

The importation method is required when a pest arises as a side effect of a

pesticide or is introduced into an area without its natural enemies. Surveys conducted by researchers regarding the centre of origin of the pest serve to locate suitable natural candidate biocontrol agents which are then released into the newly pest-colonized area to re-establish control (Waage, 1999; Shah and Pell, 2003). Often it is difficult to identify which of the natural enemies, effective at the original location, will also be as effective at the new site. The rate of reproduction of the natural enemies needs to match that of the pests in order to suppress outbreaks. Also, a new attack strategy is sometimes required to be effective at the new site e.g. attack of the larval stages at the original site might need to be replaced to attack the pupal stage in the new location (Ehler, 1998).

A few attempts have been made at classical biocontrol such as the control of spotted alfalfa aphid using *Zoophthora radicans* in Australia (Milner and Lutton, 1986). Another classical example is *Entomophaga maimaiga* which was introduced into North America from Japan in the early 1900's and was theorized to work effectively at controlling the gypsy moth. Also, an Australian pathotype of *Entomophaga grylii* was introduced into U.S.A. to control grasshoppers and significant disease levels were documented (Leathers *et al.,* 1993). However, EPF have trailed far behind for classical biocontrol and the reasons are due to the stringent testing requirements by many government organisations for microbial agents (Leathers *et al.,* 1993; Hajek, 2004).

A number of disadvantages in classical biocontrol exist and are described below. This control strategy does not offer a profit motive for the commercial market (Leathers *et al.,* 1993) and negative environmental impacts arise through the ill-considered introductions of exotic biocontrol agents. The effects of the exotic biocontrol agents may lead to non target species suffering worse consequences than from chemical insecticides (Gullan and Cranston, 1994).

1.5.3. Augmentation

Augmentation is the periodic supplementation of existing biocontrol agents with those that do not establish permanently. The augmentative release is appropriate

due to a combination of good dispersal abilities with high reproduction rates of the biocontrol agent (Shah and Pell, 2003). *V. lecanii* is used against aphids, whiteflies and thrips in greenhouses, *M. anisopliae* against locusts and *B. bassiana* against numerous pests (Cooks *et al.,* 1996) by this method. Inoculation and inundation have strong commonalities and are thus grouped in the augmentation method, yet they have different goals and strategies with respect to performance (Shah and Pell, 2003).

1.5.3.1. Inoculation

Inoculation refers to the periodic release of biocontrol agents in small quantities. These biocontrol agents are unable to survive indefinitely, and do not follow a pest range that is expanding. In the inoculation method the repeated reproduction cycles of the natural enemy will maintain the pest population below economic thresholds (Gullan and Cranston, 1994; Shah and Pell, 2003). This form of control depends on the progeny of the biocontrol agents and not the initial colonizers i.e. this form of control establishes the biocontrol agent as a sustained population in the environment (Cooks *et al.,* 1996). It provides a more long-term and self-sustained control of pests (Hajek, 2004).

Inoculative biological control has also been used frequently with fungi such as *B. brongniartii* which is used against the cockchafer, *M. anisopliae* against spittlebugs and *B. bassiana* against pine moths (Roberts and Hajek, 1992). It has been attempted for the successful control of *T. urticae* by *Phytoseiulus persimilis* (predatory mite) in glasshouses (Leathers *et al.,* 1993).

1.5.3.2. Inundation

In inundative control the adult organisms are released and not the subsequent progeny. In this strategy, the biocontrol agent with a high population density is introduced to ensure maximum suppression or death of the pest (Cooks *et al.,* 1996).

With respect to using EPF for inundative control, the process involves adding commercial mycelial/conidial suspensions in a timely manner, often in

combination with synthetic materials to a field or glasshouse crop (Dress and Krutson, 1996; Boucias and Pendland, 1998). Microbes (biopesticides) of inundative forms are sold in a similar form to synthetic chemicals as wettable powders or flowable concentrates (Hajek, 2004). This method is appealing in areas where chemical resistance has developed. *B. bassiana*, for example is a fungal pathogen inundatively applied to many pests like pine caterpillars and corn borers in China. Commercial *M. anisopliae* in Brazil is used against spittlebugs in sugarcane, cockroaches and termites in the U.S.A (Hajek, 2004). Commercial *V. lecanii* is applied against whiteflies and aphids in greenhouses in Europe (Hajek, 2004).

Inundation is used in short-term crops since the natural enemies emigrate or die due to a reduction in their host population. This method is appropriate where damage thresholds are low and rapid control is needed early (Hajek, 2004). Thus, control in the form of biopesticides is rapid but short-term (Gullan and Cranston, 1994). The inundative control strategy is chosen for the bioassay procedure in this study with a high density conidial suspension of *B. bassiana* to suppress the pest *T. urticae*.

1.6. METHODS OF SPORE CULTURE

Before a fungus may be registered as a biocontrol agent large quantities of the inoculum need to be mass produced (Goettel, 1984). EPF are widely produced *in vitro* through fermentation on a small, medium and large scale, especially in developing countries. Different fungal strains and culture conditions influence the type of product obtained i.e. mycelia, blastospores or conidia (Cherry *et al.,* 1999; Santa *et al.,* 2005). The production of fungal propagules on different artificial media will be discussed below.

1.6.1. Submerged fermentation

In the submerged culture method, mycelia and mainly blastospores are produced during the fungal log phase of growth. In some groups, such as Hyphomycetes,

under certain growth conditions low quantities of conidia are also produced but these are short-lived. Deuteromycetes grow as hyphae for a short period and then in the yeast-like growth phase produce blastospores. These blastospores are formed by the shearing forces of mechanical action in liquid medium, induced by schizolytic separation at the septa or yeast-like budding from single parent cells (Feng *et al.,* 1994).

Submerged culture is performed under controlled sterile conditions and is advantageous for scaling up the process in a simple manner (Thomas *et al.*, 1987; Goettel and Inglis, 1997). Dimorphic filamentous fungi such as *M. anisopliae, B. bassiana, B. brongniartii, V. lecanii, P. farinosus* & *N. rileyi* have been shown to produce large amounts of infectious thin-walled oval, spherical or rod-shaped blastospores that germinate within 2-6 hr but are difficult to preserve (Butt and Goettel, 2000). Submerged fermentation was successfully employed in the USSR during the 1970s to produce Boverin® (Feng *et al.,* 1994). Large inflated plastic bags using a unique submerged culture method was developed in Czechoslovakia. In this method, mycelia are produced, harvested through filtration and subsequently coated with a sugar solution. To date this method is used mainly for the culturing of fastidious species such as *Zoophthora radicans* and also *M. anisopliae, C. clavisporus, B. bassiana* and *E. neoaphidis* (Butt and Goettel, 2000).

The advantages of submerged fermentation are:

1. The growth conditions can be controlled and manipulated to increase sporulation through the accumulation of various additives during the production process.
2. Spores are produced in a short time interval.
3. Cultivation and processing take place in sterile conditions and the scaling up process is easy.
4. Any material obtained from this culture may be used as inoculum.
5. Spores produced in submerged culture have proven to be equally or more virulent than the surface cultivation method.

6. Industrial fermentors may be utilized to hold up to 200m^3 of culture media.

(Khachatourians, 1986; Rombach, 1989; Feng *et al.,* 1994; Kassa *et al.,* 2004)

The disadvantages of submerged fermentation are:

1. Strict control of factors such as pH, temperature, foaming and aeration dictates the quantity and quality of the inoculum.
2. Mycelia and blastospores are produced which are thin-walled, unstable, short-lived with a low virulence, poor field stability and do not survive extreme environmental conditions.
3. Submerged spore production yields high amounts of mycelium that affect the drying and formulation for application in the field.

(Khachatourians, 1986; Feng *et al.,* 1994; Kassa *et al.,* 2004)

1.6.2. Solid substrate fermentation (SSF)

The function of solid state fermentation is to support the growth of micro-organisms with inert substrates for the aerial growth and sporulation of EPF (Kang *et al.,* 2005). Agar based media are used primarily for routine laboratory culturing of EPF and bioassays. At a large-scale, EPF grow on cheaper natural substrates e.g. wheat, rice, potato pulp, bran, cereal grains, egg yolk, agro-industrial residues, sorghum, beans and cassava. These moistened substrates placed in wide-mouthed jars, plastic bags, pans or tin trays with plastic tubing are autoclaved. After cooling a conidial or blastospore suspension is inoculated onto the solid substrate medium and incubated at room temperature for at least 10 days. After incubation the closed containers are opened and dried. The conidia are extracted through sieving, direct scraping or washing off with water or buffer, and finally sifted (Butt and Goettel, 2000). This system is carried out in the absence or near-absence of free-liquid but requires some moisture to support the growth and metabolism of the fungus (Burges, 1998; Butt and Goettel, 2000; Santa *et al.,* 2005).

The SSF is a method commonly used in China, Brazil and U.S.A. for *B. bassiana, B. brongniartii* and *M. anisopliae* production (Bradley *et al.,* 1992). This

fermentation technology widely used in Canada, Russia and Czechoslovakia for Colorado potato beetle control, involves a starch-based substrate that absorbs a liquid phase and as the fungus grows on the substrate the gaseous phase is available for aeration (Feng *et al.,* 1994).

The advantages of SSF are:

1. It is a simple process for laboratory bioassays when small amounts of inoculum are required.
2. The method of fermentation allows the fungus to sporulate naturally i.e. via aerial mycelium.
3. Fungi can be produced in deep tank fermentors for easy mass production. (Roberts and Hajek, 1992; Butt and Goettel, 2000).

The disadvantages of SSF are:

1. It is a labour intensive and time consuming process.
2. It is the most expensive fermentation process and is thus not economical. (Thomas *et al.,* 1987; Butt and Goettel, 2000).

1.6.3. Diphasic fermentation

Diphasic fermentation is one of the most widely studied and used methods for the mass production of EPF (Feng *et al.,* 1994). This method combines the submerged and solid substrate cultures (Rombach *et al*. 1988).

The first phase (submerged culture) in the diphasic fermentation process is aimed at increasing fungal biomass to the end of the log phase (Feng *et al.,* 1994). The second phase involves the transfer of the fungus to a solid substrate medium for the production of aerial conidia (Feng *et al.,* 1994). In the second phase, inert substrates such as cellophane, avoid contamination of the non-cellulytic conidia by the nutritional substrate. It is a slurry system with insoluble substances that provide physical support for fungal growth (Butt and Goettel, 2000). The substrates used in this fermentation method eliminate the need to use defined or semi defined media, thus reducing the cost of the process (Thomas *et al.,* 1987;

Goettel and Inglis, 1997). Drying techniques considered include vacuum drying, freeze drying, spray drying, mixing with inert filler and also drying in a fluidized bed (Feng *et al.,* 1994). After the drying process the fungus is applied as a dust (Bartlett and Jaronski, 1988). The conidial powder produced by the diphasic fermentation process can also be formulated as a wettable powder (Bartlett and Jaronski, 1988). Inoculum produced by the diphasic fermentation process is of high quality, safe and easy to use (Feng *et al.,* 1994).

Goettel (1984), described a simple, inexpensive method of culturing *B. bassiana*. He used cookware wheat bran in roasting pans and cellophane autoclaved in bags and obtained uncontaminated inoculum (Goettel, 1984). When Rombach *et al.* (1988) produced conidia by this method, they noted that after 3 years of storage at 4˚C that 95% of the conidia germinated. These conidia applied caused a 65-95% mortality of *Nilaparvata lugens* in the field (Brown plant hopper) (Feng *et al.,* 1994).

Tao *et al.* (1988) in China, surface cultured the fungus with a mixture of wheat bran, bean flour, corn flour and water for 10 days at 28˚C and produced a conidial powder (cited in Feng *et al.,* 1994). Alves and Pereira (1989) soaked rice in deionized water for 1 hr in autoclavable polypropylene bags then autoclaved the bags at 121˚C for 45 min. The rice was then inoculated with a conidial powder, mixed uniformly and incubated at 25˚C for 3 days to grow mycelium. The contents from the bag was poured onto plastic trays, covered with tray lids to retain moisture and maintained for 12-15 days to allow for conidial production to occur. Thereafter, the cultures were transferred to a 3˚C cold chamber and allowed to dry without the lid. Conidia were then sieved out (cited in Feng *et al.*, 1994).

In Czechoslovakia, the polyethylene cushion cultivation method has been adopted for the mass production of *B. bassiana* (Feng *et al.,* 1994). Sterile polyethylene tubing serves as cushions which are sealed into sections, inflated with air and partially filled with a liquid medium containing peptone and sorbitol. The fungal mat grows by floating in the tube, harvested by discarding the liquid and cultivating virulent aerial conidia for 12 days (Feng *et al.,* 1994).

Thus, from the above examples it has been demonstrated that entomologists and microbiologists, in the past decade, have improved on the diphasic fermentation process for EPF production by using cheap substrates and materials. This method has been widely used in developing countries, due to its simplicity and cost-effectiveness, thus chosen in this study to produce *B. bassiana* fungal inoculum.

The advantages of diphasic fermentation are:

1. It is a simple and reliable method for the production of economical fungal spores.
2. Even coverage on the substrate surface of the fungus results in homogeneous growth.
3. The competitiveness of the fungus is enhanced due to the growth rate. Furthermore, there is minimal risk that the solid substrate is contaminated by microorganisms.
4. The products are more virulent and have high resistance to adverse environmental conditions.
5. The aerial conidia remain stable and resilient after fermentation and during drying.

(Feng *et al*., 1994)

The disadvantages of diphasic fermentation are:

1. This culture technique is labour intensive and time-consuming.
2. This method is not well adapted to conventional fermentation.

(Rombach, 1989; Feng *et al*., 1994).

1.7. FUNGAL GROWTH AND ACTIVITY

Unlike bacterial and viral pathogens which need to be ingested in order to initiate disease, the entomopathogenic fungi invade their hosts by penetrating the cuticle and are regarded as biological contact insecticides (Deacon, 1983; Goettel *et al.,* 1995; Burges, 1998). Understanding the mechanism for the biocontrol of agricultural pests requires visualization of host-pathogen interactions (Connick *et*

al., 1999). A general schematic representation of the entire infection process is described in Figure 1.3.

1.7.1. Attachment, germination and penetration of conidia on the host cuticle

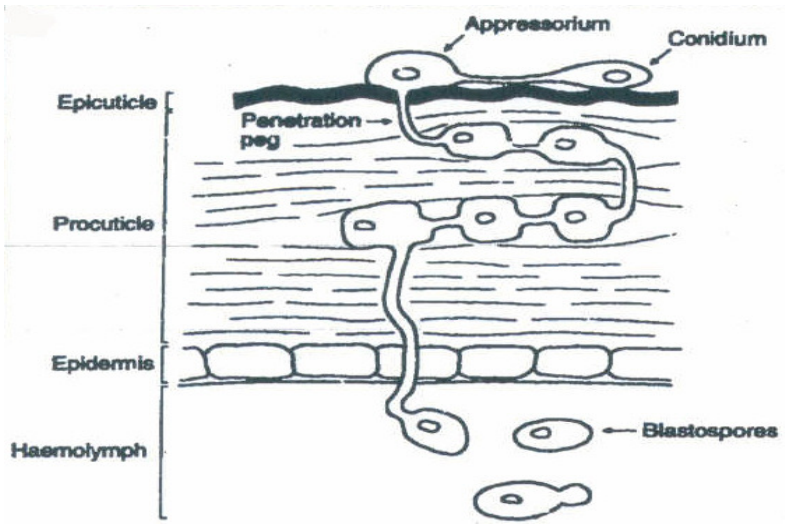

Figure 1.3: A diagrammatic representation of the fungal infection cycle in the host. Adopted from Clarkson and Charnley, (1996).

It is the surface topography as well as the chemical composition of the host cuticle which influences the attachment of the conidium to the host cuticle (Hajek and St. Leger, 1993; Lecouna *et al.,* 1997). Recently, exocellular enzymes have been detected and these include esterase, protease, lipase and N-glucosamidase. These enzymes have been suggested to attach the conidia firmly to the host cuticle and/or provide signals for germ tube formation and orientation (Boucias and Pendland, 1991; Goettel *et al.,* 1995). Due to a well organized rodlet layer the conidia of *B. bassiana* are air-borne, dry, hydrophobic and germination usually requires 8-16 hours (Burges and Hussey, 1971; Gillespie, 1988; Khetan, 2000).

The rapidity of infection depends on a number of biotic and abiotic factors, such as the nature and number of spores, as well as physical conditions of the environment (Sweetman, 1936; Deacon, 1983). Temperature, humidity and light

are critical factors for the germination of EPF infection progression. High humidity is fundamental for the germination of the spores on the host cuticle, whereas low humidity is required for spore formation and dispersal to new hosts (Coates, 1974). High humidity also favours the formation of epizootics (Ferron, 1978; Papierok and Hall, 1982). For most fungi the rate of mycelial development and hence the infection process depends on significant temperature ranges of between 20ºC-30ºC (Ferron, 1978). In Brazil, Daoust and Pereira (1986) found that exposure to sunlight for one week killed all conidia of *B. bassiana* whereas those protected from sunlight remained viable for over 3 weeks (Whipps and Lumsden, 1989).

Direct penetration of germinated hyphae through the cuticle involves a combination of both mechanical and chemical energy (Ferron, 1978). Mechanical damage occurs when the penetrating hyphae in the procuticle extend laterally to form penetration plates causing fractures in the epicuticle (Hajek and St Leger, 1993) and when a series of fungal extracellular enzymes (chitinase, protease and lipases) present in the fungus degrades the proteinaceous cuticle (Whipps and Lumsden, 1989). Antibiotic substances, such as beauvericin, produced by *B. bassiana,* may reduce competition with other fungi for nutrients during the saprotrophic stage (Whipps and Lumsden, 1989).

1.7.2. Mode of entry

Generally the mode of entry of EPF into the host is through the cuticle, anus, mouth, pores, under the elytra, the buccal cavity and the intersegmental membranes (McCauley *et al.,* 1968). Previous speculation suggests that the infection site studied in a laboratory is important to predict a field situation, as the behaviour of the pest organism influences infection (Clarkson and Charnley, 1996).

1.7.3. Melanization of the cuticle

The immune system of the host responds with humoral (phenoloxidases, lectins and peptides) or cellular (phagocytosis) immunity. Fungal components, β-1, 3

glucan and chitin are known to initiate the activation of the prophenoloxidase cascade in the host during the host's immune system response (Bidochka and Hajek, 1998). During the action of phenoloxidase phenolic substances synthesize melanin which induces blackening on the host cuticle and in the haemolymph (Ashida and Brey, 1995; Fuguet and Vey, 2004). Pasteur noted that the cuticles of healthy silkworms infected with *Nosema bombi* became dark around the injured area. Fungi *Beauveria brongnartii, Beauveria sulfurescens, Beauveria relata* and *Beauverai vermiconia* induce melanization of the host integument which is the earliest and the most obvious sign of infection (Fuguet and Vey, 2004). As the disease progresses infected pests may also exhibit changes in behaviour, such as loss of appetite and restlessness (Boucias and Pendland, 1998).

1.7.4. The blastospore stage

Entomopathogens are obligate pathogens which produce powerful insecticides that paralyze their hosts (Sweetman, 1936; Simmonds *et al.,* 1992). The enzymes seep into fractures of the procuticle created by mechanical damage mentioned in 1.7.1. This mechanical damage increases the surface area over which the enzymes act (Gullan and Cranston, 1994). *Beauveria* and *Verticillium* produce Bassionalide, a mycotoxin involved in the infection and destruction of the hosts tissue (Deacon, 1983).

The mechanical and chemical actions allow the septate mycelium to fragment in the haemocoel and are then transported throughout the body (Fig. 1.4) (Burges and Hussey, 1971). In the haemocoel the fungus grows as yeast-like blastospores without a formal cell wall but with a thin fibrillar layer of plasma membrane (Butt *et al.,* 1990). The ratio of surface area to volume of the blastospores is large and allows the fungus to obtain nutrients from various tissues and colonize the haemocoel (Sweetman, 1936; Clarkson and Charnley, 1996; Shah and Pell, 2003). The morphological transition of the fungus in the haemocoel is suggested to be an infection structure evolved to overcome host barriers and to adjust to the environment so as to colonize the host effectively (Hajek and St. Leger, 1993).

1.7.5. Death of host

EPF interfere with normal development of the host metamorphosis or the immune system causing weakening and partial paralysis (Boucias and Pendland, 1998). Once the infected pest stops feeding it slows down, becomes lethargic and dies within two to seven days (Khetan, 2000). A combination of fungal toxins, physical obstructions of blood circulation, nutrient depletion and organ invasion causes death of the pest (Hajek and St. Leger, 1993; Mietkiewski *et al*., 2000). The mycelium continues to attack the body until the cadaver is firm when touched (Khetan, 2000).

1.7.6. Production of aerial hyphae and conidiophores

The host cuticle at this stage is fragile and does not serve as a barrier anymore (Butt *et al.,* 1995). *Beauveria* produces oosporein after host death to suppress bacterial invasion of the cadaver so that the fungus is able to exploit the hosts tissues effectively (Deacon, 1983). The points where the aerial hyphae emerge are the integument, mouth, anus, intersegmental membranes on the ventro-lateral surfaces, and membranes at the base of the legs or the rest of the body (Kumar *et al.*, 1994; Kalkar, 2005). The aerial hyphae develop conidiophores which give rise to conidia (Khetan, 2000).

Water availability is important for fungal growth and small differences in the relative humidity levels will determine the effectiveness of pest control (McCauley and Zacharuk, 1968). Increased temperature and humidity produces additional infective propagules (conidia) by sporulation and conidiogenesis on the surface of the cadaver (Goettel *et al.,* 1995; Shah and Pell, 2003) thus completing the fungal life cycle (Whipps and Lumsden, 1989; Khetan, 2000). Spores that originate from germination on the cuticle of the infected pest spread to other hosts by a variety of routes such as wind and water (Whipps and Lumsden, 1989).

1.7.7. Mummification

During low humidity overwintering conidia inside the haemocoel occur (Butt *et al.,* 2001). These conidia have the ability to withstand adverse environmental

conditions and initiate growth at a later stage (Sweetman, 1936; Oduor *et al.,* 1995). The fungus transforms the cadaver into a mummy that is resistant to bacterial decay (Ferron, 1981). This mycelial persistence in cadavers is a survival mechanism for the fungus (Gillespie *et al.,* 2000).

However it is important to note that specialist (Entomophthoralean) and generalist (Hyphomycete) insect pathogenic fungi are present and also have distinct attributes regarding size of conidia and quantity produced, host range, toxin production in the host and also mummification (Butt, T. M. *pers. comm*.).

1.8. IDENTIFICATION STUDIES OF EPF

1.8.1. Morphological and biochemical analysis

In the past, fungal taxonomists have relied primarily on fungal conidial colony characteristics, vegetative compatibility and mating type to differentiate between individuals (Coates *et al.,* 2002). For example the genus *Beauveria* is generally characterised morphologically by conidiophores with whorls and dense clusters of sympodial, short, globose and flask-shaped conidiogenous cells. The apical denticulate rachi give it a zig zag appearance (Glare and Inwood, 1998). Yet, variation in identification of EPF have been based primarily on the dimensions of conidia, growth rates and enzyme activity which are dependent on the culture media used (St Leger *et al.,* 1992; Bridge *et al.,* 1993). The morphological characteristics used to classify EPF provide no isolate level characterization nor unambiguously resolve new isolates into defined species (Tigano-Milani *et al.,* 1995). The traditional microbial detection systems were useful in differentiating species or groups within species, but they generally lacked the resolution to distinguish a genet within a population. The historical based techniques also require isolation of fungi from pure cultures and propagation under a defined set of conditions to observe for reliable identification (Hegedus and Khachatourians, 1993; Entz *et al.,* 2005; Inglis and Tigano, 2006).

Isozymes are multiple molecular forms of a single enzyme. The molecular forms have similar enzymatic properties but due to differences in the nucleotide

sequences that code for the protein, have different amino acid compositions. Electrophoresis separates different isozymes based on differences in net charge and variations in size and shape. It is regarded as a simple and inexpensive method used to evaluate the taxonomy of fungi (Micales *et al.,* 1986). Isozyme analysis was used primarily to delineate morphologically similar taxa, identify factors that effect genetic variation such as environmental homogeneity and lack of sexual reproduction (St Leger *et al.,* 1992). Isozymes have also been successfully used to study inter- and intraspecific genetic variability of EPF (Tigano-Milano *et al.,* 1995).

The major drawback of using isozyme profiles includes the variation in enzyme synthesis during growth. Genetic diversity is often incorrectly assessed since only certain amino acid changes are reflected and catabolic repression events dependent on the growth medium influence isozyme production (Leal *et al*., 1994).

1.8.2. Genotypic data

Polymerase Chain Reaction (PCR) has been an essential tool in fungal biotechnology. White *et al.* (1990) described one of the first PCR applications in mycology with the fungus *Neurospora crassa*. Subsequently affordable automated sequencers have been developed and bioinformatics has provided valuable information with regards to the DNA sequences and their evolutionary relationship with different fungal species and strains (Drenth and Irwin, 2001).

Success in biocontrol programmes in the past few years has spear-headed interest in the study of taxonomy, genetic identification and molecular differences of EPF (Coates *et al.,* 2002). Research into the study of a vast number of molecular techniques has begun in order to study the sensitivity and selectivity required to track biocontrol fungi released into the environment (Uribe and Khachatourians, 2004).

Genetic variation inherent in the EPF can be detected based on genetic polymorphism, the sampling scheme and markers used (Leal *et al.,* 1994). Biological control programmes are now used to identify study and document the variation of particular fungal species (Coates *et al.,* 2002). Although studies pertaining to differentiation of EPF isolates are available in abundance, data for differentiating fungal strains is fairly recent (Coates *et al.,* 2002). A major effort in research has been devoted to describing intraspecific genetic variation of different populations from different geographic locations of the same species and to place the fungal isolate in a taxonomic rank (St Leger and Joshi, 1997; Bielikova *et al.,* 2002).

There are many molecular techniques available to study EPF. These differ in their ease of use, level of differentiation and specificity (Neuveglise *et al.,* 1997). These techniques are discussed briefly below.

1.8.2.1. Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism in nuclear or plastid DNA reflects differences in the primary sequences of DNA (Carder and Barbara, 1991). It is a DNA fingerprinting technique used to distinguish genotypic variations of EPF including *Metarhizium* and *Beauveria* species (Bidochka *et al.,* 2002). RFLP involves the identification of specific enzymes which cut the DNA at specific recognition sites. If more than one restriction enzyme is used the fragments are separated according to molecular size. The different fragment lengths reveal a pattern of DNA differences in individual organisms created by base substitutions, additions or deletions (Sreenivasaprasad and Mills, 1998). RFLP based markers have been studied extensively to address mating systems, discrimination of species, origin of strains, gene flow, host-parasite relations, symbiotic interactions and mechanisms of genetic recombination (Carder and Barbara, 1991). RFLP does not require prior sequence information, it is a simple and robust method which can be used to discriminate at a population or species level (single-locus probes) or individual level (multi-locus probes). RFLP is most suited for studies at

an intraspecific level or among closely related taxa (Bidochka *et al.,* 2002).

Mitochondrial DNA are small genomes co-evolving at their own rate in the organism in which they are lodged (Kouvelis *et al.,* 2004). mtDNA RFLP markers are maternal lineages used to track variation in EPF species and between strains (Hegedus and Khachaourians, 1993). Due to its small size, absence of methylation, ease in purification, high copy number and simple restriction enzyme patterns this fairly recent method is said to be ideal to study fungal evolutionary genetics (Laday *et al.,* 2004). Mutational changes in the length of mtDNA have shown variability of the resulting banding patterns as taxonomic characters for the purpose of cladistic and phenetic analysis of fungi such as *Tolypocladium nivea*, *M. anisopliae*, *V. lecanii*, *B. bassiana* and *P. farinosus* (Hegedus and Khachatourians, 1993).

There are a number of disadvantages in the RFLP method where large amounts of DNA are required for the time consuming and costly process (Pipe and Heale, 1990; Bridge *et al.,* 1993). Southern blot hybridization used to detect RFLPs polymorphism is laborious and incompatible with elevated levels of analytic inputs (Williams *et al.,* 1990). Finally, DNA probing methods may not exhibit the degree of sensitivity required for field samples (Destefano *et al.,* 2004).

1.8.2.2. Telomere fingerprinting

Due to difficulties experienced in the observations of chromosomes under a microscope, telomere fingerprints act as a substituted method to assess the chromosome number in EPF isolates such as *N. rileyi*, *M. anisopliae* and *B. bassiana*. The telomere fingerprinting method provides an ability to type individual isolates and assess the level of genetic polymorphism in the species. However, differences in chromosome numbers have been reported by various authors and are assumed to be attributed to chromosome number polymorphism in EPF (Padmavathi *et al*., 2003).

1.8.2.3. Random amplified polymorphic DNA (RAPD)

This technique is a PCR-based method that has been most intensively used to identify species and strains of EPF (Castrillo *et al.,* 2003). RAPD utilizes short arbitrary primers that anneal to multiple target sequences producing a diagnostic pattern. The method of polymorphic analysis does not require prior target sequence knowledge. It is easily adaptable for the study of numerous EPFs, especially those with poorly studied genomes. DNA sequencing or probe hybridization are not required (Castrillo *et al.,* 2003). RAPD is a sensitive technology that can detect single nucleotide changes between template DNA and primers (Castrillo and Brooks, 1998). The product is a spectrum of DNA fragments differing in length and nucleotide sequence (Bielikova *et al.,* 2002). RAPD detected variations in EPF such as *B. bassiana*, *M. anisopliae*, *M. flavoviride* and *P. fumosoroseus* (Castrillo and Brooks, 1998).

Since RAPD is performed under low annealing temperatures with non-specific primers, it is very sensitive to Taq DNA polymerase, quality of DNA template, primers and the thermocycle conditions during the PCR application. All the reactions need to be standardized first (Bridge *et al*., 2000). The results obtained in and out of the laboratory are not reproducible because of variability (Bidochka *et al.,* 2002). The RAPD method is susceptible to contamination by non-target DNA, thus is not useful in detecting fungal samples from the environment (Destefano *et al.,* 2004). This method has been documented to produces reliable results only when DNA is obtained from axenic cultures (Leal *et al.,* 1997).

1.8.2.4. Amplified fragment length polymorphism (AFLP)

AFLP is a novel method developed by Vos *et al.* (1995) for genetic mapping and DNA fingerprinting and is based on the selective PCR amplification of genomic DNA restriction fragments. It is a tool used to evaluate genetic variation amongst various organisms (Vos *et al.,* 1995; Bridge *et al.,* 1998). Recently, AFLP has been used to differentiate between individuals, genotypes and strains and to assess genetic diversity and phylogeny of EPF isolates (Sreenivasand and Mills, 1998).

In AFLP, the selective PCR amplification of fragments is obtained from restriction enzyme digests of genomic DNA. The procedure briefly involves three steps. First, restriction of DNA and the ligation of oligonucleotide primers occur then the selected 50-100bp fragments with primers are amplified. Finally, polyacrylamide gel analysis of the amplified fragments is performed (Vos *et al.,* 1995). To date, researchers have successfully demonstrated that AFLP analysis is useful and reproducible for a variety of applications (Terashima *et al.,* 2002).

However, AFLP cannot assess precise genotypes in diploid or polyploid organisms and is thus not useful in population genetic studies (Weeks *et al.,* 2000). AFLP markers display dominance and often cluster at the telomeres and centromeres. This method is expensive and technically demanding (Cornell University, 2003).

Most of the marker system investigations mentioned above regarding host specialization and geographic origin (Wang *et al.,* 2005) have shown variable results regarding genetic variation of EPF. This is attributed to the intrinsic polymorphism of the population used in the study, the number of samples and markers used, part of genome studied and also collection strategies (Gaitan *et al.,* 2002; Uribe and Khachtourians, 2004). Also, EPF such as *B. bassiana* and *B. brongniartii* are recognized as being a "species complex" with genetically diverse lineages. Thus more polymorphic, sensitive and reliable markers are required for strain genotyping as well as inference into the population structure (Wang *et al.,* 2005). Non destructive markers that can function effectively under field conditions are also required for the identification of similar isolates (Tigano-Milano *et al.,* 1995).

1.8.2.5. rDNA analysis

Ribosomal genes of many organisms have been studied for evolutionary, phylogenetic, developmental, ecological, phytopathological, transcriptional and replicational studies (Gasques *et al.,* 2003). A taxonomic and phylogenetic relationship of EPF such as *B. bassiana, M. anisopliae, M. album, M. flavoviride*

and V. lecanii can be established using amplification and direct sequencing of ribosomal DNA (rDNA) (Bridge *et al.,* 1998; Drenth and Irwin, 2001; Destefano *et al.,* 2004; Inglis and Tigano, 2006).

Variable and conserved regions in the genome allows for the comparison and discrimination of organisms at different taxonomic levels. For example the nuclear rDNA in fungi is organized as a tandemly repeated rDNA unit. Three rRNA genes are included in one unit i.e. the small nuclear rRNA (18S-like), the large rRNA (28S-like) and the 5.8S rRNA genes (Fig. 1.5). The genes in one unit are separated by the internal transcribed spacers (ITS1 & ITS2). Also, the two rDNA units are separated by intergenic spacers (IGS). Since the 18S rDNA evolves slowly it is useful to compare distantly related organisms. The non-coding regions, namely ITS and IGS evolve faster thus are useful to compare fungal species within a genus or strains within a species (Curran *et al.,* 1994; Bridge *et al.,* 1998).

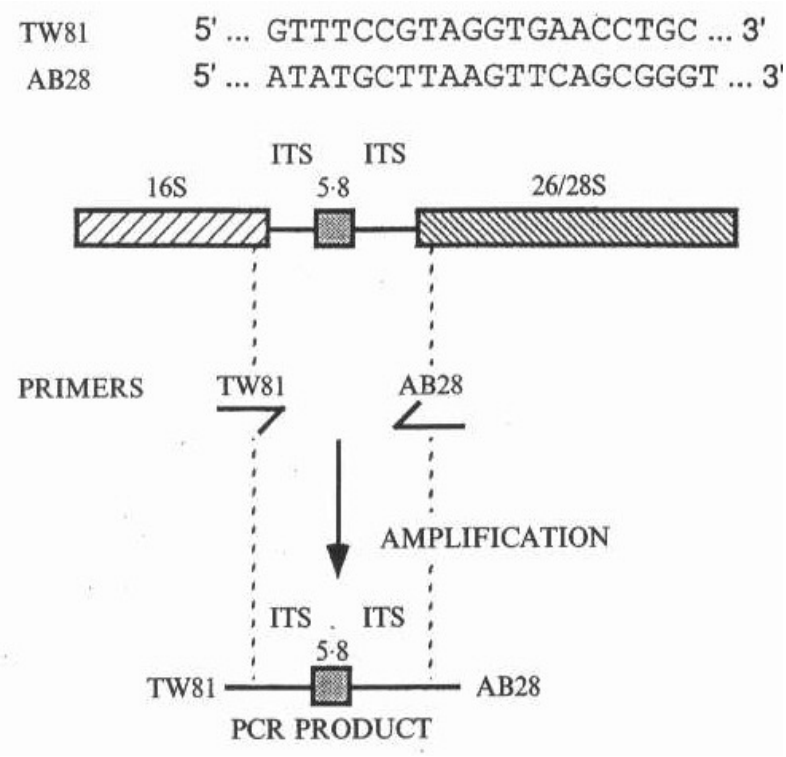

Figure 1.4: A diagrammatic representation of a ribosomal repeat extending from the 5' end of the 16s rDNA to the 3' end of 28s rDNA. Adopted from Curran *et al.* (1994).

Ribosomal DNA sequence variation is used to study phylogeny and to estimate genetic variation or relationships of closely and distantly related EPFs at a species and strain level and is thus important in research of a potential biocontrol agent (Fargues *et al.,* 2002). Sequence polymorphism in rDNA is attributed to insertions, deletions and duplications. In previous studies the analysis of partial sequences of ribosomal RNA was used to estimate genetic distances between organisms and provide nucleic support on the characterization of species (Rakotonirainy *et al.,* 1994). Based on the ITS1-5.8S-ITS2 reports on EPF from different geographic locations and hosts, polymorphisms detected were high (Curran *et al.,* 1994; Rakotonirainy *et al.,* 1994; Kouvelis *et al*., 2004).

Local fungal isolates collected from the soil of a cotton field in Rustenberg, South Africa and baited with *Galleria mellonella* were identified by the Plant Protection Research Institute (PPRI) as *Beauveria bassiana.* These isolates are lodged in the Biosystematics Culture Collection of the PPRI (MCB 121-PPRI 04306, MCB 124-PPRI 04304 and MCBG- PPRI 04305) (Appendix 1). These native *B. bassiana* isolates then formed the foundation of this study. However, it is noted that species and strain recognition within the *Beauveria* genus is difficult from morphological studies due to structural simplicity and lack of distinctive phenotypic variation (Rehner and Buckley, 2005). This ongoing difficulty in the morphological technique has prompted us to compare the South African *B. bassiana* strains with exotic *Beauveria* isolates with an additional taxonomic character. The simple rDNA sequencing technique with the ITS1-5.8S-ITS2 regions was used in the current project.

CHAPTER 2

Beauveria bassiana inoculum produced by the diphasic fermentation process

2.1. Abstract

Inoculum of the entomopathogenic fungus *Beauveria bassiana,* produced in a diphasic fermentation process in the current study comprised of an initial mycelial production stage in submerged media followed by aerial conidia production in a semi-solid fermentation stage. In the first submerged phase the impact of maltose (carbon) and yeast extract (organic nitrogen) treatments in a 1:1 ratio at differing concentrations of 3% and 4%, was investigated for mycelium dry mass and spore yield. Liquid culture media containing basic salts, trace elements, maltose and yeast extract supplementations were inoculated with fungal inoculum and incubated at 25ºC for 72 hours. A MANOVA indicated that for the majority of the strains the dry mass and spore yields were not significantly different in their responses to the 3% and 4% carbohydrate and nitrogen treatments. Overall, the 4% supplementation produced maximal mean dry mass levels with strain B3 affording the highest mean dry mass. In comparison, the lower 3% concentration yielded maximal spore yield levels overall with strain Bb 9011 displaying the highest yield. Since the 4% supplementation demonstrated maximal mycelium production overall, it was implemented in the cost-effective second phase of the diphasic fermentation process to support the formation of aerial conidia in the semi-solid phase in response to low nutrient stress. Mycelia, submerged conidia and blastospores were fungal structures observed in the first submerged phase of the diphasic fermentation process. Aerial conidial inoculum produced in the second semi-solid phase was morphologically different from the submerged phase conidia. In the current study a two-stage *in vitro* mass production process was applied to produce a microbial biocontrol agent. Nutritional parameters exploited in the present study were used to favour production of conidia for use as a potential mycopesticide.

2.2. Introduction

Recognition of the potential of the entomopathogenic fungus (EPF) *Beauveria bassiana* as a microbial biocontrol agent against various arthropod crop pests has led to intensive research into its development as a commercial mycopesticide (Deacon, 1983; Feng *et al.,* 1994). A fundamental requirement for ensuring the economical viability of a mycopesticide in the agricultural industry includes a low cost *in vitro* mass production process yielding viable, stable and virulent conidia with a shelf-life of about 12-18 months (Butt and Goettel, 2000).

Hence, various culture techniques for the mass production of *B. bassiana* have been developed (Butt and Goettel, 2000). Selection of a procedure for mass production of a mycopesticide agent depends on the type of fungal propagule most suitable for the insect pest to be controlled (Thomas *et al.,* 1987). Aerial conidia are readily produced by mycelia growing on solid and semi-solid media, whereas, production of blastospores occurs under submerged liquid culture conditions. Compared to blastospores the aerial conidia of *B. bassiana* are better suited as biocontrol agents as they are more tolerant to harsh environmental conditions. Aerial conidia are also more virulent than short-lived environmentally sensitive blastospores (Thomas *et al.,* 1987).

A two-stage or diphasic fermentation process has become one of the most widely used conidial production procedures to date (Feng *et al.,* 1994). In the diphasic fermentation process large quantities of mycelium and spores are first generated in the submerged liquid culture. After this submerged phase of growth, the fungal mycelium and spores are transferred onto a semi-solid substrate with a low nutrient value and maintained under conditions of low humidity (Rombach *et al.,* 1988). This condition promotes the formation of aerial conidia which can be readily harvested and packaged as a biocontrol agent (Butt and Goettel, 2000). In the current study, the diphasic fermentation process was applied for the production of *B. bassiana* aerial conidia.

Various studies have investigated the effects of nutrients on the growth and sporulation of EPF in culture media (Daoust and Roberts, 1983; Khachatourians, 1986; Butt and Goettel, 2000). Both the ratios and concentrations of various combinations of carbon and nitrogen sources in media cultures are documented to affect mycelial production, enzyme synthesis and spore yields (Khachatourians and Ingledew, 1986; Bidochka *et al.,* 1990). It is thus important to consider the influence of carbon and nitrogen compositions in the culture medium on the host range and specificity of EPF's when a particular strain is developed as a mycopesticide (Kassa *et al.,* 2004).

In the present study the influence of maltose (organic carbon) and yeast extract (complex nitrogen) concentrations (4% and 3%) in a 1:1 mass ratio was investigated on dry mass and spore yield in a submerged culture medium. Thereafter, mycelium from the first submerged phase served to stimulate the production of aerial conidia in the second nutrient deprived semi-solid phase of the diphasic fermentation process. Microscopy served as a tool to observe the different fungal structures produced by the submerged and semi-solid phases of the diphasic fermentation process.

2.3. Materials and Methods

2.3.1. Submerged fermentation phase

2.3.1.1. Fungal strains

The geographic origins of the different *Beauveria bassiana* strains used in the submerged fermentation nutritional experiments are listed in Appendix 2. The local fungal isolates used in the current study were collected from the soil of a cotton field in Rustenburg, South Africa and baited with *Galleria mellonella*. The fungi, identified by the Plant Protection Research Institute (PPRI) as *B. bassiana* are lodged in the Biosystematics Culture Collection of the PPRI (MCB 121-PPRI 04306, MCB 124-PPRI 04304 and MCBG- PPRI 04305) (Appendix 1). In the current project the isolates PPRI 04304 and PPRI 04306 are assumed to be *B. caledonica* based on the results in Chapter 5. However, due to the lack of

confirmation of the change in species identification, the isolates PPRI 04304 and PPRI 04306 are regarded as *B. bassiana sensu latu* with respect to these isolates.

2.3.1.2. Preparation of fungal inoculum

Cultures of *B. bassiana* were established on Merck Sabouraud-Dextrose agar (SDA) (30 g l^{-1}) supplemented with yeast extract (2 g l^{-1}) and glucose (20 g l^{-1}) and incubated at 25ºC for 5 days (Goettel and Inglis, 1997). A loopful of mycelial inoculum from the SDA plates was inoculated into sterile 100 ml Sabouraud-Dextrose broth (SDB) solutions (30 g l^{-1}), supplemented with yeast extract (2 g l^{-1}) and glucose (20 g l^{-1}) to initiate the submerged fermentation experiments. These inoculated broth solutions incubated for 5 days at 25ºC on a rotary shaker at 150 rpm (Nardini, unpublished) were used for the carbon and nitrogen nutritional experiments.

2.3.1.3. Influence of concentrations of carbon and nitrogen sources

a) Growth of the strains in the nutrient media

For the submerged fermentation nutritional experiments, the medium used was modified from Rombach *et al.* (1988). Liquid media contained the following Merck basic salts (1.5 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4$. H_2O and 0.01 g l^{-1} $CaCl_2$) and Merck trace elements (0.03 mg l^{-1} H_3BO_3, 0.04 mg l^{-1} $MnSO_4.4H_2O$, 0.025 mg l^{-1} $Na_2MoO_4.4H_2O$, 0.08 mg l^{-1} $CuSO_4.5H_2O$, 0.4 mg l^{-1} $ZnSO_4.7H_2O$, 0.75 mg l^{-1} $FeCl_3.6H_2O$ and 0.4 mg l^{-1} $CoCl_2.6H_20$). The medium was supplemented with maltose (carbon) and yeast extract (organic nitrogen) sources at concentrations of 3% and 4% (w/v) in a 1:1 ratio (L. Nardini, unpublished data). The basic salts and trace elements broth solutions (50 ml) supplemented with the carbon and nitrogen nutrients were autoclaved at 121ºC for 15 minutes. After cooling, the pH for each of the three replicates was adjusted to 6.25 with sterile 1M NaOH. Each of the flasks was then inoculated with 1 ml of the obtained fungal cultures (section 2.3.1.2). The inoculated media were incubated at 25ºC on a rotary shaker at 150 rpm for 72 hours in constant low light.

b) Determination of growth and spore yield

For the nineteen *B. bassiana* strains dry mass was determined as described by Rombach *et al.* (1988). After the 72 hour incubation period, three aliquots (3 ml) were removed from each of 3 replicates, placed on pre-weighed filter papers (Whatman No. 1, 9 cm) and de-watered by filtering via vacuum suction through a sterile Buchner funnel. These samples were then dried at 60ºC for 48 hours and reweighed for dry mass determination.

Spore yield was determined according to Goettel, (1984). From each of the 3 replicates, triplicate 3 ml aliquots were placed on sterile filter papers (Whatman No.1, 9 cm) in sterile Petri dishes, and incubated at 25ºC for 8 days to allow for the process of sporulation to take place. After the eighth day, the Petri dishes were inverted and dried in a 60ºC oven for 24 hours. Conidia were isolated for conidial counts by transferring each filter paper with dried mycelium into a test tube containing 10 ml 0.5% Tween 80®. The contents of the tube were vortexed vigorously for 1 minute and the concentration of conidia was determined using an Improved Neubauer Haemocytometer by counting five squares of 16 cells.

Statistical analysis: The statistical analysis for the effects of the nutrient treatments on dry mass production (mycelial yield) and spore yield was analyzed by Multifactorial Analysis of Variance (MANOVA) using Statgraphics 7.0 (Manugistics Inc.) at the 95% confidence level.

2.3.2. Semi-solid fermentation phase

The semi-solid substrate fermentation phase was performed as described by Goettel (1984). The procedure was modified as follows, approximately 70 grams of oats and 700 ml of distilled water combined, were placed in an aluminium roasting pan (30x26 cm). The oats were allowed to imbibe water for three minutes. A pre-cut sunbag (Sigma-pore size 0.02 µm) sheet was layered onto the oats mixture. At least 4 cm of the sunbag sheet was extended up onto the sides of the pan to prevent the inoculum from seeping below the sunbag surface into the oats. Once the sunbag was in place, the sheet was stapled to the roasting pan

(Goettel, 1984). Sunbags serve as semi-permeable membranes used to obtain conidia, free of nutritive substrate contamination.

Each pan was placed in an autoclave bag, sealed and steam sterilized for one hour at 121ºC. The pans were removed after autoclaving, cooled to room temperature, swabbed with sterile paper to absorb condensation settled on the surface of the autoclave bags and further cooled at 4ºC overnight to ensure that the sunbags settled back onto the surface of the oats.

A small area on the surface of the autoclave bag was first swabbed with 70% ethanol and then an incision made with a sterile scalpel. The 4% treatment produced a higher mean mycelial dry mass compared with the 3% supplementation in the submerged phase nutritional experiment (Table 2.3) and was thus implemented to support sporulation of fungi in the semi-solid phase (Table 2.3). Thus, broth solutions (100 ml) consisting of the 4% formulation were inoculated with 5 ml *B. bassiana* liquid culture (section 2.3.1.2) and incubated at 25ºC for 72 hours on a rotary shaker at 150 rpm. Fungal inoculum poured onto the sunbag surface through the hole made in the autoclave bag was swabbed with ethanol and sealed. The roasting pans were then gently rocked to allow for an even distribution of the inoculum and incubated at 24:0 L: D at 25˚C for a period of 15 days at constant low light.

2.3.3. Microscopy of inoculum produced in the first and second phase of the diphasic fermentation process

Cultures, collected onto isopore membrane filters and prepared by standard methods (Lindsay *et al.*, 1996) were viewed with a JSM-840 Scanning Electron Microscope (Jeol Ltd, Tokyo, Japan). For Light microscopy, a loopful of the inoculum was placed on a glass slide and observed under a BX41 microscope, using a 400x objective.

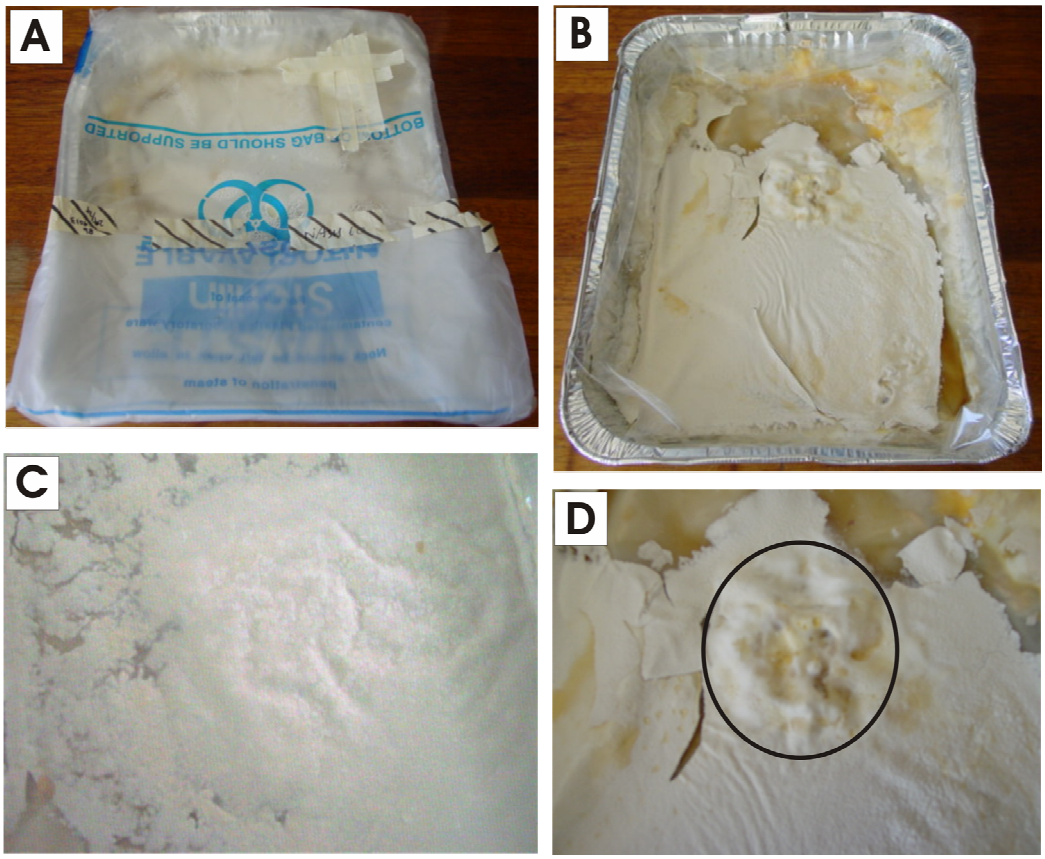

Figure 2.1: A representative view of the second phase of the diphasic fermentation process. (A) Aluminium pan with fungal inoculum in an autoclave bag after a 15 day growth period. (B) Overview of inoculum obtained through the second-phase of the diphasic fermentation process. (C) Close-up of inoculum obtained through the second-phase of the diphasic fermentation process. (D) Circle indicates sporulation of the fungal isolate on the sunbag.

2.4. Results

2.4.1. Submerged fermentation nutritional experiment

Application of maltose and yeast extract nutrients in culture media resulted in variation in dry mass production (mycelial yield) and in spore yield for the majority of the *Beauveria bassiana* strains (Table 2.1). Overall, the collective response of the strains used in the present study to the 3% or 4% concentrations displayed no significantly different trend. A comparison of the two different nutrient treatments regarding dry mass showed that only strain LRC 120 produced a significantly higher mycelium yield at $P< 0.05$ with the 4% treatment (Table 2.1). Similarly at the same concentration, a significant increase ($P< 0.05$) in spore yield was achieved by isolates LRC 95 and KVL 01-135.

The response of individual strains to the nutrient input was as follows. Strain B3 produced the highest dry mass at the 4% concentration, *B. bassiana sensu latu* PPRI 04306 the lowest. In comparison, Strain LRC 121 produced the highest whilst LRC 120 produced the lowest dry mass at the 3% concentration. The highest spore yields were obtained at 3% by strain Bb 9011, the lowest by B. ba 136. Comparatively, Strain KVL 01-135 produced high spore yields at the 4% treatment and Bb 9013 the lowest. Overall, maximal dry mass was demonstrated with the 4% supplementation whilst the 3% concentration yielded high spore levels.

Table 2.1: Influence of different media compositions of maltose: yeast extract at a 1:1 ratio on the mean dry mass production (mycelial yield) and spore yield of *B. bassiana* strains produced in the first submerged phase of the diphasic fermentation process. Values are the means of 3 replicates (± SEM).

Strain	Geographic location	Dry mass (g/3ml)				Spore yield (x10^6/3ml)			
		3%		4%		3%		4%	
LRC 120*	Bulgaria	0	±0.00	0.61	±0.11	50.44	±1.97	31.35	±10.98
LRC 107	Portugal	0.02	±0.01	0.20	±0.11	49.12	±1.55	63.55	±18.26
ALB 60	Albania	0.03	±0.00	0.11	±0.09	46.45	±9.02	29.77	±7.66
PPRI 04305	South Africa	0.04	±0.00	0.04	±0.00	41.78	±2.26	31.33	±3.79
LRC 77	Madagascar	0.07	±0.00	0.26	±0.16	44.89	±1.46	40.00	±3.68
PPRI 04304	South Africa	0.08	±0.00	0.42	±0.20	37.11	±1.17	32.00	±3.67
LRC 95*	North Dakota	0.08	±0.01	0.32	±0.18	59.12	±5.68	100.21	±20.23
KVL 01-135*	Denmark	0.10	±0.01	0.17	±0.01	54.67	±8.06	111.99	±42.56
Bb 9011	Colombia	0.12	±0.01	0.43	±0.20	81.35	±9.33	77.11	±10.63
BPIC 1333	Greece	0.12	±0.06	0.11	±0.02	50.00	±15.75	47.77	±10.65
PPRI 04306	South Africa	0.13	±0.09	0.04	±0.01	34.45	±3.89	29.99	±1.76
105	Slovakia	0.19	±0.09	0.31	±0.14	51.79	±15.50	46.9	±5.90
B ba 136	Germany	0.31	±0.23	0.39	±0.29	15.78	±0.80	29.77	±3.67
Bb 9015	China	0.34	±0.14	0.24	±0.07	48.23	±13.04	36.68	±64.69
Bb lygus	Mexico	0.38	±0.16	0.38	±0.09	74.67	±18.20	61.77	±12.95
Bb 9019	China	0.44	±0.16	0.51	±0.16	59.33	±9.45	58.00	±11.33
Bb 9013	Philippines	0.49	±0.30	0.15	±0.12	39.56	±5.63	27.11	±0.98
B3	Poland	0.53	±0.09	0.75	±0.08	43.12	±6.55	37.56	±0.44
LRC 121	Spain	0.65	±0.14	0.35	±0.08	59.12	±3.49	57.12	±19.23

Species marked * and * represent significant differences between 3% and 4% treatments at the 95% confidence interval for dry mass and spore yield respectively.

2.4.2. Microscopy of inoculum produced in the first and second phase of the diphasic fermentation process

The vegetative mycelium produced by the first phase of the diphasic fermentation process is shown under light microscope in Fig. 2.2 (A, C) and through SEM (Fig. 2.2 B). The fungal structures produced during the first phase of the diphasic fermentation process were submerged conidia recognized as small, spherical structures (Fig. 2.2 D, F, I) and large cylindrical shaped blastospores (Fig. 2.2 E, G). Inoculum of the second semi-solid phase produced aerial conidia with small, spherical, rough surfaces and a brittle appearance (Fig. 2.2 H).

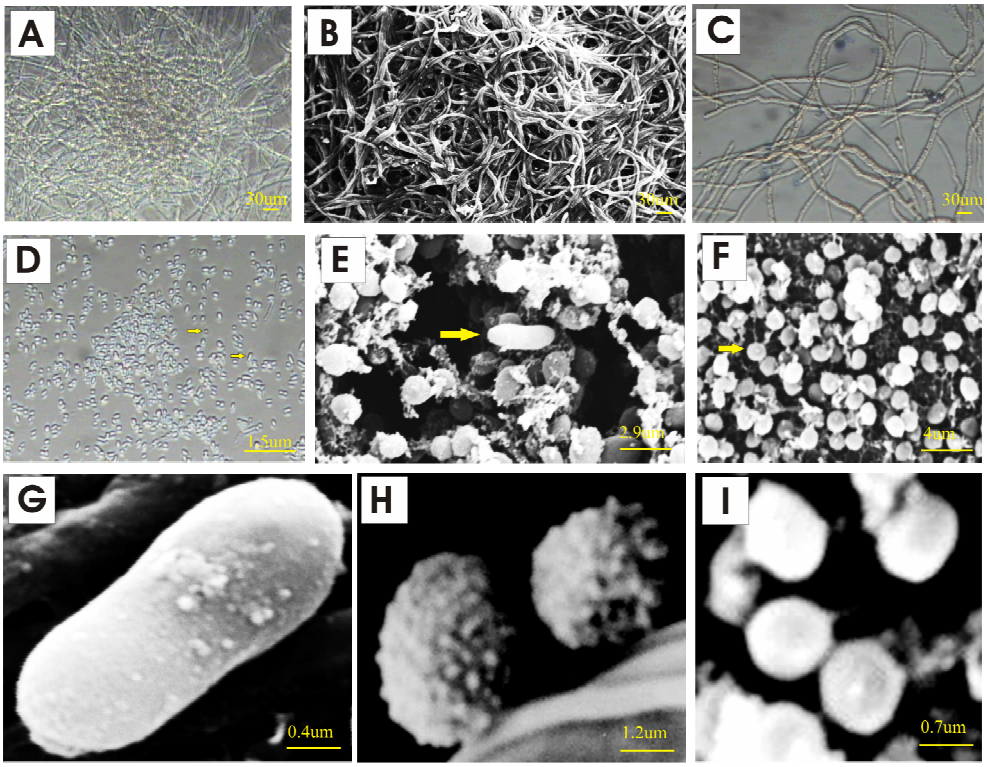

Figure 2.2: Fungal structures produced by the diphasic fermentation process demonstrated by light microscope images (A, C, D) and scanning electron micrographs (B, E-I). (A, B, C) Mycelial growth produced in the first phase of the diphasic fermentation process. (D, E, F) Submerged conidia and blastospores produced in the submerged culture medium. Morphological differences between smooth walled cylindrical blastospores (G), smooth spherical submerged conidia (I) and rough-faced aerial conidia (H) are shown.

2.5. Discussion

2.5.1. Submerged phase culture

Compared to the 4% treatment which provided high mycelial yields in the present study, the lower 3% concentration yielded high spore numbers. This trend is in agreement with that of Thomas *et al.* (1987) who also found that vigorous mycelial growth did not necessarily result in high rates of conidiation. Feng *et al*. (1994) clarify that under submerged culture conditions media rich in carbon and nitrogen substrates promote high production levels of fungal mycelial dry mass. It is thus assumed from the current study that lower levels of nutrient sources (carbon and nitrogen) promote high spore numbers.

In their preliminary experiments, Thomas *et al.* (1987) noted that after 48 h blastospores were produced in abundance in the submerged culture due to the nutrient supplementations in the medium and after 96 h blastospores germinated into submerged conidia (Thomas *et al.,* 1987). Kassa *et al.* (2004) have stated that filamentous fungi produce submerged conidia under conditions that limit vegetative growth such as the exhaustion of the carbon source or depletion of the nitrogen source. As in the studies of Thomas *et al.* (1987) and Kassa *et al.* (2004), in the current study it was demonstrated that successful submerged conidial production noted at 120 h, is dependent on carbon and nitrogen reduction or depletion in the medium.

It appears from the current study that in the submerged fermentation phase the nutrient component in the culture medium is an essential factor for blastospore germination into conidia. Kassa *et al.* (2004) state that submerged conidia production is influenced not only by carbon and nitrogen levels as tested in the present study but also by phosphorus and water activity. Further nutritional studies are required to find the factors involved in the formation of submerged conidia. Thus, if the submerged liquid phase alone was to be used to produce conidia from blastospores, this would have important implications for commercial spore production (Thomas *et al.,* 1987).

2.5.2. Semi-solid phase

In general, the 4% nutrient composition applied in the first submerged phase yielded maximal dry mass in comparison to the 3% supplementation (Table 2.1). This nutrient composition was thus implemented to support *Beauveria bassiana* sporulation during the second nutrient deprived semi-solid phase of the diphasic fermentation process. Previous research has demonstrated that the quantity and quality of the conidia produced during the second semi-solid phase depends on the carbon and nitrogen nutrition of the first submerged phase (Thomas *et al.,* 1987; Bidochka *et al.,* 1990). Complementary studies on the effects of nutritional parameters on aerial conidial yield and quality are thus warranted.

The culture method of Goettel (1984) proved cost effective, as large quantities of contaminant free conidia of various strains of *B. bassiana* were yielded with simple equipment and reusable materials such as aluminium trays and sunbags (Fig. 2.1 B). It was found in preliminary experiments in this study that the cellophane used by Goettel (1984) shrank during autoclaving, and the surface area for sporulation was reduced. As an alternative, we used transparent sunbags due to their larger surface area, and sporulation was abundant (Fig 2.1 D) and aerial conidial harvesting was made easier (Fig. 2.1 B, C, and D).

2.5.3. Microscopy of the submerged and semi-solid phases of the diphasic fermentation process

Previous research has demonstrated that aerial conidia are produced in the second phase whilst the submerged phase consisted of vegetative mycelium (Feng *et al*., 1994). In the current study, mycelium of the first submerged phase is shown in Fig. 2.2 (A-C). Also, the other fungal structures produced by the first phase of the diphasic fermentation process consisted of two types of spores namely, submerged conidia (recognized as small, spherical structures (Fig. 2.2 D, F, I)) and blastospores (large and cylindrical shaped (Fig. 2.2 E, G)). Previous research distinguished *B. bassiana* and *H. thompsonii* blastospores as large, smooth walled and cylindrical shaped, with submerged conidia as small, spherical, uniform in size and refractile (Van Winkelhoff and McCoy, 1984; Thomas *et al.*, 1987).

In the second semi-solid phase, aerial conidia were found (Fig. 2.2 D, H) as small, spherical, with a rough surface and a brittle appearance. The rough surface of aerial conidia is assumed to be due to the deposition of cell wall material (Thomas *et al.,* 1987). In the current study, it is assumed that cell wall deposition is the result of nutrient deprivation and stress in the second semi-solid phase of the diphasic fermentation process.

In conclusion, a two-stage *in vitro* mass production process was applied to produce a potential microbial biocontrol agent. Nutritional parameters investigated in the first submerged phase demonstrated that a carbon and nitrogen supplementation at the 4% concentration produced a higher mycelial dry mass and the 3% treatment yielded high spore numbers. The 4% supplementation was implemented in the second phase of the diphasic fermentation process to support the formation of aerial conidia in the semi-solid phase. The current study demonstrates the impact of nutritional parameters for growth and sporulation when producing the inoculum.

CHAPTER 3

A Preliminary study of the potential of *Beauveria bassiana* as a biocontrol agent against the two-spotted spider mite (*T. urticae*) *in vitro*

3.1. Abstract

An *in vitro* bioassay was carried out to compare the infectivity of indigenous and exotic strains of *Beauveria bassiana* against *Tetranychus urticae* adults in a single-dose experiment. Conidiospores, at a density of 10^6 conidia/ 3 ml^{-1}, were sprayed onto mite-infested bean leaves on water agar in Petri dishes. Post-infection progression was monitored over 7 days by observing for sluggish movements and melanization of the mite cuticle. Following death of the hosts it was noted that two post-mortem changes were exhibited such as blackened mummified mites with legs extending away from the cadaver or dead mites enveloped by *B. bassiana* mycelium. Cumulative mortality data recorded was analyzed at days 3, 5 and 7. Total percentage cumulative mite mortality of the controls (Tween-treated) was on average 6% at day 7 post-inoculation. Total percentage cumulative mite mortality for all the *B. bassiana* strains tested ranged from 36 -99% at day 7 and indicated a wide variation in strain pathogenicity. The average percentage cumulative mite mortality significantly increased with time, with mean percentage values of 25% at day 3, 53% at day 5 and 77% at day 7. The differences in virulence of the native South African isolates *B. bassiana* (PPRI 04305) and *B. bassiana sensu latu* (PPRI 04304 and PPRI 04306) are most likely reflections of genetic differences demonstrated in Chapter 5. Maximal mortality at all the time intervals was caused by six strains namely 105, Bb 9015, LRC 126 and 50.771. Strain ALB 60 was the most virulent isolate with 99% mortality at the end of the 7th day. In this preliminary study 98% of the *B. bassiana* isolates produced mortality above 50% suggesting that in general *B. bassiana* has the potential to be a *T. urticae* biocontrol agent. This study reinforces the need for strain selection trials to ascertain the virulence variability of different strains within *B. bassiana* before any particular strain is selected as a biocontrol agent of the two-spotted spider mite.

3.2. Introduction

Two-spotted spider mites, *Tetranychus urticae* (Koch) are regarded as major cosmopolitan polyphagous pests in the agricultural industry (Chandler *et al.,* 2005). The application of acaricides is the predominant strategy for the effective and cost efficient control of two-spotted spider mites (Sanderson and Zang, 1995). *T. urticae* outbreaks in a number of agricultural systems in the past few years has led to frequent, large-scale applications of high-doses of non-selective pesticides and acaricides, which in turn has resulted in environmental pollution as well as the decimation of the mites natural predators (Aslan *et al.,* 2004; Castagnoli *et al.,* 2005). Moreover, the impact of a number of approved chemical acaricides, such as decofol, cyhexatin, febutin oxide, pyrethoids and organophosphorus acaricides have steadily declined with the development of mite resistance (Shaw *et al.,* 2000; Shi and Feng, 2004). The effects of pesticides on *T. urticae* and its resistance to pesticide products has been widely monitored and researched, however, sustainable chemical control of this pest has not been achieved to date (Aslan *et al.,* 2004; Castagnoli *et al.,* 2005).

Biological control is a promising and environmentally acceptable alternative to chemical control to combat pests such as *T. urticae*. Farmers have been encouraged to use predators, pathogens or other known natural enemies, for the control of these sap-sucking crop pests (Chandler *et al.,* 2005).

Entomopathogenic fungi (EPF), *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycota: Hyphomycetes) is a cosmopolitan, toxin-producing fungus, having a wide host range and the potential as a mycoinsecticide because it is relatively easy to mass produce, displays a wide host range and does not infect non target organisms, such as vertebrates (Coates, 1974; Sun and Fuxa, 2003; Alves *et al.,* 2005). *B. bassiana* has not been reported as a natural pathogen of phytophagous mite populations (Shi and Feng, 2004; Alves *et al.,* 2005). Its potential however as a mycoacaricide has been recently reviewed (Alves *et al.,* 2005). Naturalis ^{TM} a commercial product containing *B. bassiana* inoculum is

known to give excellent control of *T. urticae* on glasshouse roses (Mietkiewski *et al.,* 2000). Similarly, the product Boveril® in Brazil has been used for the biocontrol of *T. urticae* in papaya and ornamental plants (Pena *et al.,* 1996; Alves *et al.,* 2002; Alves *et al.,* 2005).

Laboratory screening of fungal isolates has been an integral part in identifying virulent strains before proceeding to field trials (Cherry *et al.*, 2005). The purpose of the current study was to conduct a preliminary screening to compare the pathogenicity of indigenous and exotic *B. bassiana* strains towards mites, and to identify high, intermediate and low virulence levels in strains.

3.3. Materials and Methods

3.3.1. Mite hosts

Chrysanthemum plants from a garden in the Gauteng region, South Africa provided two-spotted spider mite, *Tetranychus urticae* Koch stock cultures. Mite-infested flowers were transferred every two weeks onto new surface sterilized flowers (0.35% sodium hypochlorite) and maintained in a growth chamber at 25 ±2˚C under natural light to provide new colonies.

3.3.2. Isolation of South African fungal isolates

The local fungal isolates used in the current study were collected from the soil of a cotton field in Rustenburg, South Africa and baited with *Galleria mellonella* (V. Gray, pers. comm.). The fungi, identified by the Plant Protection Research Institute (PPRI) as *Beauveria bassiana* are lodged in the Biosystematics Culture Collection of the PPRI (MCB 121-PPRI 04306, MCB 124-PPRI 04304 and MCBG- PPRI 04305) (Appendix 1). In the current project the isolates PPRI 04304 and PPRI 04306 are assumed to be *B. caledonica* based on the results in Chapter 5. However, due to the lack of confirmation of the change in species identification, the isolates PPRI 04304 and PPRI 04306 are regarded as *B. bassiana sensu latu* with respect to these isolates.

The native and exotic isolates (Appendix 2) used in the present study (Table 3.1) were grown in Sabouraud Dextrose Broth (SDB) (SDB 30 g; Glucose 20 g and Yeast extract 2 g) and incubated at 25˚C for 5 days. Glycerol stock cultures of the isolate are maintained at -70˚C.

3.3.3. Production of aerial conidia

The diphasic fermentation process was used to produce aerial conidia for the bioassays. For the first fermentation stage involving submerged liquid culture, sterile broth solutions (100 ml) incorporated the basal salts and trace elements as described by Rombach *et al.* (1988). This medium was supplemented with a 1:1 ratio of 4% (w/v) maltose and yeast extract. The 100 ml broth solution was inoculated with 5 ml of the obtained fungal culture (section 3.3.2). The cultures were incubated at 25ºC for 72 h on a rotary shaker.

The preparation of the semi-solid substrate was adopted from Goettel (1984) with modifications. In the modified procedure pre-cut sheets of sunbags (Sigma, pore size 0.02 um) were layered onto an oats mixture (70 g oats and 700 ml distilled water) in aluminium roasting pans (30x26 cm). Each pan was sealed in an autoclavable bag and autoclaved at 121˚C for 60 minutes. After cooling, 100 ml of the mycelium from the submerged liquid culture was inoculated onto the surface of the sunbag and incubated for a period of 15 d at 25˚C at constant light.

The conidial mat that formed on the sunbag sheet was harvested by scraping it off and suspending it in 30 ml of 0.05% Tween 80. The suspension was macerated with glass beads for 10 min and then filtered through cheesecloth to remove mycelial fragments. Conidia were counted using an improved Neubauer haemocytometer and the suspension of 10^6 conidia $3ml^{-1}$ was prepared and kept at 4˚C for 24 h before the bioassays.

3.3.4. Bioassay

3.3.4.1. Preparation of the bioassay procedure

Single leaf petioles were harvested from French bean plants grown in a walk-in

growth chamber at 25±2˚C and 12:12 (L: D) with the light illumination at 80-100 μms^{-1}. The leaves were rinsed in sterile distilled water for 10 min, surface sterilized in 0.35% sodium hypochlorite for 5 min, rinsed in sterile distilled water and air-dried under a laminar flow hood for 15 min. Sterile filter paper (9 cm) was placed onto 1% water agar (20 ml) in a Petri dish (9 cm). The detached leaf was then placed on the filter paper (Fig 3.1 A).

3.3.4.2. Bioassay procedure

The bioassay procedure was performed according to Alves *et al.* (2002) with modifications. Due to difficulties in handling the other stages of the mites life cycle a single age population of adults was used in this study. A fine tipped paint brush served to transfer twenty adult mites to sterile bean leaves. A sample of the inoculum obtained from the diphasic fermentation process was stained with Lactophenol Blue (Scharlau Microbiology) and incubated for 18 h at 25˚C on a glass slide with a coverslip to examine spore germination (i.e. the emergence of a germ-tube at half the conidial length). The Petri dishes containing the test mites were sprayed with the conidial suspensions (2 ml per spray application) (Fig. 3.1 C) using an artists airbrusher (Herbert Evans, Cp101) (Fig. 3.1 B). The distance of 50 cm employed between the airbrusher and the Petri dish in Fig. 3.1 C prevented death of the mites due to the force of the airbrusher. Leaves sprayed with 0.05% Tween 80® only, formed the controls. The experimental and control dishes were incubated at 25±1ºC for a period of seven days at constant light.

3.3.4.3. Quantification

The test and control two-spotted spider mites were observed daily under an Olympus S2-CTV Stereomicroscope (SM) (60X mag) to examine for external signs of infection for a period of seven days without interfering with the experimental procedure. Mortality was determined by prodding the mite's with a fine tip paint brush and identifying either dead mite's enveloped by *B. bassiana* mycelium or blackened mummified mite's with legs extended away from the cadaver. The mummified mites were then placed onto a new water agar plate to provide optimal conditions for fungal emergence and sporulation, and incubated

at 25ºC for a further 4-7 days to be examined for the absence or presence of sporulating *B. bassiana* (Fig. 3.2). Cadavers showing *B. bassiana* mycelial growth were recorded as being infected. Also, since mites were not incubated individually, dead mites were removed as soon as possible to prevent horizontal transmission. The experiment was repeated twice.

3.3.4.4. Data analysis

Variability was not significant between the two bioassay experiments, thus data from the initial experiment was used. The data obtained was subjected to a Two-Way Analysis of Variance (ANOVA) using Statistica 6.0 software (Statsoft inc. 99) with time and geographic source as factors, followed by the post ANOVA Turkey test ($P<0.05$).

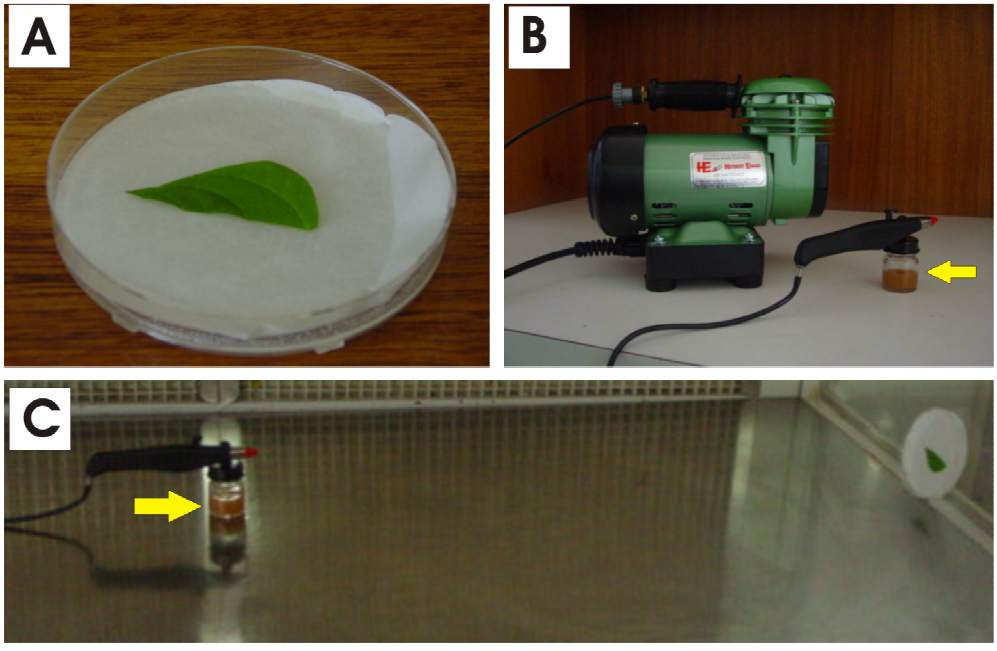

Figure 3.1: A representation of the bioassay procedure. (A) Surface sterilized single leaf petiole placed on sterile filter paper on a water agar plate. (B, C) Artists' airbrusher used to inoculate the test mites.

3.4. Results

Two-spotted spider mites infected with different *Beauveria bassiana* strains showed symptoms at 2 d. The onset of melanization of the spider mite cuticle, sluggish movements and decline in feeding were taken as the initial visible symptoms that successful infection had been established. Thereafter, dead mites appeared either deeply melanized with rigid legs extending away from the body (Fig. 3.2 A, B, C) or surrounded by the white mycelial weft of *B. bassiana* (Fig. 3.2 D, E, F).

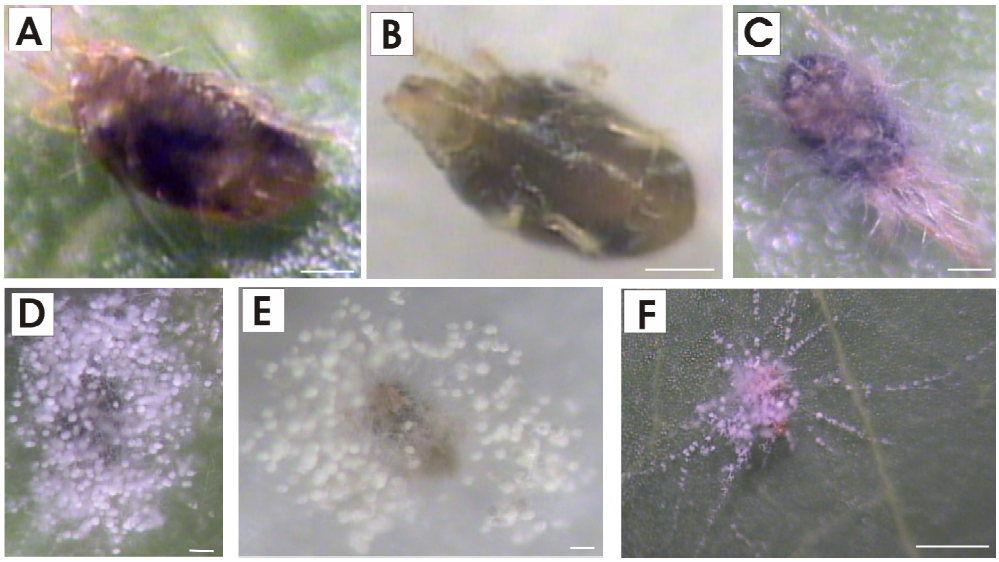

Figure 3.2: The inoculated two-spotted spider mites observed under a stereo microscope. (A, B, C) Dead blackened mummified mites with legs extending away from the cadaver and (D, E, F) mycosed mites showing fungal outgrowths. Bars represent 100µm.

The cumulative mortality in the uninfected control mites for all the bioassays ranged from 2 to 8% (n=225). A comparison of dead *Tetranychus urticae* due to spore treatment at 7 d was significantly higher at $P<0.05$ than the control mites.

In this preliminary study all the fungal strains demonstrated pathogenicity towards *T. urticae* with a significant cumulative mite mortality over the 7 day interval (Tukey's HSD, $P<0.05$). All the *B. bassiana* strains used in this study demonstrated infection levels ranging from 0.4-52% at day 3, 26-79% at day 5 and 36-99% at 7 d. The average percentage cumulative mite mortality significantly increased with time, with mean percentage values of 25% at day 3, 53% at day 5 and 77% at day 7.

Differences in total percentage cumulative mortality ranging from 0% to 99%, over the seven day interval were shown in the current study. The strains have thus been grouped into three virulence categories: ranging from low (0–69%), intermediate (70-89%) to high (90-99%) virulence. Differences within and between the virulence groups at days 3, 5 and 7 with respect to cumulative mortality were significant at $P<0.05$, but the data relating to within group differences are presented in Table 3.1.

In the low virulence group (Table 3.1) all five of the *B. bassiana* isolates displayed pathogenicity to *T. urticae*, but at a slower rate compared with the other virulence groups i.e. more than 50% mortality was only observed at 7 d. The mortality caused by the strains within this group was highly significant between strains (Tukey's HSD, $P < 0.05$). Strain LRC 121 (Spain) caused the highest mortality (29%) in the shortest time (3 d). However, strain Bb 9011 (Colombia) was the most virulent at days 5 (40%) and 7 (63%) in the low virulence category. Bb 9016 (Colombia) also produced more than 50% mortality on 7 d, although not significantly so. Strains Bb 9013 (Philippines) and 00061 (Kenya) showed the lowest mortality on 7 d (Table 3.1).

The majority of the strains fell into the intermediate mortality range. With the intermediate mortality range, more than 50% mortality was observed at 5 d. This virulence group was further subdivided into 70-79% (B1) and 80-89% (B2) categories. In the 70-79% (B1) category the seven day mortality ranged from 72-79%, with *B. bassiana sensu latu* PPRI 04306 showing the lowest and Bb 9019 the highest mortality at 7 d but with little significant variation between strains (Table 3.1). Within the B2 group (80-89%) isolate 51.1083 (Paris) displayed the highest mortality at days 3, 5 and 7. In contrast, comparatively low mite mortality was produced by B3 (Poland) at days 3 (0%) and 7 (81%) respectively, with LRC 171 (Canada) producing a low percentage mortality at 5 d (52%) (Table 3.1).

The indigenous isolates, PPRI 04304, PPRI 04305 and PPRI 04306 fell into this intermediate virulence group (Table 3.1). The *B. bassiana* isolate PPRI 04305 was the most virulent at 85% at 7 d and nested in the higher (80-89%) B2 category of the intermediate mortality range. Interestingly, the *B. bassiana sensu latu* isolates PPRI 04304 and PPRI 04306 were grouped together within the lower (70-79%) B1 category.

In the high virulence group the mite mortality increased steeply with more than 50% mortality demonstrated at 3 d for isolates 105 (Slovakia), LRC 126 (Denmark) and ALB 60 (Albania). At 7 d the strains tested in this virulence group did not differ significantly at 7 d (Turkey's HSD, $P < 0.05$) (Table 3.1). In the 90-99% group, 50.771 (Paris) produced the lowest mortality (90%) at days 3, 5 and 7. Isolate ALB 60 caused the greatest mortality (Albania) of adult spider mites at 3 d (54%), 5 d (87%) and 7 d (99%) than the other isolates at a concentration of 10^6 conidia 3 ml^{-1} (Table 3.1).

Table 3.1: Mean cumulative mortality of adult *T. urticae* infected with different *B. bassiana* strains. Similar letters in the last column indicate no significant difference between strains within a virulence group at day 7. (Tukey HSD test at P<0.05 on MCM data).

Strain	Country	n	Day 3		Day 5		Day 7		Control		sd
			MCM	se	%	MCM	se	%	MCM	se	%	
A (0-69%)												
Bb 9013	Phillippines	225	0	± 0	0	2.3	± 0.2	15	5.3	± 0.3	36	0.3 ± 0.2 2 c
OOO61	Kenya	225	2.2	± 0.4	15	4.0	± 0.6	27	6.7	± 0.5	44	0.5 ± 0.1 3 ac
Bb 9016	Thailand	225	2.4	± 0.7	16	5.1	± 0.8	34	7.7	± 0.5	51	1.3 ± 0.2 8 a
LRC 121	Spain	225	4.4	± 0.7	29	5.7	± 0.7	37	8.7	± 0.7	58	0.7 ± 0.2 5 ab
Bb 9011	Colombia	225	2.8	± 0.5	19	6.1	± 0.7	40	9.4	± 0.4	63	0.7 ± 0.2 5 b
B1 (70-79%)												
PPRI 04306	South Africa	225	2.3	± 0.3	15	6.8	± 0.6	46	10.9	± 0.6	72	0.7 ± 0.2 5 ac
KVL 01-135	Denmark	225	1.0	± 0.3	7	4.5	± 0.7	30	10.9	± 0.7	73	0.9 ± 0.2 6 a
B ba 136	Germany	225	5.0	± 0.6	33	8.7	± 0.8	58	10.9	± 0.8	73	0.8 ± 0.2 5 c
LRC 107	Portugal	225	3.9	± 0.5	26	7.0	± 0.6	47	11.1	± 0.5	74	0.7 ± 0.2 5 ac
LRC 120	Bulgaria	225	3.4	± 0.5	23	8.5	± 0.6	57	11.2	± 0.5	75	0.7 ± 0.2 4 ac
OO144	Ghana	225	1.3	± 0.3	9	3.9	± 0.4	26	11.3	± 0.4	76	1.0 ± 0.3 7 b
OO362	Benin	225	1.7	± 0.3	11	5.2	± 0.4	35	11.5	± 0.6	76	1.0 ± 0.2 7 ac
OOO38	Uganda	225	3.9	± 0.6	26	8.0	± 0.6	53	11.7	± 0.7	77	1.1 ± 0.2 8 a
PPRI 04304	South Africa	225	2.9	± 0.2	20	7.3	± 0.5	49	11.7	± 0.6	78	0.6 ± 0.2 4 ac
Bb 9019	China	225	3.7	± 0.5	24	8.7	± 0.8	58	11.9	± 0.6	79	1.3 ± 0.2 8 c
B2 (80-89%)												
B3	Poland	225	0	± 0	0	9.3	± 0.5	61	12.1	± 0.4	81	1.1 ± 0.2 7 abc
BPIC 1333	Greece	225	6.7	± 0.7	45	10.5	± 0.6	70	12.6	± 0.6	84	0.7 ± 0.2 4 abd
PPRI 04305	South Africa	225	0.1	± 0.1	0.4	8.9	± 0.7	59	12.7	± 0.5	85	0.9 ± 0.3 6 abc
Bb lygus	Mexico	225	5.9	± 0.8	39	10.8	± 0.7	72	13.1	± 0.6	87	1.2 ± 0.2 8 d
LRC 171	Canada	225	1.5	± 0.2	11	7.9	± 0.8	52	13.2	± 0.4	88	1.1 ± 0.4 7 a
51.1083	Paris	225	5.4	± 0.6	36	11.3	± 0.5	75	14.7	± 0.2	89	0.6 ± 0.1 4 b
C (90-99%)												
50.771	Paris	225	6.4	± 0.7	41	10.2	± 0.8	68	13.5	± 0.3	90	0.7 ± 0.2 5 a
Bb 9015	China	225	6.4	± 0.6	43	11.5	± 0.7	77	13.5	± 0.4	90	1.3 ± 0.3 8 a
105	Slovakia	225	7.6	± 0.6	51	10.7	± 0.8	75	13.7	± 0.3	91	0.6 ± 0.2 4 a
LRC 126	Denmark	225	7.9	± 0.6	52	11.6	± 0.5	77	13.7	± 0.3	91	0.9 ± 0.2 6 a
LRC 77	Madagascar	225	4.5	± 0.6	30	11.9	± 0.6	79	14.5	± 0.3	96	0.6 ± 0.2 4 a
ALB 60	Albania	225	8	± 1	54	13.0	± 0.8	87	14.9	± 0.5	99	1.2 ± 0.3 8 a

MCM: Mean Cumulative Mortality
se: standard error
%: percentage cumulative mortality
sd: Significant difference between isolates in the same group.

3.5. Discussion

The results from the present study indicated that all the *Beauveria bassiana* strains (i.e. exotic and local) caused mortality of the adult two-spotted spider mite under laboratory conditions (Table 3.1). Prior to field trials laboratory screening of particular isolates is required to identify the most promising virulent candidates. The present study thus served as a preliminary report in the performance screening of exotic and indigenous strains of *B. bassiana* for the control of the two-spotted spider mite adults. Additional studies on the other life stages of *Tetranychus urticae* are required as the susceptibility of arthropod pests to fungal infection is influenced by specific developmental stages due to changes in the environmental conditions and also differences in the life stages of the host (Wraight and Carruthers, 1999; Muerrle *et al*., 2006). The control experiments conducted in the present study displayed no fungus induced mortalities. Thus, the pathogenicity of the fungal isolates to the mites was directly comparable with the control data.

Adane *et al.* (1996) demonstrated in their study that the cumulative mortality of all the *B. bassiana* isolates infecting *S. zeamais* increased with time. This was also demonstrated with the time-mortality data in the current study with the host *T. urticae* (Table 3.1). Adane *et al.* (1996) also showed in their study that the number of deaths increased more rapidly with the more virulent isolates and the number of deaths for less virulent isolates occurred at a slower rate. Similarly, in the current study the highly virulent isolates demonstrated more than 50% mortality at 3 d, the response in the intermediate group was at 5 d and within the low virulence group at 7 d.

Differences in the virulence of fungal species and strains have been documented in other studies using different arthropods (Puterka *et al.,* 1994, Muerrle *et al.,* 2006). Although all the strains tested in the present study caused mortality, the results obtained support the information mentioned above since the *B. bassiana* strains exhibiting variability in virulence were placed into the low, intermediate

and high virulence groups (Table 3.1). This variation in virulence suggests that a level of control of the chosen host is dependent on the strains host specificity. According to Cherry *et al.* (2005) influences on the virulence capabilities of the isolates include recovery of the fungus from the host species and also the culture history. Complementary studies are thus required since the restriction in host range demonstrated in the current study is assumed to be attributed to one or both factors mentioned above.

In the current study the higher mortality percentage of 99% compared to the 94% for Alves *et al.* (1994) and a 97% for Barreto *et al.* (2004) could be attributed to factors such as size of mites, high humidity in the Petri dish chamber or passaging through the host. Shaw *et al.* (2000) found in their study that relative humidity (RH) was an essential external factor that promoted infection by fungal isolates. Fungi were also less virulent at 40% RH than at 100% RH in their study (Shaw *et al.*, 2000). Since humidity is a key variable, it should be considered for further studies. Butt and Goettel (2000) recommended passaging of isolates through the host before culturing or bioassays in order to compare virulence effectively (Cherry *et al.,* 2005). Previous work has demonstrated that a single passage through the mosquito *Culex pipens* increased the virulence of *Metarhizium anisopliae* strains against the host (Smith *et al.,* 2000). The findings from the present study also suggest that the single passaging of the *B. bassiana* strains through the *T. urticae* host prior to the bioassay experiment allowed for a comparison to be made on the virulence of different *B. bassiana* strains.

In a study by Barreto *et al.* (2004), mortality of the mite pest *Monychellus tanajoa* varied from 13-97% at a 10^8 conidia/ ml^{-1} spore application concentration of *B. bassiana* isolates. Also, in another study at the same concentration, mortality of the two-spotted spider mite ranged from 43-78% using *B. bassiana* isolates (Alves *et al.,* 2002). Comparatively, the present study showed that with a lower spore concentration of 10^6 conidia. 3 ml^{-1}, 27 strains of *B. bassiana* caused a higher total mortality range from 36 to 99% in *T. urticae* adults (Table 3.1). The citrus mite, similar to the two-spotted spider mite, has a widespread distribution requiring a

large volume for foliar coverage. Thus, lower concentrations of fungal inoculum (10^6 and 10^7 per ml) according to Alves *et al.* (2005) allow for more application of an EPF at an economical cost price similar to a single application of the highest concentration. Conidia application concentrations of 10^6 3 ml^{-1} used in the current study gave a high 90-99% mortality range for the most pathogenic isolates (Table 3.1). These isolates would therefore be economical to use if considered for use as possible mycoacaricides.

Ansari *et al.* (2004) stated that differences in virulence of isolates are reflections of genetic and physiological differences between isolates. The current study concurs with Ansari *et al.* (2004) because the phenotypic separation of the native *B. bassiana* (PPRI 04305) and presumed *B. caledonica* (PPRI 04304 and PPRI 04306) isolates based on the bioassay results in this study supports the genotypic separation of the isolates demonstrated in Chapter 5. The native South African isolates produced high mortalities of adult *T. urticae* with PPRI 04305 at 85%, PPRI 04304 at 78% and PPRI 04306 at 72% at the end of the experiment. In this study, the use of the indigenous isolates as mycoacaricides against *T. urticae* is optional based on the results in Table 3.1. For further studies, detailed dose-mortality bioassays may be attempted and additional work is required to evaluate arthropod hosts as well.

In previous studies on arthropod pests the post-infected pests showed symptoms of melanization, spots on the cuticle, sluggishness and uncoordinated movement (Adane *et al.,* 1996; Krutmuang and Mekchey, 2005). The current study concurs with the above observations. Mycelium emergence from dead mites has also been previously demonstrated (Adane *et al.,* 1996; Krutmuang and Mekchey, 2005). In the present study, dead mites were mummified with hyphal outgrowths of *B. bassiana* on the cadaver as shown in Fig. 3.2 C-F or stiff intense melanized cadavers with rigid legs extending away from the body (Fig. 3.2 A, B). The mite-fungus relationship is explored further in Chapter 4.

In the current study all the strains were virulent against *T. urticae* suggesting that in general *B. bassiana* is highly virulent and has the potential as an adult two-spotted spider mite biocontrol agent. Isolate ALB 60 (Albania) is a good example of the capacity of an individual isolate to produce high mortality rates which increased over time. This isolate is of particular interest for further laboratory studies against other sap sucking pests. Its considerable potential as a biocontrol agent can also be assessed in field trials for adult *T. urticae* control at a low economical concentration. Further studies in this regard are a way of meeting the growing demand in the agricultural industry for non-chemical control (Alves *et al.,* 2005).

CHAPTER 4

The infection cycle of a South African isolate of *Beauveria bassiana* in the two-spotted spider mite

4.1. Abstract

In this study scanning electron microscopy (SEM), stereo microscopy (SM), phase contrast microscopy (PCM) and confocal scanning laser microscopy (CSLM) were used to monitor the infection and colonization of the two-spotted spider mite *Tetranychus urticae* Koch by an indigenous entomopathogenic fungus (EPF) *Beauveria bassiana* (PPRI 04305). Adult spider mites were placed onto young, sterile, detached French bean leaves in 1% water agar plates. Inoculum of 10^6 conidia. 3 ml^{-1} produced by the diphasic fermentation process was sprayed directly onto the spider mite infested French bean leaves. The infection cycle was monitored over a seven day period. *B. bassiana* entry was displayed by PCM as being *per os*. SM showed that the initial antifungal defense response of the spider mites was an intense melanization on the dorsal surface of the mite cuticle induced by the EPF isolate. CSLM demonstrated the morphological transition of the fungal infection structures from hyphal growth form into blastospores in the haemocoel. Furthermore, PCM revealed the transition of blastospores to resting spores in the mummified cadaver which is a survival mechanism of the EPF. Aerial hyphae were shown by SEM to emerge through the setal annulum on the dorsal surface of the cadaver. Although isolates of *B. bassiana* have been commercialized for use against polyphagous mites, to our knowledge observational reports of mite diseases by EPF are scarce and fragmentary. Based on visual observations, the current study shows the infection process of *B. bassiana* in two-spotted spider mites. New and interesting observations made in the current study will provide valuable information in further research in the development of a mycoacaricide in South Africa.

4.2. Introduction

The advantages of chemical insecticides and acaricides for crop pest control include availability, potency, rapid action, management flexibility and ease of application (Shi and Feng, 2004). However, the main disadvantages include health hazards to humans and other vertebrates as well as the rapid selection of pesticide resistance resulting in a resurgence of the pest problem (Burges, 1981; Shi and Feng, 2004).

Roberts and Hajek, (1992) state that the disadvantages arising from a dependence on chemical pesticides outweigh the advantages. It is therefore important to promote research into the potential utilization of biological control agents as an alternative method of crop pest management. Biological control relies on natural enemies to reduce and maintain the crop pest population below the economic threshold level (Lacey and Goettel, 1995). Predacious spiders (*Linyphiidae, Theridiidae*), mites (*Phytoseiulus, Amblyseius*) and other insects (*Orins, Oligota*) have received tremendous attention as agents to control Acari pests including the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) (Magdalena and Meyer, 1996). However, entomopathogenic fungi (EPF) studied as potential biocontrol agents against mite pests have demonstrated advantages over predators due to a short generation time required to produce a vast number of the infection structures and the ease of application of this biocontrol agent (Gaitan *et al.,* 2002). Many acarian crop pests have been reported as being susceptible to infection by EPF (Puterka *et al.,* 1994). *Beauveria bassiana,* an opportunistic EPF, is cosmopolitan and since it infects the host by direct penetration of the cuticle without the need to be ingested, it has advantages over other entomopathogens such as viruses and bacteria (Ravellec *et al.,* 1989).

The recent investigations of Oduor *et al.* (1995) as well as Lacey and Brooks, (1997) into the infectivity of *B. bassiana* against various Acari pests support the potential of this EPF as a mycoacaricidal agent. Although isolates of *B. bassiana* have been commercialized for use against polyphagous mites, to our knowledge to

date, no observational reports have been published which show in detail the infection process of *B. bassiana* in two-spotted spider mites. Most studies have concentrated on bioassays of the different stages in the mite's life cycle or comparison of different EPF isolates against *T. urticae* (Clarkson and Charnley, 1996; Van der Geest *et al.,* 2000; Alves *et al.,* 2002).

This investigation thus focused on monitoring over time the external and internal signs of post-infection of *B. bassiana* strain PPRI 04305 on and in two-spotted spider mite adults by scanning electron microscopy (SEM), stereo microscopy (SM), phase contrast microscopy (PCM) and confocal scanning laser microscopy (CSLM).

4.3. Materials and Methods

4.3.1. Mite hosts

Chrysanthemum plants from a garden in the Gauteng region, South Africa provided two-spotted spider mite, *Tetranychus urticae* Koch stock cultures. Mite-infested flowers transferred every two weeks onto new surface sterilized flowers (0.35% sodium hypochlorite) and maintained in a growth chamber at 25±2˚C under natural light provided new colonies.

4.3.2. Isolation of South African fungal isolates

The local fungal isolate used in the current study was collected from the soil of a cotton field in Rustenberg, South Africa and baited with *Galleria mellonella* (V. M. Gray, pers. comm.). The fungus, identified by the Plant Protection Research Institute (PPRI) as *Beauveria bassiana* is lodged in the Biosystematics Culture Collection of the PPRI (MCBG- PPRI 04305) (Appendix 1). The phylogenetic analyses conducted in Chapter 5 also confirmed the identity of PPRI 04305 as *B. bassiana*.

The native isolate used in the present study (Table 3.1) was grown in Sabouraud Dextrose Broth (SDB) (SDB 30 g; Glucose 20 g and Yeast extract 2 g) and incubated at 25˚C for 5 days.

4.3.3. Production of aerial conidia

The diphasic fermentation process was used to produce aerial conidia for the bioassays. For the first fermentation stage involving submerged liquid culture, sterile broth solutions (100 ml) incorporated the basal salts and trace elements as described by Rombach *et al.* (1988). This medium was supplemented with a 1:1 ratio of 4% (w/v) maltose and yeast extract. The 100 ml broth solution was inoculated with 5 ml of the obtained fungal culture (section 4.3.2). The cultures were incubated at 25ºC for 72 h on a rotary shaker.

The preparation of the semi-solid substrate was adopted from Goettel (1984) with modifications. In the modified procedure pre-cut sheets of sunbags (Sigma, pore size 0.02 µm) were layered onto an oats mixture (70 g oats and 700 ml distilled water) in aluminium roasting pans (30x26 cm). Each pan was sealed in an autoclavable bag and autoclaved at 121˚C for 60 minutes. After cooling, 100 ml of the mycelium from the submerged liquid culture was inoculated onto the surface of the sunbag and incubated for a period of 15 d at 25˚C at constant light.

The conidial mat that formed on the sunbag sheet was harvested by scraping it off and suspending it in 30 ml of 0.05% Tween 80. The suspension was macerated with glass beads for 10 min and then filtered through cheesecloth to remove mycelial fragments. Conidia were counted using an improved Neubauer haemocytometer and the suspension of 10^6 conidia. $3ml^{-1}$ was prepared and kept at 4˚C for 24 h before the bioassays.

4.3.4. Conidial observation

B. bassiana conidia, collected onto isopore membrane filters and prepared according to Lindsay *et al.* (1996) were examined with a SEM JSM-840 microscope (Jeol Ltd, Tokyo, Japan). Furthermore, a loopful of the conidial suspension was examined with a LSM 410 Invert Laser Scan Microscope (Carl Zeiss) at 50% laser intensity, 488 excitation wavelengths, using the 63x oil-immersion objective.

4.3.5. Bioassay

4.3.5.1. Preparation of the bioassay procedure

Single leaf petioles were harvested from French bean plants grown in a walk-in growth chamber at 25±2˚C and 12:12 (L: D) with the light illumination at 80-100 μms^{-1}. The leaves were rinsed in sterile distilled water for 10 min, surface sterilized in 0.35% sodium hypochlorite for 5 min, rinsed in sterile distilled water and air-dried under a laminar flow hood for 15 min. Sterile filter paper (9 cm) was placed onto 1% water agar (20 ml) in a Petri dish (9 cm). The detached leaf was then placed on the filter paper.

4.3.5.2. Bioassay procedure

Adult mites, used for the bioassay procedure (Alves *et al.,* 2002) were transferred to the sterile bean leaves and sprayed with 2 ml conidial suspensions using an artist's airbrusher (Herbert Evans, Cp101) (Fig. 4.1. A-B). Leaves sprayed with 0.05% Tween 80® only, formed the controls. The Experimental and control dishes were incubated at 25±1ºC for a period of seven days at constant light.

4.3.6. Observation of inoculated and uninoculated (control) *two-spotted spider mites*

Four different microscopy methods (SEM, SM, PCM and CSLM) were implemented to observe for internal and external signs of infection at time intervals of 0, 1, 2, 4 and 7 days.

For SEM, four drops of 100% ether were added on the control and infected spider mites to kill them after transferral from the leaf disks onto SEM stubs. The stubs which held the spider mites in position were covered by 12 mm spectro tabs (Ted Pella Inc). Thereafter, E. de Lillo advised (pers. comm.) to view the mites at 5 and 20 kV within 15 min to avoid their desiccation. The etherized mites were also placed on a cover slip in a 55 mm petri dish and examined under CSLM (B. Fabian, pers. comm.). Test mites were directly examined with an Olympus S2-CTV stereo microscope at the 60 x magnification (mag). Test mites were prepared for PCM using a standard method (Krantz, 1978) and examined with an Olympus BX41 light microscope (100 x mag).

4.4. Results and Discussion

4.4.1. Attachment onto and penetration of conidia through the host cuticle

The morphologies of uninoculated (control) mites are shown in Fig. 4.1(A-D) with no evidence of any spore-like structures or damage to the cuticle and appendages as shown in the micrographs of the inoculated mites (Figs. 4.2 and 4.3).

SEM showed adherence of spores to the mite host at day 0 after a period of 4 hours (Fig. 4.1 E, F). This observation concurs with those demonstrated by Alcides *et al.* (2002) that *Beauveria bassiana* and *Metarhizium anisopliae* infection for termite control follows after attachment of spores to the target pest's cuticle within 0-6 hours after inoculation.

Conidia (Fig. 4.2 A, B) were observed after inoculation primarily on the dorsal surface of the host on the cuticle (Fig. 4.1 F), gnathosoma (Fig. 4.1 E) and legs (Fig. 4.1 F). Previously, conidia of EPF were demonstrated to attach firmly to all surfaces of the arthropod cuticle especially under wings, on appendages and on the segmental cuticle of the host (Schreiter *et al.,* 1994). This study is in concordance with Schreiter *et al.* (1994) that higher conidial densities concentrate in areas of the cuticle with setae where the conidia are trapped (Fig. 4.1 F).

By days 1 and 2, hyphal growth over the surface of the cuticle was observed before penetration through the cuticle occurred (Fig. 4.2 C). According to previous work, hyphomycetes such as *B. bassiana, M. anisopliae* and *Hirsutella thompsonii* penetrated the cuticle after limited hyphal growth on the surface which is regarded as a sign of degradation and dissolution of the cuticular surface (Gerson *et al.,* 1979; St. Leger *et al.,* 1991; Schreiter *et al.,* 1994).

4.4.2. Mode of entry

Generally the mode of entry of EPF in the host through vulnerable sites includes the cuticle, anus, mouth (*per os)* or pores (McCauley and Zacharuk, 1968). *B.*

bassiana strain PPRI 04305 entry into the host was observed as being *per os* under SEM (Fig. 4.2 D) and PCM (Fig. 4.2 E). Infection through the pest's cuticle was also noted by SEM in this study; however no evidence could be obtained to reveal the cuticular fracture created by the fungal isolate at the penetration site.

4.4.3. Melanization of the cuticle

In the current study, the cuticle of the healthy mite (Fig. 4.1 C) after infection (days 2 and 4) with *B. bassiana* melanized (Fig. 4.2 F). According to Alves *et al.* (2002) as well as Fuguet and Vey (2004), melanization of the host's cuticle is the earliest and most prominent sign of host infection.

4.4.4. The blastospore stage

CSLM revealed that large numbers and different sizes of blastospores formed within the mite at days 4 and 7 (Fig. 4.3 A). Blastospore formations within the host pest are common in EPFs' such as *M. anisopliae, B. bassiana, Nomurea riley, Tolypocladium cylindrosporum* and *Entomophthora coronata* (Schreiter *et al.,* 1994; Fuguet and Vey, 2004). Septate mycelium elongated from germ tubes in the haemocoel fragment and form yeast-like blastospores that float and scatter throughout the host's body (Burges and Hussey, 1971). A large surface area to volume ratio facilitates nutrient absorption from various tissues and allows the fungus to colonize the host effectively (Hajek and St. Leger, 1993; Clarkson and Charnley, 1996; Shah and Pell, 2003).

4.4.5. Death of host

A combination of fungal toxins, physical obstructions of blood circulation, nutrient depletion and organ invasion causes host death (Hajek and St. Leger, 1993; Mietkiwski *et al*., 2000). The mite cadavers were swollen and rigid with legs extending away from the body due to a continual internal fungal growth (Fig. 4.3 B, C, D).

4.4.6. Lateral and aerial hyphal development

Upon death of the mite, *B. bassiana* vegetative hyphal development in the two-spotted spider mite was observed by CSLM to be parallel to and under the cuticle

(Fig. 4.3 E). McCauley and Zacharuk, (1968) found extensive lateral vegetative hyphal development in elatrid larvae in the procuticle or between the epicuticle and procuticle before the hyphae started to grow out the body through the cuticle. In the current study it was not obvious where the lateral hyphae were observed, but we support their findings that lateral hyphal development occurs in the area where the least amount of resistance is encountered.

Generally, after lateral hyphal growth EPF are reported to exit the cadaver as aerial hyphae from the mouth, anus, intersegmental membranes on the ventro-lateral surfaces and membranes at the base of the legs (McCauley and Zacharuk, 1968). However, in the current study, aerial hyphae emerged through the annulum on the dorsal surface of the mite cadaver (Fig. 4.4 A). These aerial hyphae were perpendicular to the host cuticle.

4.4.7. Conidiophore development

The aerial hyphae apparent as a "white fur" on the surface of the cadaver (Fig. 4.3 D) developed conidiophores (days 4 to 7) which gave rise to conidia (Fig. 4.4 B) completing the fungal life cycle (Khetan, 2000). The white muscardine hyphal growth was noted on the entire dorsal surface in the current study (Fig. 4.4. B) with conidial growth similar to previous studies (Kumar *et al.,* 1994; Srisukchayal *et al.,* 2005).

4.4.8. Mummified T. urticae

Butt *et al.* (1990) state that water regulates conidiogenesis on the pest surface. High humidity causes external conidiogenesis on the host cadaver and with low humidity overwintering mycelial filaments occur inside the haemocoel of a mummified mite (Delalibera and Hajek, 2004).

In the current study a change in the developmental mode in some cadavers occurred when the blastospores formed rust coloured scattered hyaline bodies best detected by PCM in the mummified mite (Fig. 4.4 C). This transformation in structural forms was assumed to be stimulated by a decrease in the humidity of the

bioassay chamber due to evaporation of the water agar used in the chamber. Oduor *et al.* (1995) suggested that preparations of the mummies may be potential biopesticides due to the ability of the fungus to survive in this form. However, the potential of using *B. bassiana* inoculum in the form of spider mite mummies as the basis of a mycoacaricide still needs to be evaluated.

In conclusion, this study illustrates the morphological stages of a native *B. bassiana* isolate as it progressed through its infection cycle which is similar to those described in a number of agricultural pests (Clarkson and Charnley, 1996). However, to our knowledge aspects not observed before with mites included limited hyphal growth on the cuticle surface before penetration, *per os* mode of entry, cuticular melanization, lateral hyphal development under the cuticle and aerial hyphal emergence through the setal annulum on the dorsal surface of the cadaver. New and interesting observations made in the current study will provide valuable information in further research in the development of a mycoacaricide in South Africa.

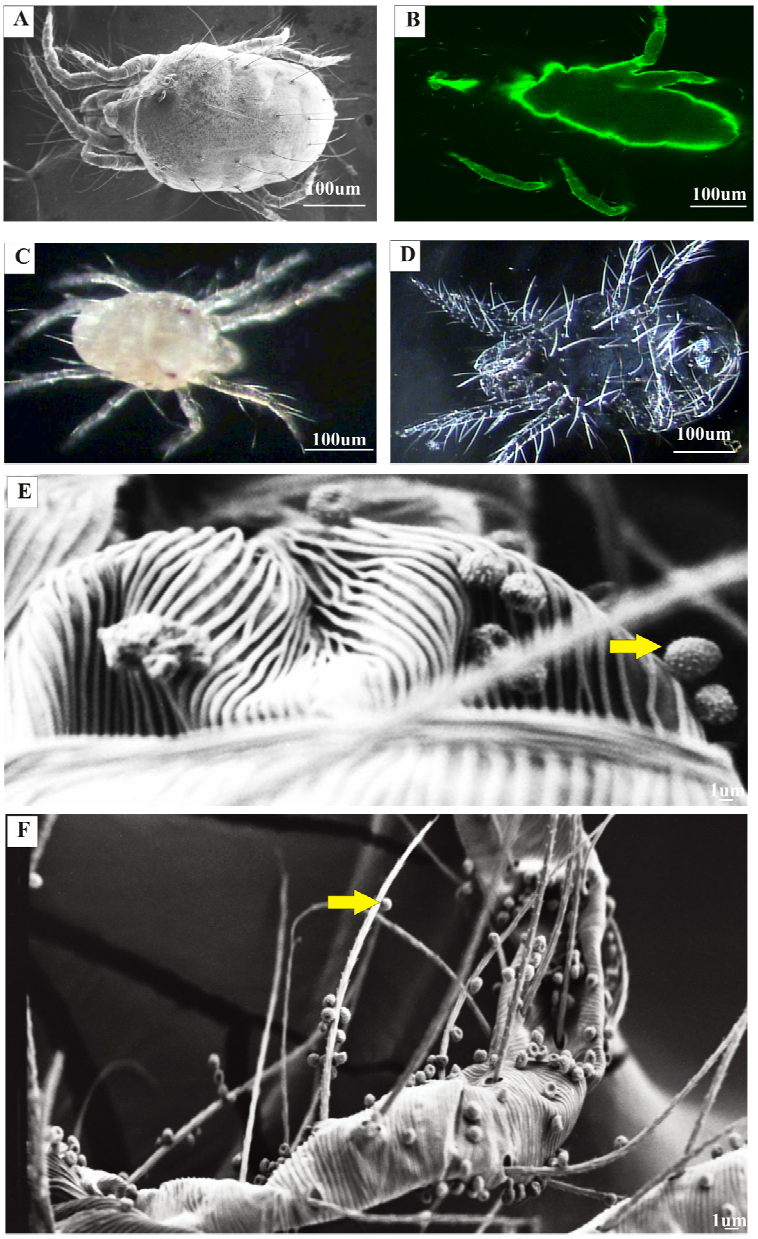

Figure 4.1: (A-D) Untreated two-spotted spider mites demonstrated by SEM, CSLM, SM and PCM. (E) Attachment of *B. bassiana* conidia to the mite's gnathosoma, (F) legs and cuticle (SEM). (CSLM figure fluoresced green in the green channel)

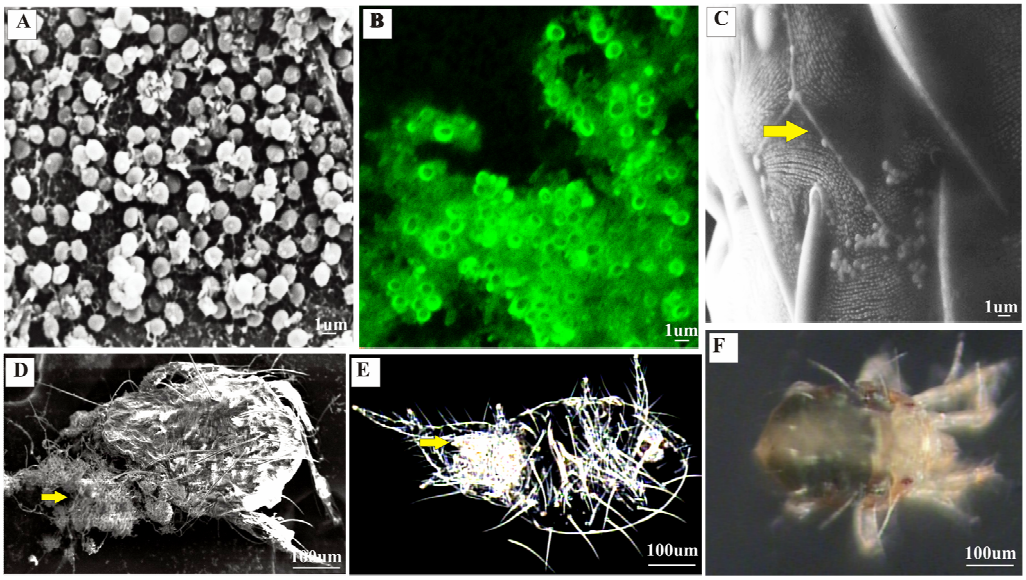

Figure 4.2: The infection process of *B. bassiana* against the two-spotted spider mite. (A, B) Conidia of *B. bassiana* PPRI 04305 used as inoculum as seen with SEM and CSLM. (C) Lateral hyphal development on the cuticle (SEM). (D, E) Fungal mode of entry *per os* noted with SEM and PCM. (F) Melanization of mite cuticle (SM). (CSLM figure fluoresced green in the green channel)

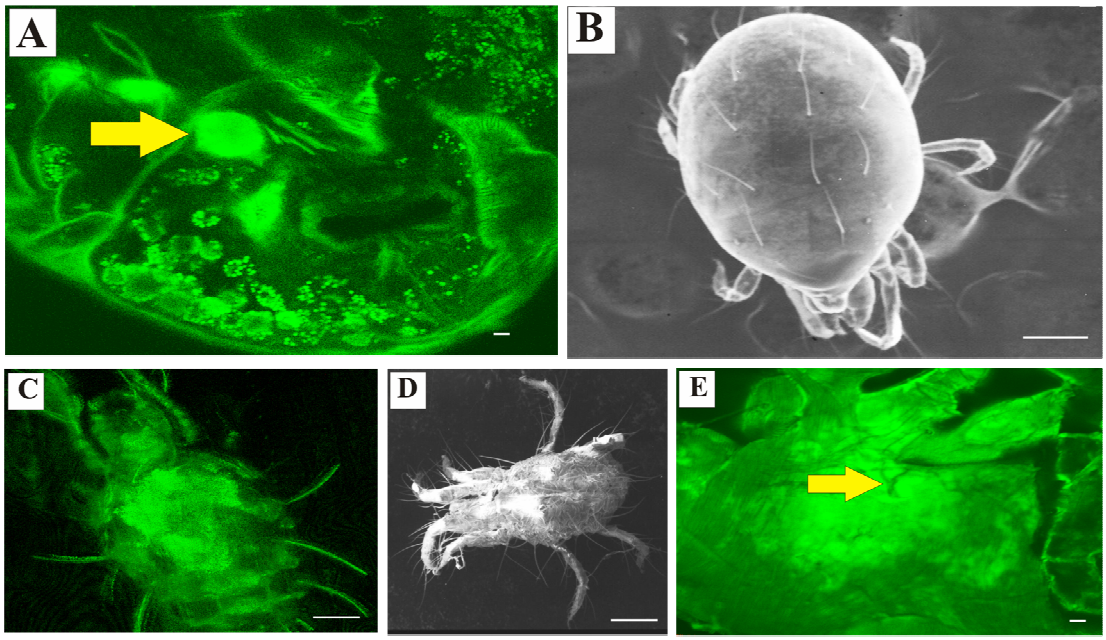

Figure 4.3: Infection of the two-spotted spider mite demonstrated by SEM (B, D) and CSLM (A, C, E). (A) Large blastospores produced *in vivo*. (B, C) Swollen cadaver of mummified two-spotted spider. (D) Cadaver of mycosed mite. (E) Vegetative hyphal development in the cuticle. Bars represent 100µm. (CSLM figures fluoresced green in the green channel)

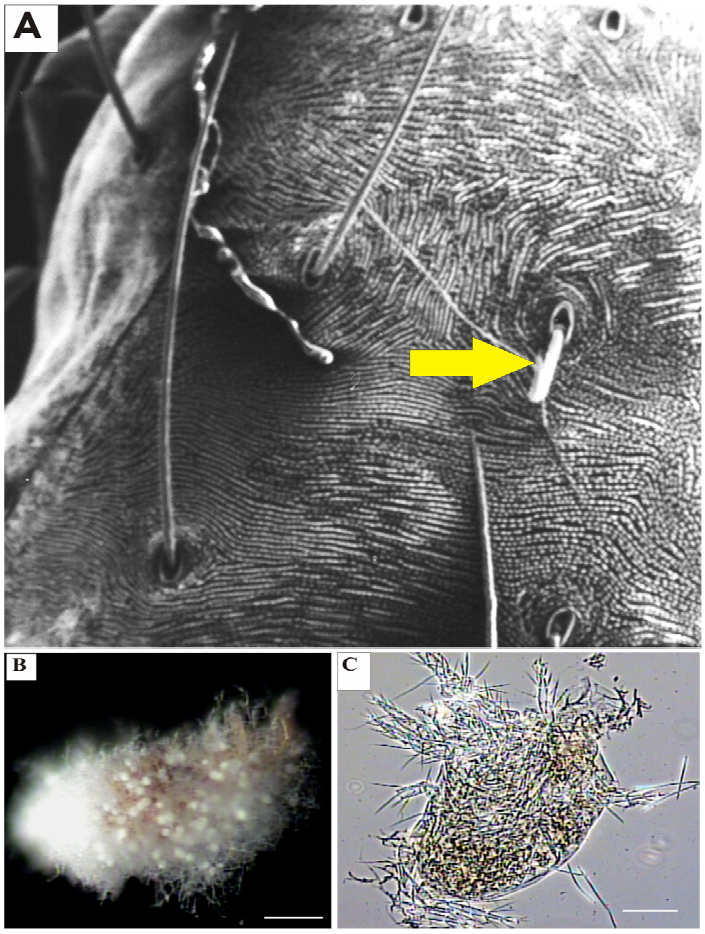

Figure 4.4: Two-spotted spider mite cadavers. (A) Emergence of aerial hyphae through the annulum of setae as seen with SEM. (B) Mycosed mites with conidiophore formation (SM). (C) Mummified cadaver with rust coloured hyaline bodies (PCM). Bars represent 100 µm.

CHAPTER 5

Phylogenetic analyses of *Beauveria bassiana* by ribosomal DNA sequence data

5.1. Abstract

Fitch parsimony and neighbour joining analyses of the ITS1-5.8S-ITS2 regions of different *Beauveria* species from different geographic origins and the *Engyodontium album* isolate were assessed to characterise genetic variability and relationships. rDNA analysis confirmed that *B. bassiana* is not a monophyletic group but a species complex. Distinct clades in the phylogenetic analyses in the current study were matched to four species of *Beauveria*: *Beauveria bassiana*, *B.* cf. *bassiana, Beauveria brongniartii* and *Beauveria caledonica* species. Isolates 154/02 and 183/02 from Norway were placed in the *B. brongniartii* group. The South African isolates PPRI 04304 and PPRI 04306 morphologically placed in *B. bassiana* in the current study, nested with *B. caledonica*. Within the clade comprising mainly of *B. bassiana* strains, a distinct feature was displayed when three of the *B. brongniartii* reference taxa namely, DQ153029, DQ153032 and DQ153038 nested in this group. Results from the current study indicate that rDNA analysis is a valuable technique in the biocontrol industry for population studies and species differentiation. However, there was no clear correlation between the phylogenetic pattern and geographic origin of the fungal isolates. The limited variation demonstrated at a strain level in the current study suggests further investigations of more variable regions for population level studies are warranted.

5.2. Introduction

Beauveria bassiana (*Bals.*) Vuill. is a cosmopolitan, soil-borne, haploid and mitosporic fungus with a host range of approximately 750 insects (Bridge *et al.,* 1990). *B. bassiana* has been formulated and registered as a commercial product against agricultural pests (Wang *et al.,* 2003). Castrillo *et al.* (1999) state that the microbial control agent i.e. the fungal strain to be released in the field, must be differentiated from other *B. bassiana* strains that may be native to the environment. Accurate identification of the isolates used for biocontrol is a prerequisite for registration and patenting to monitor the isolate released into the environment (Hegedus and Khachatourians, 1996; Wang *et al.,* 2005).

Phenotypic data i.e. standard bioassays of fungi with pests are not sufficient to characterise and select strains at an infraspecific level to be used for experimental purposes. The morphological characteristics used to characterise and select strains for laboratory and field studies used in the past by fungal taxonomists in biocontrol studies include spore dimensions, reproduction, colonial morphology, pathogenicity and host specificity. The morphological approaches have had some success determining species within a genus (Uribe and Khachatourians, 2004). This historical based technique has disadvantages including the lack of resolution in distinguishing a genet within a population, the need for isolation of fungi from pure cultures and lack of reliability (Uribe and Khachatourians, 2004; Entz *et al.,* 2005). Also, Entomopathogenic Fungi (EPF) such as *B. bassiana* are recognized as forming a "species complex" with genetically diverse lineages. Thus more polymorphic, sensitive and reliable markers are required for strain genotyping as well as inference into the population structure (Wang *et al.,* 2005).

Success in biocontrol programmes in the 21st century of EPF has spear-headed interest in molecular genetics to study fungal taxonomy, genetic identification and variation (St. Leger *et al.,* 1992; Coates *et al.,* 2002). Deoxyribonucleic acid (DNA) based methods are regarded as the best possibility to distinguish between

different species and strains grown under different conditions based on the genetic material being a constant feature. Genomic fingerprints are used increasingly to study relationships at an intraspecific and interspecific level. The genetic distances are interpreted by differences between individuals (Wurff *et al.,* 2000). Some molecular approaches attempted in previous studies to detect polymorphism include restriction fragment length polymorphisms (Maurer *et al.,* 1997), isozyme profiles (St Leger *et al.,* 1992; Bidochka *et al.,* 2002), random amplified polymorphic DNA (Maurer *et al.,* 1997; Berretta *et al.,* 1998), telomere fingerprintings (Padmavathi *et al.,* 2003), sequence characterised amplified regions (Castrillo *et al.,* 2003), inter-simple sequence repeats (Wang *et al.,* 2005), single-strand conformational polymorphisms (Hegedus and Khachatourians, 1996) and amplified fragment length polymorphisms (Muro *et al*., 2003). All the above molecular methods have provided some information on the taxonomy and population structure of EPF (Uribe and Khachatourians, 2004). However, they have to date, provided contradictory results with respect to correlating molecular structure with host range and geographic locations (Wang *et al.,* 2005). Thus, Wang *et al.* (2005) state that more sensitive and reliable markers are required to obtain strain genotypes.

In the current study an understanding of the genetic basis of evolution of pathogenicity is of interest thus it is important to study the molecular phylogeny of *B. bassiana*. Moreover, to monitor the efficiency and persistence of the strains for mite control requires detection assays. This is essential for the entomopathogenic fungus *B. bassiana* since the fungus has a wide host range and is common in nature (Castrillo *et al.,* 2003). In order to develop markers to study genetic variation, rDNA information is first required (Drenth and Irwin, 2001). Ribosomal genes have been analysed for evolutionary, phylogenetic, developmental, ecological, phytopathological, transcriptional and replication studies (Gasques *et al.,* 2003). Strain identification would be a valuable tool in differentiation of highly virulent strains used against *Tetranychus urticae* in comparison to mildly virulent ones. In this study we utilized the simple rDNA sequencing technique with the ITS1-5.8S-ITS2 regions to screen the population

structure of the South African strains of the *B. bassiana* complex, which is unknown, and to provide EPF phylogenies of exotic and native strains.

5.3. Materials and Methods

5.3.1. Fungal strains

Beauveria species used in the current study were procured from a collection of institutes around the world (Appendix 2) and reference taxa were obtained from Genbank (Appendix 2). The local fungal isolates used in the current study were collected from the soil of a cotton field in Rustenburg, South Africa and baited with *Galleria mellonella*. The fungi, identified by the Plant Protection Research Institute (PPRI) as *Beauveria bassiana* are lodged in the Biosystematics Culture Collection of the PPRI (MCB 121-PPRI 04306, MCB 124-PPRI 04304 and MCBG- PPRI 04305) (Appendix 1).

5.3.2. Cultivation of strains

The fungal isolates were inoculated onto Merck Sabouraud-dextrose agar (30 g/l) supplemented with yeast extract (2 g/l) and glucose (20 g/l) and incubated at 25ºC for 5 days. A loopful of culture from these plates was then inoculated into sterile 100ml Sabouraud-dextrose broth solutions (30 g/l), supplemented with yeast extract (2 g/l) and glucose (20 g/l) and incubated at 25ºC for 5 days on a rotary shaker at 160 rpm. The broth was used to obtain high density mycelium which was harvested by vacuum suction using a Buchner funnel with sterile filter paper (Whatman No. 1), washed thrice with sterile distilled water (Gaitan *et al.,* 2002) and placed in 10 cm tissue culture Petri dishes (Corning). The Petri dishes were placed at -70ºC overnight, freeze dried (Modylyo, Edwards) for seven hours and stored at -70ºC until required for DNA extraction.

5.3.3. DNA extraction

Genomic DNA was extracted from the freeze dried mycelium according to Wang *et al*. (2003) with modifications. The sample (30-60 mg) was ground with a sterile mortar and pestle in liquid nitrogen to a fine powder. The extraction buffer (500

µl) (100mM Tris, pH 8.0, 10mM EDTA, 2% SDS) and 2 µl of ß mercaptoethanol was added to the fine powder in an Eppendorf tube. The mixture was incubated at 65˚C for 60 minutes with occasional gentle mixing. The salt concentration was adjusted to 1.4 M with 5 M NaCl. Ten percent CTAB (1/10 volume of the salt concentration) was added to the contents in the Eppendorf and incubated at 65˚C for 1 hour. The aqueous layer containing the TNAs was extracted once by adding 1 volume of 24:1 chloroform: isoamyl alcohol, mixed gently, incubated for 30 minutes at 4˚C, centrifuged for 10 minutes at 4˚C at maximum revolutions per minute (max rpm) and the supernatant transferred to a fresh tube. The nucleic acid was precipitated with a 0.55 volume isopropanol, inverted gently as the DNA precipitated, placed on ice for 30 minutes and centrifuged at max rpm for 10 minutes at 4˚C. The supernatant was aspirated off, the pellet washed with cold 70% ethanol, centrifuged at 12 000 rpm for 15 minutes, vacuum dried, the pellet resuspended in 500 µl of TE buffer and 3 µl of RNAse A and incubated at 30ºC for 30 minutes. The resuspended pellet was placed at -4ºC overnight and the DNA concentration determined by running the DNA extract against a molecular mass ladder (Inqaba, 1kb Mass Ruler).

5.3.4. DNA amplification

The primer pairs used for the PCR reactions were TW81 (universal primer- GCG GAT CCG TTT CCG TAG GTG AAC CTG C) and AB28 (universal primer- GCG GAT CCA TAT GCT TAA GTT CAG CGG GT) which amplify the ribosomal repeat from the 3' end of the 16s rDNA across the internal transcribed spacer 1, the 5.8s rDNA, and internal transcribed spacer 2, to the 5' end of the 28s rDNA (Curran *et al.,* 1994). The primers were obtained from Inqaba Biotechnology and the original sequence information was obtained from Chambers *et al.* (1986). The 50 µl PCR reaction mixture contained 0.25 µM of primer (forward and reverse) (Inqaba), 2x PCR buffer (Inqaba), 10 ng fungal DNA, 25 uM Master mix (Inqaba) and nuclease free water. The amplification parameters were programmed in a thermal cycler (Gene Amp PCR system 2700) as follows: initial denaturation at 94°C for 5 minutes; followed by 30 cycles of: denaturation, 1 minute at 94°C; annealing, 1 minute and 30 seconds at 55°C;

extension, 2 minutes at 72°C with a final extension of 5 minutes at 72°C (Curran *et al.*, 1994). The controls contained no fungal DNA. The DNA samples were amplified by PCR as above. The PCR product was visualized on a 1.5% TBE (Tris/ Boric Acid/ EDTA) (Sambrook *et al.,* 1989) agarose gel stained with ethidium bromide and run at 4V. cm^{-1} for 2 hours. The gels were visualized and photographed under UV light (Bio Doc IT System).

5.3.5. Nucleotide sequencing

PCR amplified products of the *B. bassiana* strains were purified and sequences were generated on the ABI Prism 310 automated sequencer at the School of Molecular and Cell Biology, University of the Witwatersrand using the same primers as used for PCR. Both forward and reverse DNA strands were sequenced. Electropherograms of all the sequences were assembled into contigs, proof-read manually and the consensus sequences aligned using Sequencher (version 4.1.2, Genecodes Corp). Apomorphies were confirmed by referencing back to electropherograms.

5.3.6. Data handling

Selected sequences from Genbank were included in the analysis and the accession numbers appear in Appendix 2. The alignment was checked visually and minor changes made manually. All the sequences used in the current study are deposited in Genbank. Four samples (Bb 9013, Bb 9015, Bb 9016 and Bb 9019) supplied by Dr. A. Gaitan (CENICAFE) were excluded from the analysis as a huge portion of the ITS1 region was unalignable with the other ingroup taxa. The alignments were exported in Nexus format into PAUP (version 4.0b10, Swofford, 2001). Nucleotides were moved to the left if alternative alignments were possible due to insertion or deletion events. Non-homologous insertions were aligned and coded separately. Non-homologous deletions were aligned together, but coded separately. All insertion or deletion events were treated as missing data. An extension to the data matrix coded each indel for presence or absence.

For the outgroup, taxa *Verticillium lecanii* and *Fusarium oxysporum* were initially tested in the alignment analysis. However, the sequences were so divergent as to prevent alignment with ingroup taxa with any confidence. Thereafter, *Beauveria brongniartii* (*Sacc.*) Petch was selected but it nested within the ingroup. *Beauveria alba,* used as the outgroup in the study of Huang *et al.* (2002), was obtained from Genbank and used as the final outgroup taxon (Fig 5.1 and 5.2) in the current study. However, it should be noted that isolate *Beauveria alba* identified in 1912 was moved to the genus *Titirachum* in 1940 (Augustinsky *et al.,* 1990). Finally, in 1978, Hoog transferred it to the genus *Engyodontium* as the species *Engydontium album* (Limber) de Hoog (Rehner and Buckley, 2005). It is surprising that the name *B. alba* is still being used to date.

The aligned matrix of sequence data of ITS1-5.8S-ITS2 regions of three *Beauveria bassiana* native strains, 20 exotic strains and reference taxa from Genbank (15 strains of *B. bassiana*, 5 strains of *B. brongniartii* and 1 strain of *E. album*) is presented in Appendix 3.

5.3.7. Phylogenetic analysis

PAUP was used to estimate the phylogenetic trees using parsimony and neighbour joining methods. Fitch parsimony analyses with rigorous heuristic searches, 100 random addition replicate analysis, Tree Bisection Reconnection (TBR) swapping with ACCTRAN options were conducted under equal character weighting. Multiple parsimonious trees were combined as strict consensus trees. The indels matrix was included for the phylogenetic analyses because resolution decreased considerably when indels were excluded. Branch support was estimated using bootstrap support values (Felsenstein, 1985), based on 100 replicates using the same settings as the general heuristics search mentioned above. Neighbour-joining analysis using the Kimura two-parameter method (γ=0.5) constructed an alternative phylogenetic hypothesis with bootstrap values based on 200 replicates to provide confidence estimates for tree topologies. *E. album* was used as the outgroup taxon in both analyses (Fig. 5.1 and 5.2). Tree statistics are reported for each resulting tree.

5.4. Results

5.4.1. Sequence characteristics

The multiple sequence alignments of the 550 base pair (bp) nucleotide sequences of the ITS1-5.8S-ITS2 regions of the *Beauveria* isolates investigated by us with the Genbank sequences for the same regions are shown in Appendix 3. The consensus length of ITS1 region was 189bp, 156bp for the 5.8S rDNA gene region and 190bp for the partial ITS2 region. The sequences contained 29 parsimony informative characters of 96 variable characters out of a total of 550 characters. In addition 57 coded indels were included, 13 of which were phylogenetically significant, out of a total of 607 characters. Ingroup variability occurred with 56 point mutations.

5.4.2. Phylogenetic analyses

Neighbour joining (Fig. 5.1) and parsimony (Fig. 5.2) analyses produced almost identical tree topologies that differed primarily in minimum branch lengths and bootstrap support values. Phylogenetic analyses excluding indels resulted in considerably less resolution than with indels. Results presented here are from analyses including indels. The phylogeny resulting from the neighbour joining analysis displayed slighter higher resolution than the parsimony analysis with 184 MPTs of 185 steps with a higher rescaled consistency index (RC) of 0.75. The parsimony analysis yielded 174 MPTs of 206 steps with a lower RC of 0.61.

The *Beauveria* isolates formed two well supported clades (bootstrap values of 97% and 90%) with *Engydontium album* as the outgroup based on the genetic variation between the strains (Figs. 5.1 and 5.2). The outgroup *E. album,* was distinguished from the *Beauveria* species by 46 point mutations and 29 indels.

Within *Beauveria,* seven isolates tested in this study fell within clade 1 with five Genbank reference taxa. Within clade 2, 16 of the 23 strains nested with 15 reference sequences obtained from Genbank (Figs. 5.1 and 5.2). There is some branching evident in clade 1, but in clade 2, very little resolution is evident (Figs.

5.1 and 5.2). The formation of the clades in the phylogenetic analyses in the current study confirms that *Beauveria bassiana* is not a monophyletic group but a "species complex".

5.4.2.1. Clade 1

Clade 1 comprises two clades designated as A and B in Figs. 5.1 and 5.2, respectively.

Clade A is further subdivided into A1 with *Beauveria brongniartii* isolates and A2 with *B.* cf. *bassiana* isolates from Europe and Africa nested together. However, the branch supporting the *B. brongniartii* and *B.* cf. *bassiana* clades is not supported well (56%) by these analyses (Figs. 5.1).

The two *B. brongniartii* strains obtained from genbank in clade A (AB258369 and AY24628), are grouped with isolates 154/02 and 183/02 from Norway with a fairly high bootstrap support (71%). It was also interesting to note that the BLAST searches for the 3 regions used in the current study yielded a 100% homology of the strains 183/02 and 154/02 to *B. brongniartii*. It is suggested by the present study that isolates 154/02 (Norway) and 183/02 (Norway) are actually *B. brongniartii* strains thus all the *B. brongniartii* strains grouped together in clade 1.

Clade A2, well supported at a level of 95% (Figs. 5.1) consists of strains LRC 100 (Togo), LRC 121 (Spain), 50.771 (Paris), AY334541 (Philippines) and AY532009 (Mexico). In this study these strains are assumed to be *Beauveria* cf. *bassiana* based on the reference taxon AY532009 identified in the study of Rehner and Buckley, (2005).

Also in clade 1, clade B contained the South African strains PPRI 04304 and PPRI 04306 grouped together with a reference taxon *B. caledonica* (AY532003) from Brazil with a strong bootstrap support of 100% (Fig. 5.1). The taxonomic identities of the South African strains were determined morphologically by the Plant Protection Research Institute, a credited institute in South Africa, as *B.*

bassiana (Appendix 1). This reference taxon accessioned under the name *B. bassiana* in Genbank, was then referred to as *B. caledonica* by Rehner and Buckley in their study based on morphological and phylogenetic (EF1-α and ITS1) studies. However the phylogenetic analyses (Figs. 5.1 and 5.2) conducted in the current study suggests that the South African isolates might be *B. caledonica* or a near relative.

Also, the South African isolates, PPRI 04304 and PPRI 04306 grouped together in clade 1B, are not monophyletic with the other native isolate PPRI 04305 found in clade 2C due to point mutations displayed in the alignment (Appendix 3). Since the South African strains do not group together in the same clades, the indication is that they are different species.

5.4.2.2. Clade 2

Clade 2 is matched to the clade of *B. bassiana* isolates (Figs. 5.1 and 5.2) with very little resolution of relationships displayed. Within clade 2 a global distribution of the isolates was demonstrated suggesting no correlation of geographic origin with phylogenetic analyses with the *B. bassiana* isolates.

Only in the neighbour joining analysis (Fig. 5.1), and with weak bootstrap support at 52%, did strains LRC 130 (Australia), LRC 120 (Bulgaria), 51.1083 (Paris), ALB 60 (Albania), PPRI 04305 (South Africa), AY532015 (Commonwealth of independent states), AY532015 (Australia) and AY465430 (North America) nest together in clade C. This is supported due to a point mutation in the conserved 5.8S region (Appendix 3). Despite the same point mutation shared in the 5.8S region, the isolates PPRI 04304, PPRI 04306 and AY532003 (Brazil) in Clade 1B these isolates do not group with the *B. bassiana* isolates nested in Clade 2(C). Clade C is assumed not to be apparent in the parsimony analysis due to parallel evolution of the point mutation. C1 nested in clade 2 at a weak 52% bootstrap support with strains LRC 106 (Brazil) and LRC 77 (Madagascar), clustering with reference taxa AY336939 (China) and AJ560666 due to an A-G transition in the ITS2 region at position (p) 493 (Appendix 2).

The rest of clade 2 (Figs. 5.1 and 5.2) is unresolved except for strain LRC 126 (Denmark) which is grouped sister to AJ564484 with a strong bootstrap support of 94%. Also, isolate 105 (Slovakia) groups with reference taxon AY531999 with a weak support of 65% (Figs. 5.1 and 5.2). Strains B3 (Poland), 00061 (Kenya), LRC 171 (Canada), KVL 01-135 (Denmark), 00362 (Benin), 00144 (Ghana), LRC 171 (Canada), AY334540 (Philippines), AY532044 (Brazil), AF345539, AY334536 (Colombia), AJ564484 (Kyrgyz republic), AY336938 (China) and AY531999 (France) demonstrated a high degree of homology in their sequences. Within clade 2, comprising mainly of *B. bassiana* strains, a distinct feature was displayed when three of the *B. brongniartii* reference taxa namely, DQ153029, DQ153032 and DQ153038 nested in this group. It is thus proven in this study that these isolates actually belong to the *B. bassiana* species group. However, within this monophyletic grouping some branching was observed.

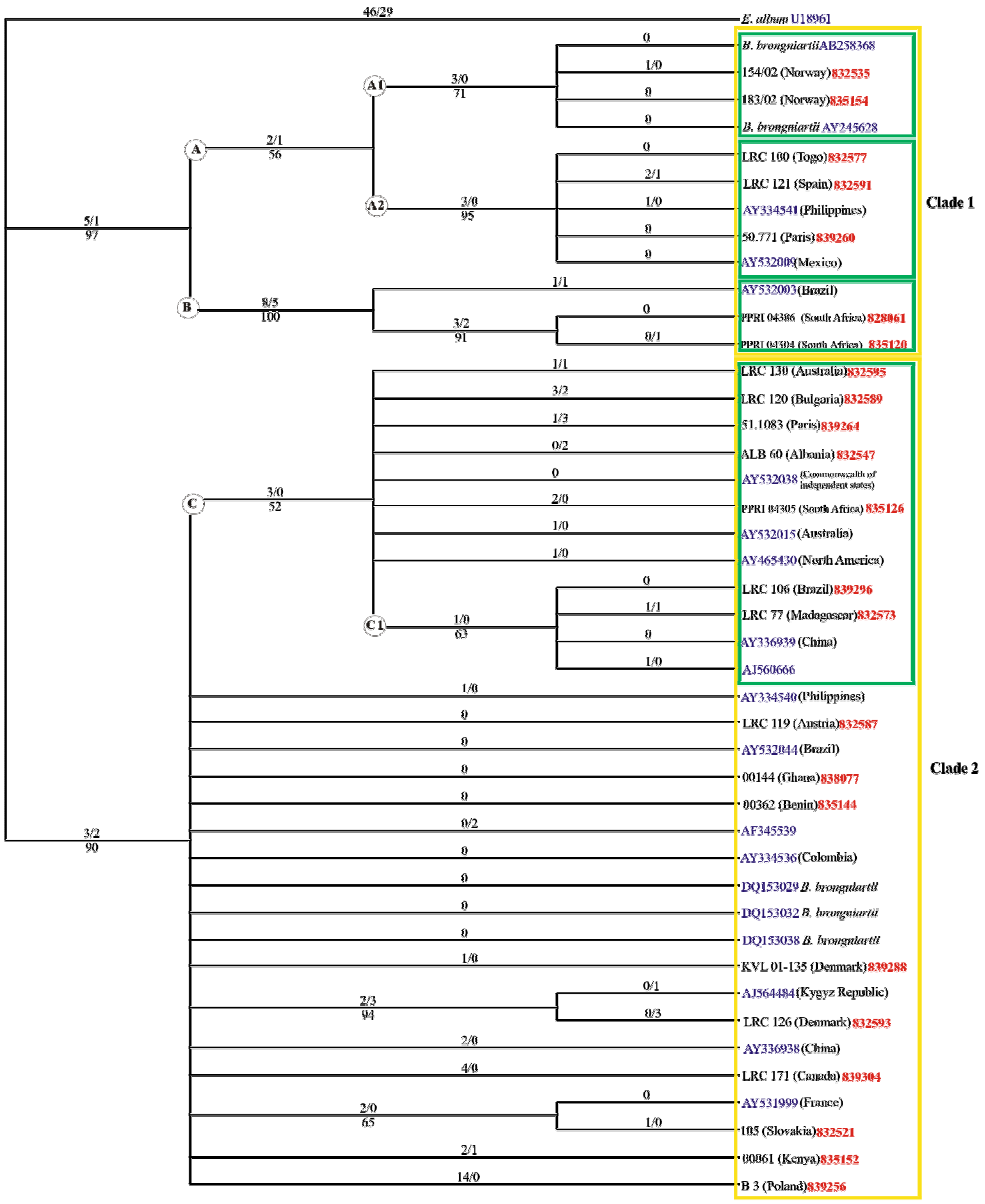

Figure 5.1. Neighbour joining analysis of sequences of the ITS1, 5.8s rRNA gene and ITS2 regions of 23 *Beauveria bassiana* strains from different geographic locations (184 MPTs, 185 steps, CI= 0.64, RI= 0.88, HI= 0.37). Minimum branch lengths (point mutations/ indels) are indicated above the branches and bootstrap values > 50% are displayed below. The fungal samples used in the experiment (shown in red) are displayed with the accession numbers. Sequences of isolates with the accession numbers in blue were obtained from Genbank. *Engydontium album* serves as the outgroup for the analysis. Blocks displayed in yellow show the clades, and in green the groups.

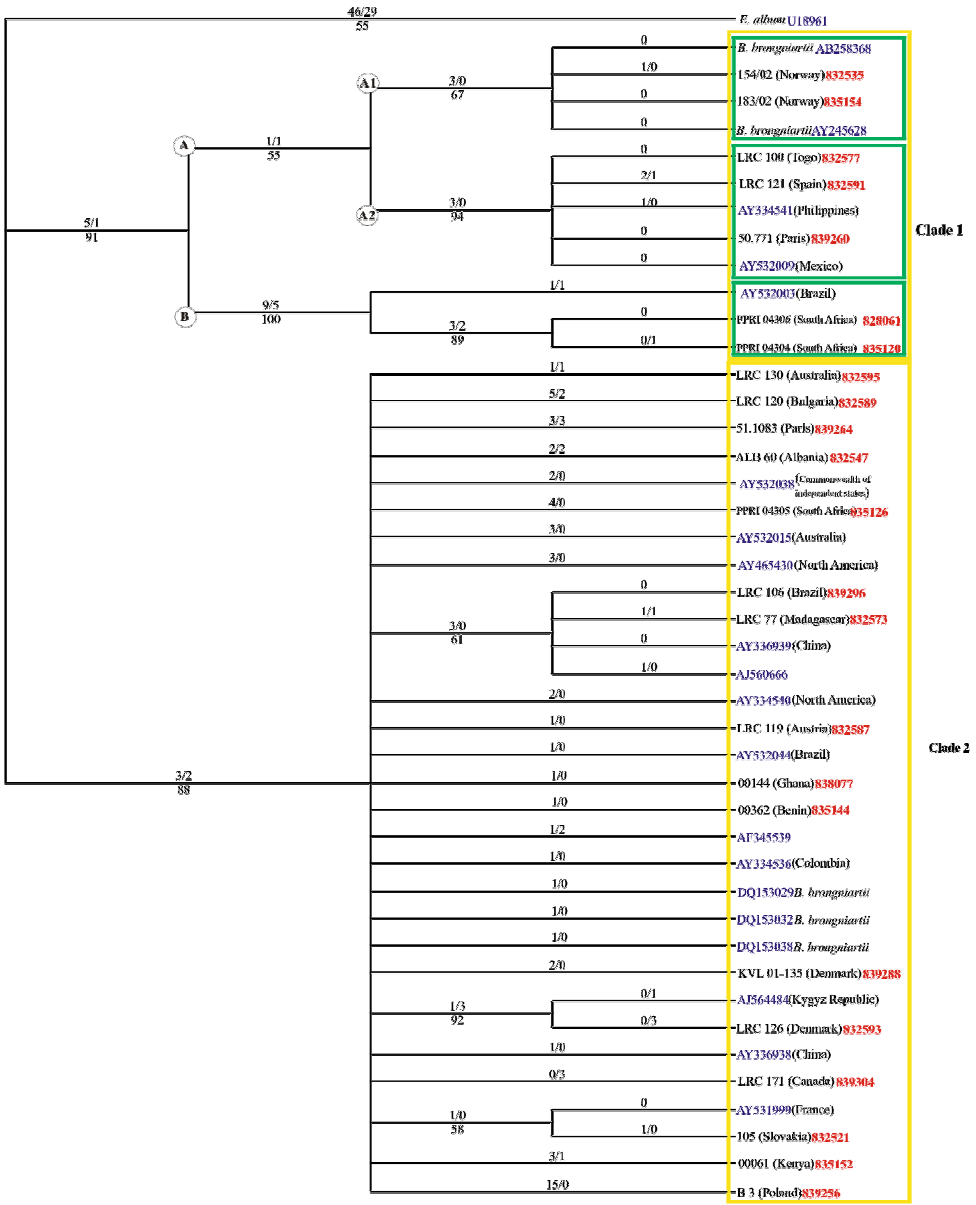

Figure 5.2. Strict consensus tree of 174 MPTs (206 steps, CI= 0.49, RI= 0.79, 0.51) resulting from unweighted Fitch parsimony analysis of sequences of the ITS1, 5.8s rRNA gene and ITS2 regions of 23 *Beauveria bassiana* strains from different geographic locations. Minimum branch lengths (point mutations/ indels) are indicated above the branches and bootstrap values > 50% are displayed below. The fungal samples used in the experiment (shown in red) are displayed with the accession numbers. Sequences of isolates with the accession numbers in blue were obtained from Genbank. *Engydontium album* serves as the outgroup for the analysis. Blocks displayed in yellow show the clades, and in green the groups.

5.5. Discussion

In the present study, differentiation between *Beauveria* isolates was demonstrated by the phylogenetic analysis of the ITS1-5.8S-ITS2 regions.

5.5.1. Monophyly of Beauveria

Phylogenetic analyses of ribosomal DNA (rDNA) sequences of *Beauveria* isolates in the current study showed two clades confirming that the *Beauveria bassiana* species complex is not monophyletic and identifying other species amongst the isolates. This result is in agreement with Rehner and Buckley (2005), who determined the ubiquitous *B. bassiana* species to be non-monophyletic and deduced that the diversity indicates a history of cryptic diversification. EPF isolates such as *B. bassiana* have been previously recognized as being a "species complex" with genetically diverse lineages (Hajek and St Leger, 1993; Huang *et al.,* 2002). This analysis thus supports the conclusion and indicates that the clades 1 (A1), 1 (A2), 1 (B) and 2 represent different species.

5.5.1.1. Clade 1

Clade 1 comprised of *Beauveria brongniartii* (clade A1), *B.* cf. *bassiana* (clade A2) and *Beauveria caledonica* (clade 2).

In the present study two isolates 154/02 and 183/02 from Norway, originally classified as *B. bassiana* (Clade 1, A1) were nested within the *B. brongniartii* clade. The species recognition of the reference taxa (AY334541 and AY532009) as *B. brongniartii* were confirmed in another study by morphology and phylogenetic analysis (Rehnner and Buckley, 2005). The apparent lack of difference with the *Beauveria* species in this clade with the phylogenetic analyses indicated in the current study that the Norway strains are *B. brongniartii*.

Clade A2 in the current study provided a dilemma regarding the designation of the isolates that nested within it. On one hand, it formed a sister taxon with *B. brongniartii* but on the other hand it contained *B. bassiana* isolates. In the current

study the isolate AY532009 nested with AY334541 (Philippines), LRC 100 (Togo) and LRC 121 (Spain) in clade 1A2 and not with the other *B. bassiana* isolates in Clade 2. Isolate AY532009 (Mexico) was identified by Rehner and Buckley (2005) as *B.* cf. *bassiana*. In their study this taxon did not fall into the *B. bassiana* clade based on the ITS and EF1-α phylogenetic analyses. There were also no conspicuous morphological or cultural characteristics in their study to differentiate the two clades formed by *B. bassiana* isolates. Thus they designated the isolate AY532009 (Mexico) as *B.* cf. *bassiana.* For this reason the isolates within clade 1(A2) are regarded as being *B.* cf. *bassiana.* Rehner and Buckley (2005), demonstrated in their study that North American and European isolates grouped into the *B.* cf. *bassiana* clade. Similarly, in the current study North American and European isolates grouped together but also with an African isolate.

In clade 1(A), sister *B. brongniartii* strains (clade A1) grouped with *B.* cf. *bassiana* strains (clade A2) with a weak support (Figs. 5.1 and 5.2). In previous studies, *B. brongniartii* grouped with *B. bassiana* based on ITS sequencing, ITS-Restriction Fragment Length Polymorphism and Random Amplified Polymorphic DNA analysis (Gaitan *et al.,* 2002; Muro *et al.,* 2003). However, the current study showed that the *B. brongniartii* and *B.* cf. *bassiana* formed sister taxa, whilst *B. bassiana* fell within another clade.

The isolates within clade B in the phylogenetic analyses, grouped with a reference taxon AY532003 (Brazil). This Brazilian isolate was accessioned under the name *B. bassiana* in Genbank. However, morphological studies conducted by Rehner and Buckley (2005), led the researchers to believe that this isolate was misidentified. They first suspected the isolate to represent *Beauveria amorpha* then, the phylogenetic and morphological analyses led them to believe that isolate AY532003 was consistent with that of *B. caledonica*. Previous chemotaxonomic and molecular studies had placed *B. caledonica* as a subspecies of *B. bassiana* (Hegedus and Khachatourains, 1993). However, the current study concurs with Rehner and Buckley, (2005) that although these species are related, *B. caledonica* is not a subspecies of *B. bassiana*.

In the current study the South African isolates, PPRI 04304 and PPRI 04306 grouped together with *B. caledonica* AY532003 in clade 1(B), but are not monophyletic with the other native *B. bassiana* isolate PPRI 04305 found in clade 2(C) due to point mutations displayed in the alignment (Appendix 3). Since, the South African strains do not group together in the same clades they support the deduction that the PPRI 04304 and PPRI 04306 designated morphologically by a credited institute in South Africa as *B. bassiana* (Appendix 1) have probably been misidentified. Thus, for the purpose of this project in a broad sense, for the isolates PPRI 04304 and PPRI 04306 the taxa *B. bassiana* and *B. caledonica* will be regarded as *sensu latu*. PPRI 04305 was identified correctly according to the current study but will be discussed later.

The nesting of the S. A. isolates with the *B. caledonica* reference taxon AY532003 suggests that the discovery of this lineage in Africa demonstrates that *Beauveria* displays biodiversity in Africa according to S. Rehner (pers. comm.). He considers continentally disjunct lineages as separate species, at least when supported by molecular data. To date, a description of the *B. caledonica* reference taxon (AY532003) is being conducted by S. Rehner (pers. comm.) and will thus be useful for further studies. Also, since the *B. bassiana* fungus has no type specimen, the usage of a reference neoisotype specimen prepared from ARSEF 1564 (Italy) which is currently being designated (R. Humber, pers. comm.) will be the most appropriate option for further studies. Morphological studies coupled with molecular work is suggested for complementary work since it is impossible to distinguish *B. bassiana* and *B. caledonica* using only morphological characters due to difficulties in obtaining taxonomically informative characteristics.

5.5.1.2. Clade 2

The majority of the isolates used in the current study were grouped within the well supported clade 2, however little resolution was displayed. In the current study the taxonomic recognition of the isolates was based on phylogenetic studies with no morphological studies (except for PPRI 04305: Chapter 4). Reference taxa (AY532038, AY532015, AY532044 and AY531999) that nested in clade two in

the current study were also used by Rehner and Buckley, (2005). In their study these reference taxa fell within the *B. bassiana* clade with phylogenetic analyses (separate and combined) of the EF1-α and ITS regions. These isolates also displayed the traditional conidial morphology of *B. bassiana* of globose to subglobose conidia with the diameter of 2.3-3.2µm. Thus, based on the study by Rehner and Buckley (2005), results of the current phylogenetic study prove that the *Beauveria* isolates within clade 2, including the S. A. isolate PPRI 04305, are *B. bassiana*.

Information pertaining to the reference *B. brongniartii* isolates (DQ153029, DQ153032 and DQ153038) nesting in clade 2 made up of globally distributed *B. bassiana* isolates was unavailable from Genbank. However, the phylogenetic analyses in the current study suggest that these *B. brongniartii* isolates are actually *B. bassiana*.

5.5.2. Usefulness of the ITS1-5.8S-ITS2 regions

Noonan *et al.* (1996) state that ribosomal DNA is well suited for use in phylogenetic studies for different levels of discrimination as the ITS spacers evolve rapidly and the 5.8S coding region evolves slowly.

The analysis of the more conserved gene region such as 5.8S clarifies phylogenetic relationships only at higher taxanomic levels (Inglis and Tigano, 2006). Redecker *et al.* (1999) state that the limited phylogenetic signal in the short 5.8S subunit is sufficient to assign the sequences to defined groups. Point mutations in the South African strains PPRI 04304 and PPRI 04306 seen at positions 225, 227, 234, 335, 344 and 357 in the alignment (Appendix 3) placed the strains in clade 2 (B) with a high bootstrap support of 91% (Fig. 1). Also, the T-C transition at position (p) 245 (Appendix 1) resulted in the monophyletic grouping of the *B. bassiana* isolates LRC 130 (Australia), LRC 120 (Bulgaria), 51.1083 (Paris), ALB 60 (Albania) and PPRI 04305 (South Africa) nested in Clade 2 of the neighbour joining analysis.

The sequence variation of the ITS region makes it useful for studies at the intraspecific and interspecific levels (Chen *et al.,* 2001). The pattern of evolution within the ITS regions in Eukaryotes is a mosaic of base substitutuions and insertion or deletion events not uniformly distributed throughout the spacers (Inglis and Tigano, 2006). The current study concurs with Donnell (1992) who demonstrated that the ITS2 region in *Fusarium sambucinum* was more divergent than the ITS1 spacer. In the current study, sequence heterogeneity in the ITS regions also clustered strains closely together in the different clades based on transitions (G-A at p394 and p483) and a single transversion event (A-C at p479) (Appendix 3). Point mutations demonstrated in the ITS1 region grouped the isolates in clade 1 together. For example, Group A and B (Fig. 5.1) were separated based on C-A transversion at p32. A1 consisted primarily of *B. brongniartii* strains based on a C-T transition at p50. The A2 clade with the *B. bassiana* isolates was based on transversions in the ITS1(C-A at p133) and ITS2 (T-A at p478) regions.

The low level of genetic variability in the ITS regions between exotic and native *B. bassiana* strains is demonstrated by the lack of resolution in the current study. Rehner and Buckley, (2005) found in their study that ITS regions do not provide sufficient variation in order to show within species relationships compared to the more variable EF1-α region. Thus, for complementary studies molecular work should be conducted on other variable regions of the genome which are more likely to elucidate the evolutionary relationships within *Beauveria*.

5.5.3. Geographic variation

Previous studies showed evidence that an association between *B. bassiana* strains with geographic regions existed (Glare and Inwood, 1998, Castrillo *et al.,* 1999). The current study indicates that the South African *B. bassiana* isolates are grouped together with strains from different habitats thus contradicting the groupings based on geographic location. Entomopathogenic Fungi (EPF) genotypes have however been reported to be associated with host species. In studies with *Metarhizium* genotypes, although limited evidence of association

with particular ant species was described there was no association with habitat (Hughes *et al,* 2004). Also in another study, *Entomophthora muscae* isolates from a particular host species appeared to be clonal due to very little variability in the sequences despite that fact that the isolates were obtained from different geographic locations (Chen *et al.,* 2001). In the present study the poor resolution of the *B. bassiana* strains displayed could be related to host-specificity; however, this information was unavailable for analysis.

5.5.4. Conclusion

Sequencing of the ITS1-5.8S-ITS2 regions in the current study has been effective in partitioning *B. bassiana, B. brongniartii, B.* cf. *bassiana* and *B. caledonica* species within the genus *Beauveria*. The phylogenetic analyses also demonstrated that two of the South African isolates PPRI 04304 and PPRI 04306 could have been misidentified. However for the authors to confirm that these isolates are *B. caledonica* or a close relative, complementary studies are required. The results from this study suggest that in order to assess the diversity of the *Beauveria* "species complex" traditional morphology studies must be coupled with molecular studies.

CHAPTER 6

Summary and Conclusions

The detrimental effects of pesticides on the environment and human health have led to the worldwide recognition that a replacement of synthetic chemical pesticides is necessary (Hajek, 2004). Also unsustainable cropping systems have been demonstrated with *Tetranychus urticae* Koch pests where chemicals have been banned or are in the process of being phased out due to the mites developing resistance to them. Thereby, control of this pest with natural biocontrol agents is warranted (Van der Geest *et al.,* 2000; Ansari *et al.,* 2004). To date, much of the research on the biocontrol of phytophagous mites with microbial pathogens focused on the potential of entomopathogenic fungi (EPF) (Sztejnberg *et al.,* 1997). EPFs' have demonstrated the capacity to regulate mite populations and EPF epidemics have the potential to decimate mite populations (Chandler *et al.,* 2005). South Africa seems to have lagged behind in this research endeavour.

Beauveria bassiana a cosmopolitan EPF, is reported to be non-toxic to humans and other mammals and is therefore a potential candidate for the biocontrol of phytophagous mites (Muerrle *et al.,* 2006). *B. bassiana* is not an obligate entomopathogen of *T. urticae* (Shi and Feng, 2004). However, current *B. bassiana* products have been commercialized (Alves *et al.,* 2002; Alves *et al.,* 2005). This prompted us to also to investigate indigenous and exotic *B. bassiana* strains as biocontrol agents against the two-spotted spider mite pest since the applications of EPFs' such as *B. bassiana* have not been researched to our knowledge in South Africa.

Considering the requirements mentioned above, this project has focused on the following objectives:

1. Growth and development of the fungus by the diphasic fermentation process in response to nutrients (carbon and nitrogen).
2. The virulence of *B. bassiana* against *T. urticae* Koch;
3. Observation of *B. bassiana* infection cycle in *T. urticae*.
4. The study of phylogenetic relationships between isolates of the *Beauveria* genus.

6.1. *Beauveria bassiana* inoculum produced by the diphasic fermentation process

In the potential commercialization of an EPF, emphasis should be placed on whether a product can be developed at a minimal cost to compete with chemicals in order to return an acceptable profit on the research and development investment (Bartlett and Jaronski, 1988). The generally non-fastidious nature of the pathogenic deuteromycete *B. bassiana* has made it amenable to mass production. For many years the technology for mass production of *B. bassiana* in America, Europe and Asia has been employed (Feng *et al.,* 1994; Wraight and Carruther, 1999). Industrial units namely, Biological Control Products (South Africa) and LUBILOSA (Benin), are appearing in Africa as *B. bassiana* mycopesticide producers. The ideal production system in a developing country such as South Africa involves a system with low capital investment and high labour inputs with the usage of local equipment, resources and workers (Cherry *et al.,* 1999). This study made use of a simple diphasic fermentation system with a high labour input appropriate for a developing country situation. The product i.e. contaminant-free virulent conidia were economically mass produced at a small scale.

B. bassiana grown in the submerged phase of the diphasic fermentation process in the 4% (1:1) ratio of maltose and yeast extract supplementation promoted high levels of mycelial dry mass production. Comparatively, spore production was maximal in the 3% supplementation assumed to be related to the low levels of nutrient sources supplemented in the medium. Knowledge of nutrient supplementation is suggested to have implications for the further development of the fungus as a biocontrol agent because of the effects of the nutritional factors on growth of the fungus in media and virulence of spores (Daoust and Roberts, 1983, Bidochka *et al.,* 1990).

The fungal structures produced during the first phase of the diphasic fermentation process were submerged conidia recognized as small, spherical structures with a smooth form. In comparison, inoculum of the second semi-solid phase produced aerial conidia with small, spherical, rough surfaces and a brittle appearance assumed to be related to nutrient deprivation.

Further research

Production process parameters such as temperature, humidity and media composition play crucial roles in determining the yield and quality of EPF inoculum, affecting the cost of production and decisions concerning commercialization (Cherry *et al.,* 1999).

Butt and Goettel, (2000) state that the criteria for a commercially viable EPF product for use in the agricultural sector include optimization of storage properties such as longevity and maintenance of viability. Formulation of the EPF product can improve longevity under storage and field efficacy by protecting against desiccation and harmful UV radiation as well as enhance fungal virulence by improving spore attachment to the host surface (Butt and Goettel, 2000). Since formulation has been recognized as an important key to the success of an EPF it needs to be addressed in further studies.

The warty brittle outer layer of aerial conidia observed in the current study by microscopy (Chapter 2) was suggested by Thomas *et al.* (1987) to confer extra protection of the conidia making the conidia more resistant to adverse environmental conditions. Complementary ultrastructure studies are required to observe the outer wall of the aerial conidia.

The 10^6 conidial yield produced in the current study by the diphasic fermentation process is not competitive with other new existing technologies which produce conidial yields of 10^{13}. Since new technologies are being developed worldwide, mass production of EPF for complementary studies using a more advanced diphasic fermentation method needs to be attempted. Submerged conidiation of *B. bassiana* demonstrated in the current study (Chapter 2) has also been illustrated previously by Thomas *et al.* (1987). Ideally, submerged fermentation should be explored in future studies for the production of infective conidia since it is reported to fit the framework of current industrial microbiology which may significantly lower production costs of EPF used for pest control (Alves *et al.,* 2002).

6.2. A preliminary study of the potential of *Beauveria bassiana* as a biocontrol agent against the two-spotted spider mite (*T. urticae*) *in vitro*

An *in vitro* bioassay was carried out to compare the virulence of *B. bassiana* strains isolated from wide geographic origins against the two-spotted spider mite. Strain selection is essential for the successful application of EPFs' in pest control programmes (Butt *et al.*, 2001). All the *B. bassiana* strains were pathogenic to *T. urticae* adults with mortality increasing with time demonstrating the effectiveness of the inoculum. Since differences in the virulence of the *B. bassiana* isolates in the current study were displayed, this study is in concordance with Mueller *et al.* (1999) that *B. bassiana* exhibits restricted host ranges and contains a diverse assemblage of genotypes and a "species complex". The preliminary *in vitro* results obtained support the hypothesis that *B. bassiana* can be developed as a mycoacaricide against *T. urticae*. Isolate ALB 60 (Albania) is the most likely candidate for use as a mycoacaricide.

The bioassay protocol was adopted from Alves *et al.* (2002) in view of the fact that failure to understand the importance of the bioassay design is reported to lead to inconsistent results, high control mortalities and poor assessment of fungal virulence (Butt and Goettel, 2000). Since the dose-mortality assays were not performed in this study, the control mortalitites were not corrected for in the statistical analysis (Chapter 3). Loss of virulence in the EPF isolates have been documented to be attributed to serial passaging in artificial media (Butt and Goettel, 2000; Alves *et al.,* 2002) thus *B. bassiana* strains were passed through *T. urticae* prior to the bioassays. Bioassays were conducted on adult mites only due to the other stages of the mites' being smaller in size making it difficult to handle them.

Further research

Assaying of *B. bassiana* against all *T. urticae* life stages and other arthropod pests is essential for the development of the EPF as a mycopesticide. Also, commercial preparations should be used for comparison of virulence levels. For further studies, screening of more native fungal isolates from *T. urticae* is necessary.

The results from this study are not considered an accurate indicator of which isolates can be successful as biocontrol agents under field conditions, thus further studies would involve improved protocols designed to predict performance of the isolates under field conditions. Field studies will ultimately determine the place of *B. bassiana* in *T. urticae* management programmes in South Africa.

6.3. The infection cycle of a South African isolate of *Beauveria bassiana* in the two-spotted spider mite

The different stages of the life-cycle of *B. bassiana* infecting *T. urticae* were observed since reported observations on mite diseases caused by EPF are scarce, and knowledge is fragmentary. Mietkiewski and Balazy, (2003) state that the diagnostic description of fungal diseases in mites is difficult due to the mite's small size. This study thus dealt with the preparation of the diseased mites' by the four different microscopy methods using either modified or new protocols. The observations in this study confirmed the findings of earlier reports regarding the nature of physical characteristics of arthropod fungal infections (McCauley and Zacharuk, 1968; Whipps and Lumsden, 1989; Boucias and Pendland, 1991; Khetan, 2000). However, aspects not observed before with *T. urticae* infection included limited hyphal growth on the cuticle surface before penetration, *per os* mode of entry, cuticular melanization, lateral hyphal development under the cuticle and aerial hyphal emergence through the setal annulum on the dorsal surface of the cadaver. These observations will stimulate further research in the development of *B. bassiana* as a mycoacaricide.

Further research

Alternative microscopy techniques are available to study fungal infection in the host such as transmission electron microscopy and fluorescent microscopy but did not form part of the objectives of the project. These techniques are suggested for further studies.

A comparison of different inoculation methods must be included in complementary studies with the inclusion of some methods which simulate field conditions (Butt, T. M. *pers. comm.*).

Since not all areas on the insect cuticle are equally vulnerable to penetration by EPF the location where the inoculum lands on the cuticle is theorized to influence the probability of infection and speed of mite mortality following exposure to spores (Butt and Goettel, 2000). The carrier, Tween used for the bioassays could have influenced the adhesion of the inoculum as well. Complementary research thus would include spore adhesion characteristics. Studies on the involvement of fungal enzymes in the infection process are also recommended for future work.

The mummified cadavers observed in Chapter 4 present a method according to Oduor *et al.* (1995) for the storage or transportation of EPF. They also suggest that preparations of whole or macerated mummies formulated with suitable adjuvants may be used as biocontrol agents. The observation of mummies in this study warrants complementary studies which may accelerate the process of obtaining a biocontrol agent.

6.4. Analysis of *Beauveria bassiana* by ribosomal DNA sequence data

The rDNA analysis was chosen since the rDNA sequence information is required to develop molecular markers for further studies. The ITS1-5.8S-ITS2 regions of different *Beauveria* species were assessed for strain genotyping and population studies.

Distinct clades in the phylogenetic analyses in the current study were matched to four species of *Beauveria*: *B. bassiana, B.* cf. *bassiana, Beauveria brongniartii and Beauveria caledonica*. Ferrer *et al.* (2001) state that the ideal marker should be present in all fungal genera but should contain enough internal variation in its sequence to define a given species. They also further state that the molecular method to be implemented must be a rapid and inexpensive DNA extraction protocol with a sensitive and precise method of identifying the EPF. The amplification of ITS1-5.8S-ITS2 implemented in the current study is rapid, technically simple, broadly applicable, and inexpensive.

Fitch parsimony and neighbour joining analyses confirmed that *B. bassiana* was not a monophyletic group suggesting that the strains are a “species complex”.

Intraspecies relationships are essential for screening potential biocontrol agents to better understand the population structure, gene flow and isolate typing (Wang *et al.,* 2005). Also, strains selected for development as biocontrol agents need to be identified when applied to the environment (Castrillo and Brooks, 1998).

In this study the low level of genetic variability in *B. bassiana* with regards to geographic origins demonstrated implies that genetic relationships may be influenced more by ecological habitats then by man-made boundaries or man related interventions (i.e. different countries).

The taxonomic placement of the South African isolates within the *Beauveria* genus with the 5.8S gene and ITS region analyses was achieved in the current study. Two South African isolates PPRI 04304 and PPRI 04306 morphologically assigned to *B. bassiana*, are assumed to be either *B. caledonica* or a close relative of *B. caledonica* based on the rDNA analysis (Chapter 5). Traditionally *B. caledonica* were grouped as a subspecies of *B. bassiana* in the *Beauveria* genus (Hegedus and Khachatourians, 1996). However, recent studies including the current one proved that that they have a different species (Hegedus and Khachatourians, 1996; Glare and Inwood, 1998) but related to *B. bassiana*. The South African isolates were identified by PPRI as *B. bassiana* morphologically prior to this project (Appendix 1). However, due to the lack of confirmation of the change of species identification of these native isolates, they are regarded as *B. bassiana sensu latu*. From previous studies it is evident that the taxonomy of *Beauveria* which is still being reviewed consists of species with difficult and controversial characterisation (Rehner and Buckley, 2005; Wang *et al.,* 2005). Research and development of highly specific and sensitive fungal detection systems would benefit South Africa to differentiate native isolates from exotic ones.

Further research

The limited variation observed at the strain level suggests further investigations of more variable regions with morphological methods are warranted to delineate the

diverse and complex *B. bassiana* species.

The discovery of the native isolates PPRI 04304 and PPRI 04306 as either *B. caledonica* or a new species suggests that *Beauveria* displays biodiversity in Africa and thus further investigations regarding these isolates are warranted. Also, host range is reported to be associated with genetic diversity of *B. bassiana* (Castrillo *et al.,* 1999; Gaitan *et al.,* 2002) thus warranted in further studies.

6.5. Conclusions

The material in this dissertation highlights some important properties relevant to the biocontrol of *T. urticae* by *B. bassiana*.

Aerial conidia produced by the diphasic fermentation process (Chapter 2) demonstrated the capacity to infect a host (Chapter 4) and were also effective in causing *T. urticae* mortalities (Chapter 3). *B. bassiana* has been shown in this study to be a generalist pathogen with strain-dependent differences in nutrient preferences and virulence against a single host pest.

The molecular analyses were included since we are interested in understanding the genetic basis of evolution of pathogenicity. The differences in the virulence of the native isolates (Chapter 3) are reflections of genetic differences (Chapter 5) suggesting that there was a correlation between the genotype and pathogenic phenotypic characterisation of the EPF at a species level. The results from this study suggest that in order to assess the diversity of the traditional morphology studies must be coupled with molecular studies. Characterisation of an EPF in this study has recognized the genus *Beauveria* as forming a “species complex”.

Fungal biocontrol is seen by Butt *et al.* (2001) as an exciting and rapidly developing research area with implication for plant productivity, animal and human health and food production. Developing countries, such as South Africa, offer advantages for low-cost production of the biocontrol agent and manual methods of pesticide application which allows the necessary contact delivery. Although natural methods of pest control in South Africa may be more labour

intensive and less efficient than chemical pesticides, it will create employment and income generation for the grower because of the premium on pesticide-free and organic produce and return an acceptable profit for research and development (Cherry *et al.,* 1999).

Since commercial biocontrol products are currently accepted in the agricultural market the future for the research and development for a potential *B. bassiana* biocontrol agent in South Africa is promising based on the material from this project. Although the development of mycoinsecticides is prevalent it is undeniable that the commercial production process is slow. The biocontrol production market cannot compete economically with chemical control therefore, the biocontrol agents currently represent only 1% of the global market (Hajek, 2004). EPF will not be cure-alls for pest problems on all crops and in all agricultural settings, and it is unlikely that they will totally supplant the management of insect pests with chemical insecticides. Nevertheless they will contribute significantly to reductions in chemical pesticide use. (Butt *et al.,* 2001).

CHAPTER 7

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CHAPTER 8

Appendices

Appendix 1: Information pertaining to the identification of the native isolates.

Colin Straker

From: "Elna Van Der Linde" vrehevdl@plant5.agric.za
To: colin@biology.biol.wits.ac.za
Sent: Friday, August 13, 2004 2:31 PM
Subject: Identification of Fungi

11/10-5
E.J. v.d. Linde

13.08.2004

ATTENTION: DR COLIN STRAKER
School of Molecular and Cell Biology
University of the Witwatersrand
P/Bag 3
WITS
2050

Tel 011 717 6322
Fax 011 339 7377

Dear Colin,

IDENTIFICATION OF CULTURES

Three of your cultures have been identified:

Gray 1 (our ref. no. 04305): Beauveria bassiana (Bals.) Vuill.
121 (our ref. no. 04306): Beauveria bassiana (Bals.) Vuill.
124 (our ref. no. 04305): Beauveria bassiana (Bals.) Vuill.

The fourth culture will be identified as soon as possible.

Regards,
Elna J. van der Linde

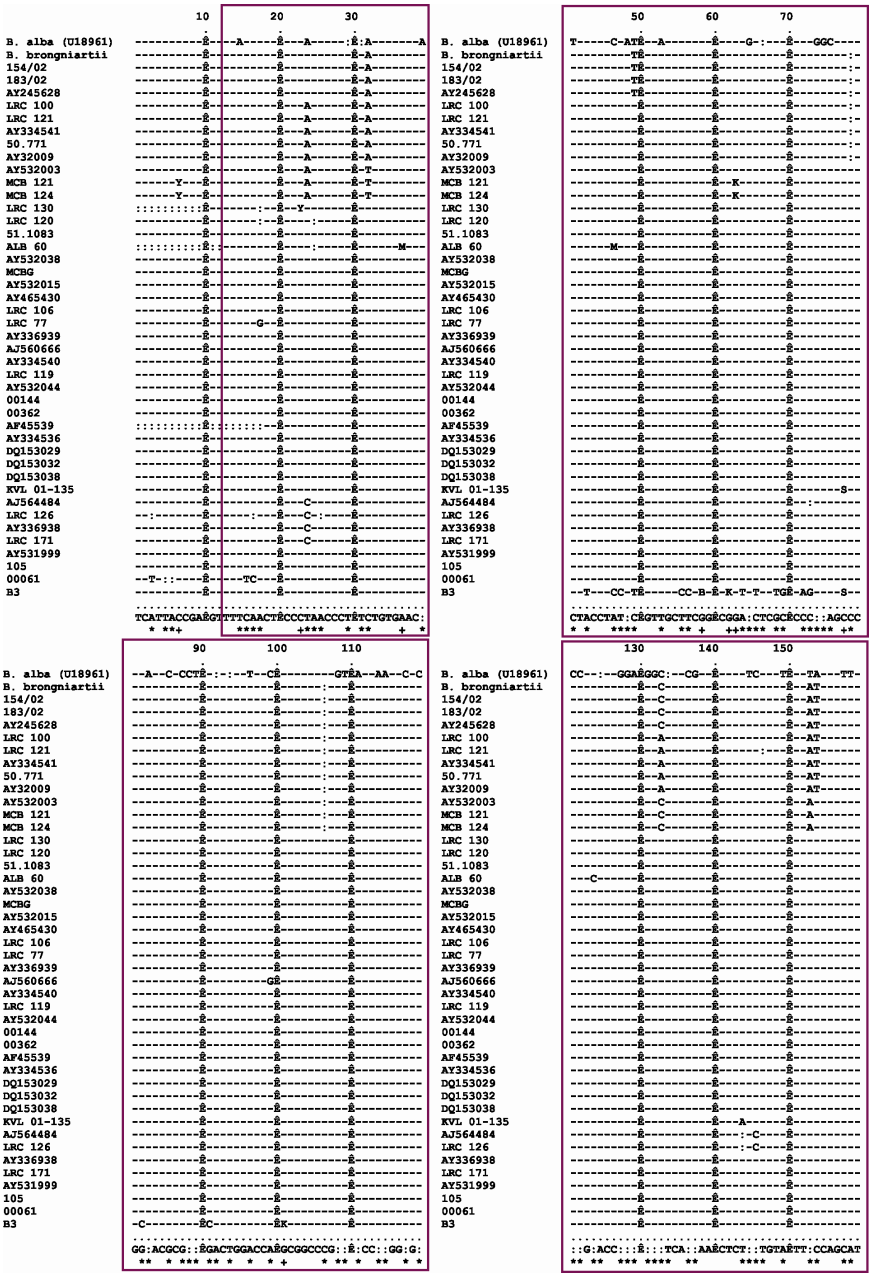
ELNA J. VAN DER LINDE
Project leader: Services
Biosystematics Division: Mycology, ARC-PPRI, P/Bag X134 QUEENSWOOD 0121
Pretoria South Africa
Tel. +27 12 3049586
Fax +27 12 3256998

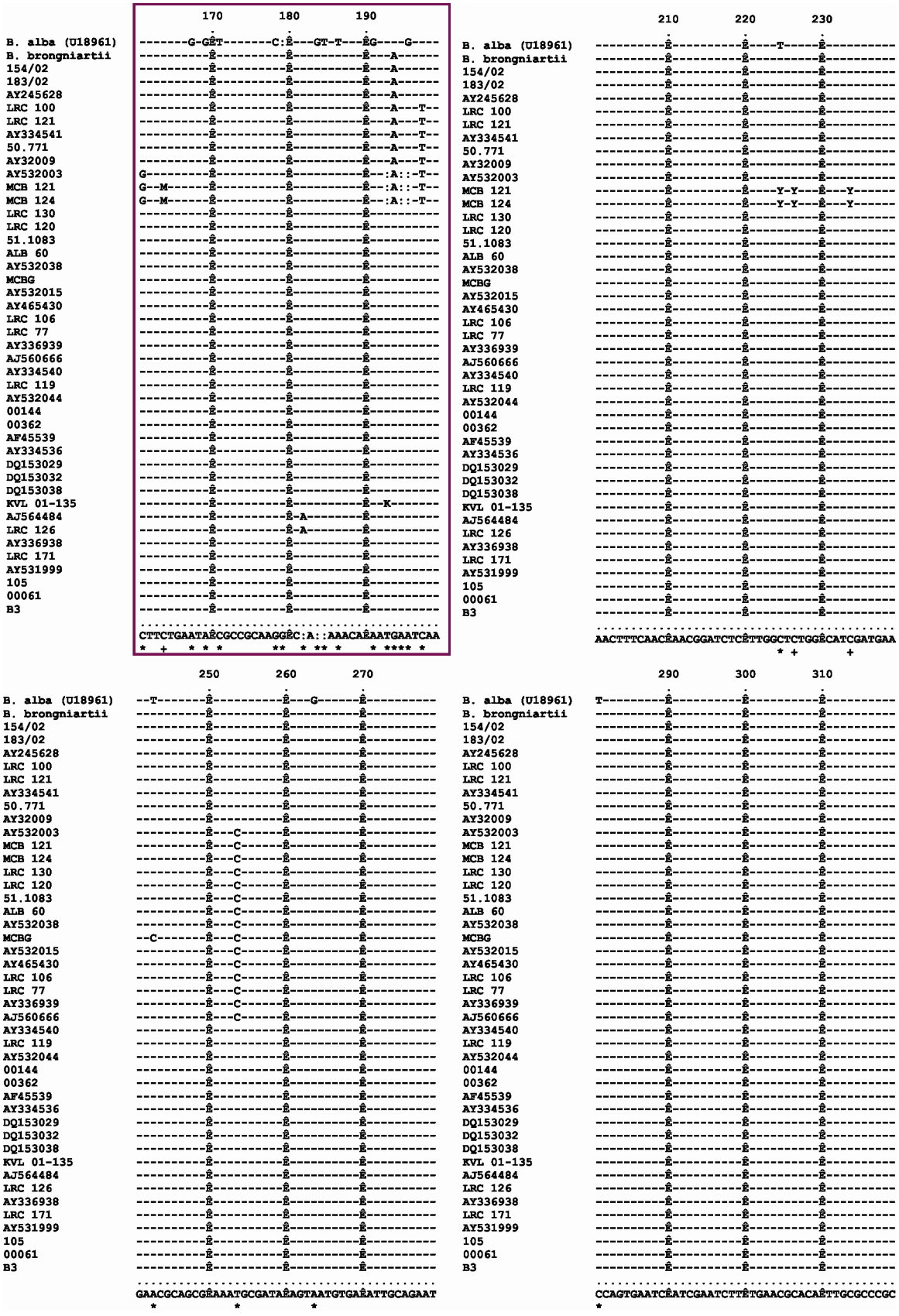
Appendix 2: Genotype information of *Beauveria* isolates from worldwide sources and Genbank used for the phylogenetic analyses in this study with their accession numbers and origins. Accession numbers shown in red are submitted to Genbank but have not been released.

Isolate	Genbank Accession Number	Authors Sources	Country of Origin
Engydontium album	U18961	Shih, H. and Tzean, S	China
B. brongniartii			
IFO5299	AB258368	Yokoyama, E.., Arakawa, M., Yamagishi, K. and Hara, A.	Unavailable
ATCC 66779	AY245628	Huo, H. C.	Unavailable
CNPMS 67	DQ153029	Carneiro, A. A. et al.	Unavailable
CNPMS 72	DQ153032	Carneiro, A. A. et al.	Unavailable
CNPMS 79	DQ153038	Carneiro, A. A. et al.	Unavailable
B. caledonica	AY532003	Rehner, S. A. and Buckley, E.	Brazil
B. bassiana			
LRC 120	832589	Dr. M. Goettel (Agriculture and Agri-food Canada)	Bulgaria
LRC 107		Dr. M. Goettel (Agriculture and Agri-food Canada)	Portugal
ALB 60	832547	Dr. O. Triggiana (University Degli Studi Di Bari)	Albania
LRC 77	832573	Dr. M. Goettel (Agriculture and Agri-food Canada)	Madagascar
LRC 95		Dr. M. Goettel (Agriculture and Agri-food Canada)	North Dakota
KVL 01-135	839288	Dr. K. Eilenberg (The Royal Veterinary and Agricultural University)	Denmark
Bb 9011		Dr. A. Gaitan (CENICAFE)	Colombia
BPIC 1333		Dr. K. Elna (Benaki Phytopathological Institute)	Greece
105	835251	Dr. L. Cagan (Slovak Agriculture University)	Slovakia
B ba 136		Dr. G. Zimmerman (Federal Biological Research Center)	Germany
Bb 9015		Dr. A. Gaitan (CENICAFE)	China
Bb lygus		Dr. E. S. Solis (Instituto de Ciencias Agricolas)	Mexico
Bb 9019		Dr. A. Gaitan (CENICAFE)	China
Bb 9013		Dr. A. Gaitan (CENICAFE)	Philippines
B3	839256	Dr. C. Tcaczuk (University of Podlasie)	Poland
LRC 121	832591	Dr. M. Goettel (Agriculture and Agri-food Canada)	Spain
Bb 9016		Dr. A. Gaitan (CENICAFE)	Thailand
00144	838077	Dr. C. Kooyman (IITABE)	Ghana
00362	835144	Dr. C. Kooyman (IITABE)	Benin
00038		Dr. C. Kooyman (IITABE)	Uganda

LRC 171	839304	Dr. M. Goettel (Agriculture and Agri-food Canada)	Canada
51.1083	839264	Dr. E. Bury (Museum National D' Histore Naturella)	Paris
50.771	839260	Dr. E. Bury (Museum National D' Histore Naturella)	Paris
LRC 126	832593	Dr. M. Goettel (Agriculture and Agri-food Canada)	Denmark
154/02	832535	Dr. I. Klingen (Norwegian Crop Research Institute)	Norway
183/02	835154	Dr. I. Klingen (Norwegian Crop Research Institute)	Norway
LRC 100	832577	Dr. M. Goettel (Agriculture and Agri-food Canada)	Togo
LRC 130	832595	Dr. M. Goettel (Agriculture and Agri-food Canada)	Australia
LRC 106	839296	Dr. M. Goettel (Agriculture and Agri-food Canada)	Brazil
LRC 119	832587	Dr. M. Goettel (Agriculture and Agri-food Canada)	Austria
00061	835152	Dr. C. Kooyman (IITABE)	Kenya
Bb 9023	AY334541	Cruz, L. P., Gaitan, A. L. And Gongora, G. C.	Philippines
2857	AY532009	Rehner, S. A. and Buckley, E.	Mexico
681	AY532038	Rehner, S. A. and Buckley, E.	Commonwealth of independent states
300	AY532015	Rehner, S. A. and Buckley, E.	Australia
OR2S2	AY465430	Ganley, R. J. and Newcombe, G.	North America
Bb 126	AY336939	Wang, C. and Butt, T. M.	China
IMI 386702	AJ560666	Aquino de Muro, M.	Unavailable
Bb 9020	AY334540	Cruz, L. P. , Gongora, C. E. And Gaitan, A. L.	Philippines
733	AY532044	Rehner, S. A. and Buckley, E.	Brazil
B 252	AF344539	Yi-Mattila, T. and Mitina, G.	Unavailable
Bb 9005	AY334536	Cruz, L. P. , Gongora, C. E. And Gaitan, A. L.	Colombia
SPT KR 452	AJ564884	Wang, C. and Butt, T. M.	Kyrgyz Republic
Bb 95	AY336938	Wang, C. and Butt, T. M.	China
1975	AY531999	Rehner, S. A. and Buckley, E.	France

Appendix 3: Sequence alignment of ITS1-5.8S-ITS2 regions of *Beauveria bassiana* strains from different geographic locations. Hyphens (-) indicates identical bases, point mutations are noted with ∗, synapomorphic mutations with † and every 10 base pairs are separated by an Ê. Species names represented as numbers indicate the sequences were obtained from Genbank.

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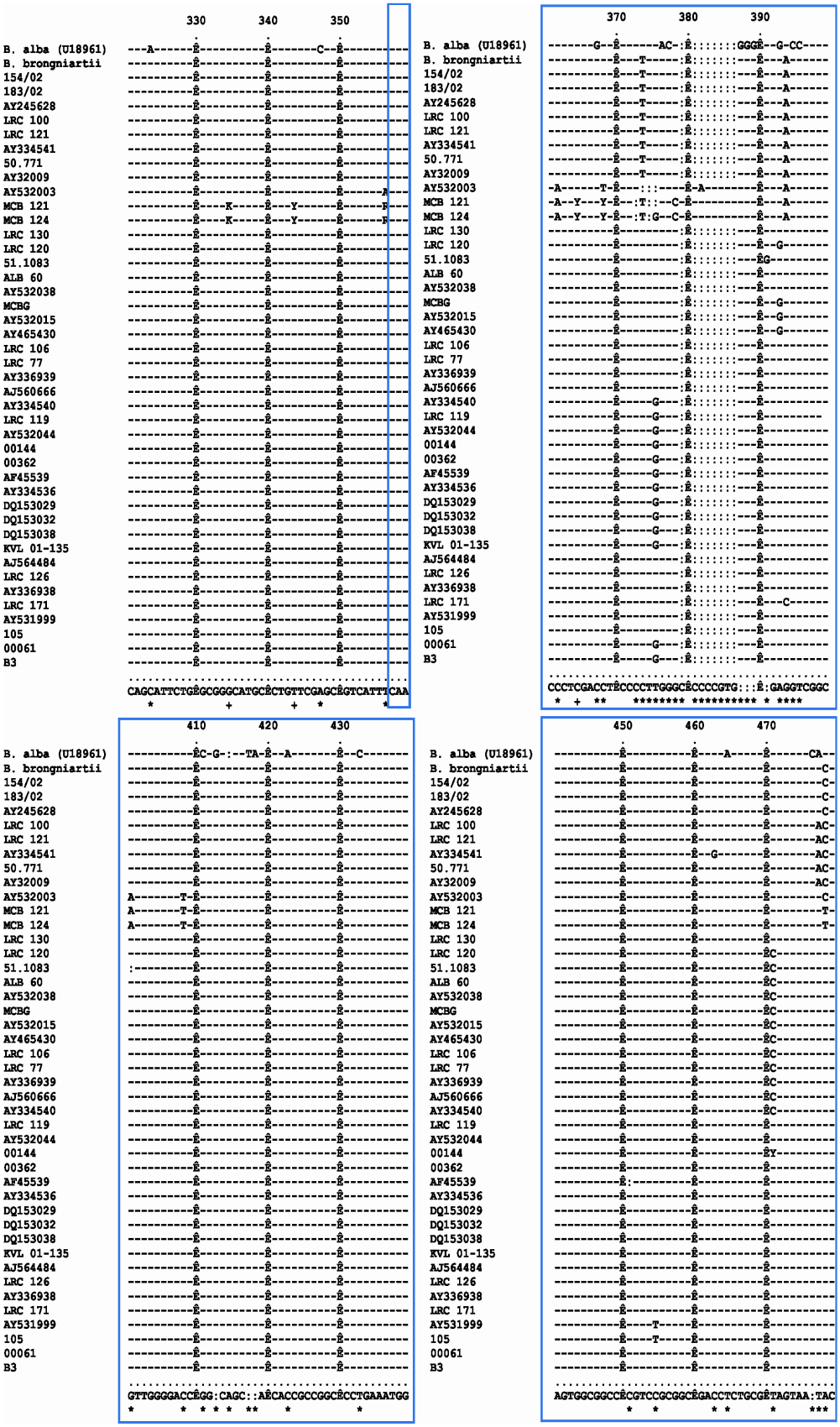
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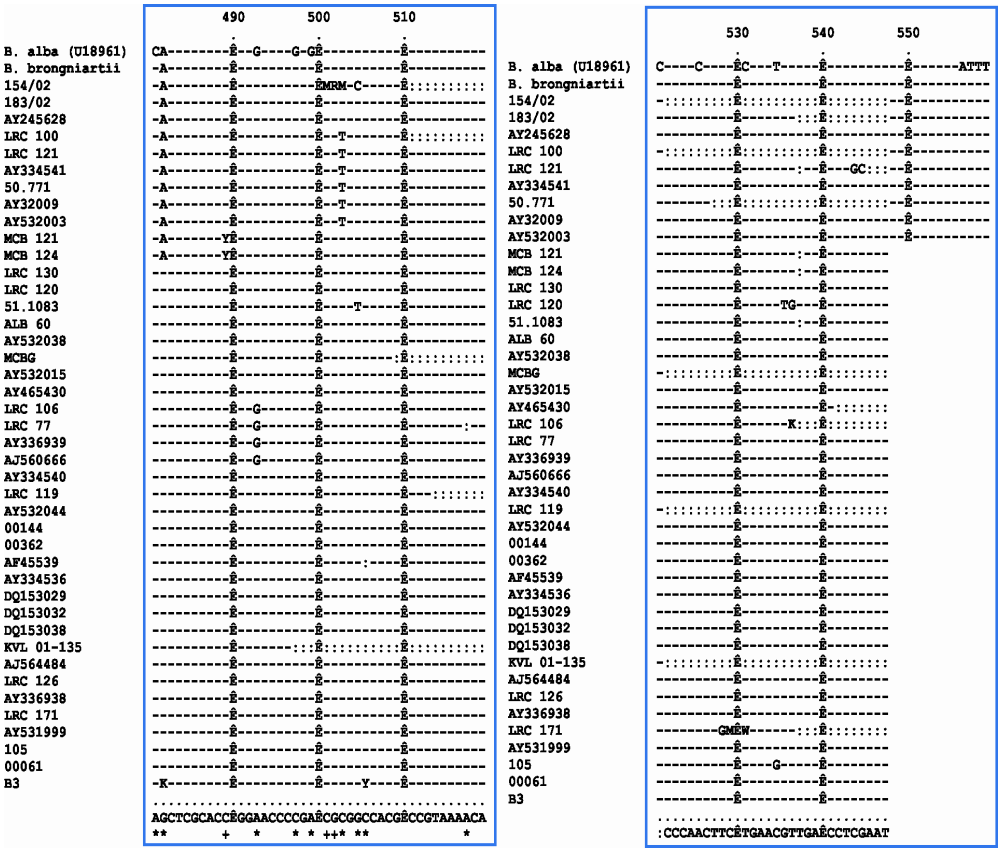
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