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Efficacy of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* against *Uvarovistia zebra* (Orthoptera: Tettigoniidae) and *Eurygaster integriceps* (Heteroptera: Scutellaridae)

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Abstract

The potential for the control of insect pests by entomopathogenic fungi has been studied for decades, and *Metarhizium anisopliae* and *Beauveria bassiana* are the best known of these entomopathogenic fungi. In this study the pathogenicities of *B. bassiana* DEBI 001 and *M. anisopliae* 715C obtained from the Iranian Research Institute of Plant Protection were evaluated for the first time against a long-horned (tettigoniid) grasshopper *Uvarovistia zebra*. *Uvarovistia zebra* is a univoltine grasshopper, distributed on the southern slopes of the Alborz Mountains in the north of Qazvin province in Iran. In some years, following good conditions for population growth, they can invade and damage field crops, and rangeland grass.

Initially the efficacy of the two fungal isolates was assessed using topical application and ingestion. Experiments were carried out in the laboratory with field collected *U. zebra* to determine the effect of the fungi on food consumption by and mortality of the insect. Both fungi caused significant mortality of *U. zebra* by both contact and ingestion and both caused a significant reduction of food consumption and faeces production by the insects.

The effect of fungal spore formulations in oil or water on lettuce discs was evaluated. Both oil and water formulations had lethal effects on *U. zebra* nymphs, but the spores formulated in oil on lettuce discs were more effective than those in water.

Pathogenicity of the fungi was also evaluated against the Sunn pest *Eurygaster integriceps*. Experiments were conducted in the laboratory with different nymphal instars of *E. integriceps* to determine the relative susceptibility of nymphal instars and adults to the fungal isolates. Results showed that the fifth-instar nymph was the most susceptible with 60% and 46% mortality caused by *B. bassiana* and *M. anisopliae* respectively.

The viability, mycelial growth and conidia production of the *B. bassiana* and *M. anisopliae* isolates preserved in different storage media were assessed. Assessments were done before and after storage to determine the extent of change and degeneration of the fungi caused by storage in PDA, distilled water, or sand. A significant decrease in germination of both fungal isolates was observed after 6 months storage in all media. Colony growth of the fungi preserved in sand and water media was significantly reduced after 6 month storage. There were no significant differences in conidia production before and after storage for *B. bassiana* preserved in PDA and sand and for *M. anisopliae* preserved in PDA.

Virulence of the *B. bassiana* and *M. anisopliae* isolates was evaluated when they were passed through artificial media and through an insect host *U. zebra*. The fungi were subcultured *in vitro* subcultures and passaged *in vivo* to determine the possibility of changes in their virulence. The virulence of the fungi was measured using mortality monitored before

subculturing *in vitro*, after subculturing *in vitro* and after passage *in vivo*. The virulence of both fungi reduced after the fourth subcultures *in vitro*, but this reduction was not quite significant for *B. bassiana*. Although there was no significant enhancement in virulence of the fungi passaged through the insect, the virulence of fungi was increased.

The pathogenicities of the isolates of *B. bassiana* and *M. anisopliae* were not unexpected, but the demonstration that tettigoniid species are susceptible to entomopathogenic fungi is novel. The isolates tested have potential for use in management programmes against *Uvarovistia zebra* and other pests, such as *Eurygaster integriceps*. Further work is now required to identify more virulent strains of the fungi, examine methods for mass production and finding the best formulation for application of these entomopathogenic fungi.

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Chapter 1. General introduction

1.1. Historical perspective on pests and chemical pesticides

The planet Earth is a communal habitat for humans and millions of different species of living organisms. There are complex interrelations and dependencies between organisms living on the Earth. With increasing population and rising demands for food, fibre and timber, humans have inescapably been led to more extensive use of natural resources such as land and water (Howard, 1967). With new technology, human have been able to exploit natural resources more effectively. Most habitats have been altered following human influences, consequently diversity in communities of plants and animals have suffered great changes, insofar as ecological balances have been disrupted in most ecosystems.

Accordingly some species may be reduced or replaced with others appearing as pests. Throughout the world man's demand for food has led him into competition with many organisms styled as "pests" (Hajek, 2004). Several descriptions and definitions have been given for "pests". A pest is defined as any organism that reduces the availability, quantity, quality, or value of some human resource (Flint & van den Bosch, 1981). In another definition, Fenner & Fantini (1999) stated "Microorganisms, plants or animals are regarded as pests when they reduce agricultural and pastoral productivity and/or threaten the natural environment."

Since time began man has been plagued by "pests" that devastate his crops, cause injury to his domestic animals, destroy his property or may attack him directly causing disease (DeBach & Rosen, 1966). The most serious problems are caused by the organisms translocated deliberately or accidentally by humans into new habitats (Fenner & Fantini, 1999). Translocated organisms can emerge as serious pests in new habitats where natural enemies do not exist. In 1986, nearly 50,000 alien organisms were intercepted at ports of entry by the US Department of Agriculture (DeBach & Rosen, 1991). Indeed, translocation of living organisms is one of the most serious ecological changes caused by humans.

In the developing world, agricultural diversity has been mostly converted to intensive monocultures. Monoculture cultivation makes agro-ecosystems simpler as they lack diversity in species of plants and animals. Diversity of living organisms is linked to ecological stability; with reductions in species such as competitors, predators and parasites, some species may outbreak and emerge as pests.

Pests are diverse taxonomically, ranging from microorganisms to mammals. The Arthropoda is the largest Phylum that comprises most species known as pests. A majority of the pest are insects (DeBach & Rosen, 1991), but it does not mean that most insects or other arthropods are pests. The order Insecta comprises nearly one million recognized species (May, 2000) which constitute approximately 67% of the world's described fauna and flora. Most insects are not considered as pests, on the contrary, many of them, such as insect pollinators, are beneficial. With the exception of a few species such as vectors that are always considered pests, most insects and other arthropods such as mites are not regarded as pests until their populations exceed economically damaging levels.

Examples of pest invasions

According to Pimentel (2004), around 15% of world food production is consumed by agricultural pest insects annually. Sailer (1983) listed 837 insects and other arthropods species that have invaded the United States during the period 1920-1980. The invasion of pests can be seen in most parts of the world. The Mediterranean fruit fly *Ceratitidis capitata* Wiedemann (Diptera: Tephritidae), the Mexican rice borer *Eoreuma loftini* Dyar (Lepidoptera: Crambidae), the black parlatoria scale *Parlatoria zizyphi* Lucas (Hemiptera: Diaspidida) and the Russian wheat aphid *Diuraphis noxia* Mordviko (Hemiptera: Aphididae) are examples of invasive pests in United States. The latter species alone has caused annual damage estimated at US \$100 million in 11 USA states and some parts of Canada. In England, the grape phylloxera *Daktulosphaira vitifolii* Fitch (Homoptera: Phylloxeridae) and western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae) invaded in 1985 and 1986 respectively (DeBach & Rosen, 1991).

Locusts and grasshoppers, especially species belonging to the family Acrididae are important pests causing significant damage in agro-ecosystems and rangelands throughout Africa, the Middle East, Australia, parts of Asia, North and South America (Baker et al., 1994; Geddes, 1990; Jenkins, 1994; Lomer & Prior 1992; Milner, 1997; OTA, 1990; Steedman, 1990). The desert locust, *Schistocerca gregaria* Forskal (Orthoptera: Acrididae) in Africa and western Asia (Showler, 1993; Steedman, 1988), the Central American locust, *Schistocerca piceifrons* ssp. *piceifrons* Walker (Orthoptera: Acrididae) in Mexico and Central America (Barrientos-Lozano et al., 2002), *Rhammatocerus schistocercoides* Rehn (Orthoptera: Acrididae) and *Schistocerca pallens* Thunberg (Orthoptera: Acrididae) in Brazil

(Faria et al., 1999) cause severe damage particularly during years with locust outbreaks. In the USA, the damage caused by grasshopper infestations on rangeland was estimated \$400 million in one year (Hewitt & Onsager, 1982). The outbreak of desert locust (*Schistocerca gregaria*) beginning in 1988 affected 23 nations in Africa, and more than \$250 million was spent on pest control and relief of damage caused by the infestation (Showler & Potter, 1991; Showler, 1995). During the last century, agricultural production over more than 20% of the world's land surface has been threatened by eight major plagues of desert locust varying in length between 1 and 22 years (Steedman, 1990).

The Sunn pest, *Eurygaster integriceps* Puton (Heteroptera: Scutelleridae), is the most harmful pest of wheat and barley in throughout the Middle East, Western and Central Asia, and Southeast Europe (Anonymous, 1966a, b; Brown & Eralp, 1962; Brown, 1963; El Bouhssini et al. 2002). Around 15 million hectares are annually affected by Sunn pest (Moore & Edgington, 2006). The olive fruit fly *Bactrocera oleae* Rossi (Diptera: Tephritidae) was translocated into Iran in 2003-2004 (Jafari & Rezaei, 2004) and caused serious damage particularly in the first few years after translocation.

Apart from the damage caused directly by pests feeding on plants, some of them such as aphids and whiteflies are virus vectors responsible for transmitting plant viral diseases. There are many aphid species that can acquire and transmit phytopathogenic viruses to horticulture crops. The Green peach aphid *Myzus persicae* (Homoptera: Aphididae) is the most important worldwide vector of plant viruses (Harrison, 1984) and can transmit more than 100 phytopathogenic viruses such as potato leafroll virus (PLRV).

1.2. Background and history of chemical pesticides

From ancient times, humans have always needed to control pests affecting their health directly, such as mosquitoes or bed bugs, or competing with them for agricultural crops. Different methods have been used for pest control, for example cultural controls include altering dates for planting and harvesting, using trap crops, planting mixtures of crops, and removing crop residues harbouring pests amongst others. Although there have been some advances in the implementation of integrating cultural and other methods for control of pests, existing control strategies still rely largely on the use of chemical pesticides.

Since World War II, chemical pesticides have become the most important applied form of pest management. Since the 1940s, chemical pesticides have been developed as a major tool to control pests and weeds in both developed and developing countries (Flint & Van den Bosch, 1981; Van den Bosch, 1978; Whitten & Oakeshott, 1991). In the early 1950s, synthetic chemical insecticides included organochlorine and organophosphorous compounds. These chemical compounds were effective against a broad spectrum of pests, they had high efficacy and killed pests very quickly, they were also relatively cheap and easy to apply using spray equipment, therefore they were used extensively. Once the high persistence of organochlorine insecticides in the environment and their subsequent residue in the food chain, water and animals bodies was identified, they were banned for most uses. Consequently, the use of DDT and the use of aldrin and dieldrin was banned in the USA and Europe in 1973 and 1975 respectively (Römbke & Moltmann, 1996), but, in 1988 dieldrin was still frequently used against locust swarms in Africa (Crick, 1990).

However new generations of synthetic chemical pesticides were produced and are still used to protect crops from invertebrate pests and diseases. About £500 million of pesticides are applied annually to farmland in the Unites States (Altieri & Nicholls, 2003). According to Smits et al. (1999) between 1985 and 1991, in order to control grasshopper infestations, 7.7 million ha were sprayed in Saskatchewan and Alberta in Canada. Since 1986, about 3 million hectares were treated against locusts every year in Africa and the Middle East (Van der Valk, 1999). Since 1997, the value of insecticides used for control of the locust *Locusta migratoria* (Orthoptera: Acrididae) swarm in Madagascar was estimated more than US\$35 million (Thomas et al., 2000). About US\$40 million is annually spent on chemical insecticides for control of Sunn pest (El Bouhssini et al., 2002). In the late 1980's, around 30,000 tons of 4,000 registered pesticides were used annually in UK for controlling of agricultural pest

insects (Best & Ruthven, 1995). Between 1980 and 2000, the use of the pesticides in the world was estimated 2.5 million tons each year at a cost of \$20 billion (Hajek, 2004). A continuous increase in weight and value of pesticides being applied can be observed during this period. It is acknowledged that humans have become excessively dependent upon synthetic insecticides. The use of chemical pesticides to manage agricultural pest insects has increased dramatically. In the last decade about US\$25-\$30 billion being spent globally on around 2.56 billion kg synthetic chemical applied per annum (Pretty & Hine, 2005). However, chemical pesticides have been responsible for maintaining agricultural productivity against some major pests.

Despite the advantages of chemical pesticides and their role to protect the crops, they have been one of the most important environmental and human health issues in recent times. The extensive use of chemical pesticides has resulted in a variety of problems which have led to changes in chemical control strategies and replacement of them with alternative plant protection techniques (mechanical, physical, cultural and biological control). The application of biological control with other methods will achieve crop protection with much less harm to the ecosystem. Natural enemies represent a large component of the world's biodiversity, but only a small proportion of the available species have been investigated as commercial products for crop protection. For example, the market for biopesticides is less than 1% of the agrochemical pesticides sales globally and a main proportion of this is represented by *Bacillus thuringiensis* (Lisansky, 1997). Although the development of biological control agents especially microbial control agents such as entomopathogenic fungi has been slower than expected, the use of these products is going to play an important role as an alternative in pest control.

1.3. Why we need biological alternatives?

Although synthetic chemical pesticides are still used widely for control of pests, there are serious reasons to consider alternatives. The major drivers for seeking alternatives to synthetic chemical pesticides are presented below.

1.3.1. Human health and environmental issues

Synthetic chemical pesticides have long lives and their residues can be found as pollutants in air, water, wildlife and food supplies. In the 1960s, increasing concern about the effect of synthetic pesticides to human health, livestock and wildlife was debated (Peterson & Higley,

1993; Snelson, 1977). The risk of synthetic chemical pesticides in the environment and their residues in human tissues was first raised by Rachel Carson in her book “Silent Spring” in 1962 (Hajek, 2004).

According to the World Health Organization about 25 million cases of chemical pesticide poisoning and 20,000 death caused by pesticides occur each year (WHO, 1992). Chronic diseases caused by chemical pesticides are also a concern. Ames et al. (1990) reported that some natural and synthetic chemicals are carcinogens. Accumulation and residues of chemical pesticides in soil and ground water have caused serious concern for environmental pollution and human health. The extensive use of the chemical insecticides in agriculture raises the risk of ground water contamination (Peterson & Higley, 1993). According to Hajek (2004) in 1991, approximately 3 kg of pesticides per hectare were applied annually to 160 million hectares in the USA. It is certain that this level of application pollutes the environment and ground water, exposing many species of flora and fauna to these pesticides.

1.3.2. Pesticide effects on non-target animals

Chemical pesticides are generally effective against a wide range of insects and other invertebrates which each have a role and place in the ecosystem, therefore local extinction cannot occur without disrupting the balance of nature. For instance, many species such as predators and parasites are essential as population regulators. Numerous studies have described the negative impact of chemical pesticides on non-target organisms. Extensive mortality, due to massive use of chemicals has been reported among the various animals (Hajek, 2004). Murphy et al. (1994) reviewed the toxicities of some chemical pesticides and found that in 45%–55% of the records, pesticides caused more than 90% mortality in non-target species. When chemical pesticides are applied for pest control, natural enemies living by consuming a pest are no longer frequent. Subsequently, in the case of the pest reinvasion, natural enemies are not present and the target pest population increases to higher densities than initially and become a pest outbreak.

As a result of elimination of natural enemies, sometimes secondary pests of crops become important because their natural enemies are severely affected. For example the European spruce sawfly *Gilpinia hercyniae* Hartig (Hymenoptera: Diprionidae) which was under natural biological control appeared as a secondary pest when DDT was applied against spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae) (Hajek, 2004).

1.3.3. Resistance to chemical pesticides

The most serious problems occur when pests develop resistance to chemical pesticides. The development of resistance to chemical pesticides is best illustrated with insect pests (Ambethgar, 2009). Studying the literature shows that more than 600 species of plant feeding insect pests had developed resistance to insecticides by 1996 (Sharma et al., 2001). Resistance to pesticides among insect populations occurs when multiple applications of the same insecticide are used over multiple generations of the pest, consequently, susceptible individuals are removed from the population and resistant individuals remain to reproduce generations that can no longer be controlled with that insecticide (Riley & Sparks, 2006). Dose of pesticide, frequency of application and pest characteristics are factors affecting resistance development (Regupathy, 1995). Arthropods such as mites, aphids and whiteflies with many generations per year and numerous offspring are more likely to develop resistance to chemical insecticides (Metcalf, 1982). Some insects develop resistance to more than one pesticide within a chemical family, which is known as “cross resistance”. Cross resistance occurs when resistance to one pesticide confers resistance to another insecticide, even where the pest has not been exposed to the latter pesticide (Zhang et al., 2000).

Development of resistance to an insecticide usually leads to application of higher doses of the same insecticide, increase in the number of pesticide applications or use of new products. Increase in the use of pesticides not only raises the cost of pest control, but also causes environmental pollution and hazards to human health.

1.4. An introduction to biological control

1.4.1. History and definition of biological control

All living organisms are subject to a large number of parasites, predators, and pathogens (fungi, viruses, and bacteria) known as “natural enemies”. Natural enemies are responsible for the natural control of populations. This process unaided and often unrecognized by humans is termed “natural control”, however in case of controlling pests it is also known as “biological control” (Hajek, 2004). Human can use biological control to suppress pest populations by exploiting or supporting their natural enemies. Biological control has been defined many times, and some common definitions are provided below.

- Biological control was defined by De Bach (1964) as “the action of parasites, predators, or pathogens in maintaining another organism’s population density at a lower average than would occur in their absence”.
- Eilenberg et al. (2001) described biological control as the use of living organisms to suppress the population of a specific pest organism, making it less abundant or less damaging than it would otherwise be.
- According to Lazarovits et al. (2007) biological control is defined as “the use of living organisms (natural enemies) to manage a pest population”.

Wisniewski et al. (2007) suggest that for plant pathologists, the entomologist’s definition does not work for plant pathology. Thus, these authors define biological control of a plant disease as “control of a plant disease by a biological process or the product of a biological process”.

The ancient Chinese exploited ant predators to control caterpillars and borers on citrus trees. They had also discovered the fungus diseases of silkworm over two thousands year ago (Moore, 2001). Although biological control had been known and applied since ancient times, there has been increasing interest by entomologists in recent years. Natural enemies have recently been studied for exploitation in biological control of pests and their successes or failures have been extensively reviewed (DeBach 1964; Huffaker & Messenger 1976; DeBach and Rosen 1991; van Driesche & Bellows 1996; Collier & van Steenwyk 2004; Stiling & Cornelissen 2005).

The first successful biological control in recent times occurred when the cottony-cushion scale *Icerya purchasi* Maskell (Hemiptera: Margarodidae) in California was controlled permanently with the vedalia beetle, *Rodolia cardinalis* Mulsant (Coleoptera: Coccinellidae) which was imported from Australia in 1888, (van Driesche & Bellows, 1996).

Biological control is implemented using two main strategies described below:

Classical biological control

Classical biological control is defined as “the intentional introduction of an exotic biological control agent for permanent establishment and long-term pest control” (Eilenberg et al., 2001). In this case, an exotic natural enemy (predator, parasite or pathogen) is released into a new environment so that it will become established permanently in the new place and adjust a pest population for a lengthy period without any further human intervention.

Augmentation biological control including inundative and inoculative control

In this case, permanent establishment of the released organism (biological control agent) is not a goal. These two strategies have different goals, but both are used when natural enemies are absent or their population are not sufficient to provide control of the pest. Inundative biological control is defined as “the use of living organisms to control pests when control is achieved exclusively by the organisms themselves that have been released” however, inoculative control is “the intentional release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not that it will do so permanently” (Eilenberg et al., 2001). The strategy of inundative control is used when rapid control of pests in a short time is required, however inoculative biological control provides more long term and continual control than the former (Hajek, 2004).

1.4.2. Types of natural enemies in biological control

The agents used for biological control of invertebrate pests are diverse. They are functionally grouped into parasites, predators and pathogens and taxonomically into insects, mites, nematodes and microorganisms (fungi, bacteria and viruses) (Hajek, 2004). The pathogens causing diseases in insect are called entomopathogens, such as entomopathogenic fungi or entomopathogenic nematodes.

Where the biological control agent is a microorganism, it is referred to microbial control, and the name of biopesticide or microbial pesticide is used for products of microbial agents produced and formulated for application. The active agent in biopesticides may be a fungus, bacteria or virus; when the active agent is a fungus the biopesticide is called a mycopesticide or mycoinsecticide (Lomer et al., 1999). Biopesticides are normally applied inundatively for biological control of pests (Hall & Menn, 1999).

Since the current study uses entomopathogenic fungi, this group of microbial agents and the fungi *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) and *Metarhizium anisopliae* (Metchnikoff) Sorokin (Ascomycota: Hypocreales) in particular, are reviewed below.

1.5. Entomopathogenic fungi

1.5.1. Overview

Fortunately, most insect pests have pathogenic microorganisms associated with them. Among pathogens of insects, fungi are important and unique because unlike other pathogens they do not need to be eaten to infect their host, they invade their host directly through the cuticle. Entomopathogenic fungi as biological control agents are viewed as promising microbial agents to control insect pests and are becoming an interesting alternative to chemical control (Clarkson & Charnley, 1996; Maniania et al., 2008; Shah and Pell, 2003; Vega et al., 2008). In appropriate conditions, entomopathogenic fungi are capable of causing epizootics among the insects, so that they may eliminate the insect population (Clayton et al., 1985; Tanada & Kaya, 1993).

Observations of the effects of entomopathogenic fungi date back thousands of years, when mycosis was observed in honey bees and silkworms (Castrillo et al., 2005). Various aspects of entomopathogenic fungi such as the history and use of them as microbial control agents have been reviewed by many authors (Boucias & Pendland, 1998; Hajek and St. Leger, 1994; McCoy et al., 1988; Roberts & St. Leger, 2004; Steinhaus, 1949, 1975; Tanada & Kaya, 1993).

More than 700 species of fungi from about 90 genera are known as insect pathogens (Charnley, 1989; Inglis et al., 2001; Roberts & Humber, 1981), they have had a place in the management of a wide range of insect pests (Ferron, 1978; Glare & Milner, 1991; Lacey and Goettel, 1995; Lacey et al., 2001; Milner, 1991; 2000; Milner & Pereira, 2000). Among entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* Wize Brown & Smith (Ascomycota: Eurotiales) are the best known fungi (Faria & Wraight 2001; Feng et al. 1994; Roberts & Leger 2004) that have been widely applied for insect control (Feng et al. 2004a, b; Langewald et al. 1997; Pu et al. 2005; Wraight et al. 2000; Wraight & Ramos 2002).

1.5.2. The fungi *Beauveria bassiana* and *Metarhizium anisopliae*

For more than 120 years, *B. bassiana* and *M. anisopliae*, in addition to *B. brongniartii*, have been used to control pest insects (Zimmermann, 2007).

They are anamorphic fungi (asexual reproduction) distributed widely and have a wide range of host insect species (Clark et al., 1968; Robert, 1970; Roy et al., 2006; Scholte et al., 2003a, b). These two fungi have been developed as commercial biopesticides, for example Mycotrol® O, is produced by Mycotech using the virulent isolate of *Beauveria bassiana* strain GHA and Green Muscle® developed by the LUBILOSA project, is based on *Metarhizium anisopliae* var. *acridum* strain IMI 330189.

1.5.3. History and general characteristics

Beauveria bassiana is a well-known entomopathogenic fungus, which has a wide host range (Clarkson & Charnley, 1996) and is currently under intensive study as a promising biocontrol agent (Akello et al. 2009; Quesada-Moraga et al. 2007). Approximately 700 species of insects in almost all taxonomic orders have been recorded as hosts of *B. bassiana* (Inglis et al., 2001; Li, 1988). This is a cosmopolitan fungus that can be isolated from insects, mites, and soil. According to Meyling & Eilenberg (2007), *B. bassiana* is only found above ground while associated with the host; however *M. anisopliae* is associated with hosts settled on or below the soil.

Discovered nearly 200 years ago, *B. bassiana* has several advantageous characteristics and could be used as one of the principal micro-organisms in research on fungal insect pathology (Steinhaus, 1963). A wide genetic diversity is observed in different isolates of *B. bassiana*, also, various isolates differ in their pathogenicity and virulence to different arthropods (Almeida et al., 1997). The insect disease caused by *B. bassiana* is called white muscardine disease, as infected hosts are covered by a white mould. The white mould is caused by the fungal spores germinating inside of the infected insect's body and then growing back out through the cuticle. Studying, white muscardine disease in silkworm, the Italian scientist Agostino Bassi in 1835 identified *Beauveria bassiana* as the disease agent. This detection not only was the basis for microbial pest control, but also affected the research of Louis Pasteur, Robert Koch and other microbiologists (Ainsworth, 1956; Porter, 1973; Van Driesche & Bellows, 1996). The Nutrilite Company prepared the first powder formulation of *B. bassiana* in 1962 and it was recommended for insect control.

Metarhizium anisopliae is one of the most studied entomopathogenic fungi applied against agricultural insect pests (Alston et al., 2005; Enserink, 2004) and vector insects of human

diseases (Blanford et al., 2005; Lazzarini et al., 2006; Scholte et al., 2005). This fungus formerly known as *Entomophthora anisopliae*, is extensively distributed in soils throughout the world. It was first recognized as a biocontrol agent in the 1880's (Genthner & Middaugh 1995). Elie Metchnikoff in 1879 first used this fungus as a microbial agent against the wheat grain beetle, *Anisoplia austriaca* Herbst (Coleoptera: Scarabaeidae) (Steinhaus, 1949). The fungus was renamed *Metarhizium anisopliae* by Sorokin in 1883. *Metarhizium* is the current name of genus, although from 1880 to 1969 at least eight other names were suggested for this genus (Tulloch, 1976). It is a pathogen of many insect species (Leal et al., 1997; St. Leger et al., 1992; Vey et al., 1982), and has been reported from different groups of insects such as Orthoptera, Dermaptera, Hemiptera, Lepidoptera, Diptera, Hymenoptera and Coleoptera (Latch, 1965).

The spores of *M. anisopliae* are green and the disease caused by it, known as green muscardine disease. In May, 1993, the U.S. Environmental Protection Agency registered the fungus for control of nuisance flies and cockroaches (Genthner & Middaugh 1995).

Metarhizium anisopliae, produced under the name Metaquino® is widely used as a biological agent to control the spittle bug, *Mahanarva posticata* Stal (Homoptera: Cercopidae) in sugar cane fields in Brazil (Moscardi, 1989). Green Muscle® was developed as a biocontrol agent to control grasshoppers in Africa (Bateman et al, 1996; Douthwaite et al., 2000; Gelernter & Lomer, 2000; Lomer et al., 2001). The project LUBILOSA abbreviated from the French "Lutte Biologique contre les Locustes et Sauteriaux (biological control of locusts and grasshoppers) was started late in 1989 by Prior & Greathead (1989) to develop biological control of locust and grasshopper using *M. anisopliae* var *acridum* (Lomer et al., 2001). The dry spore powder of the fungus formulated in oil was applied using Ultra- Low Volume (ULV) spraying against locust and grasshopper in Africa.

1.5.4. Classification

Entomopathogenic fungi are commonly classified based on their life cycle and morphological attributes (Castrillo et al., 2005). For greater precision to distinguish species, DNA characteristics are used to as well. These fungi are found in phyla Zygomycota, Ascomycota, Deuteromycota, Basidiomycota and Chytridiomycota (Castrillo et al., 2005; Samson et al., 1988), as well as Oomycota previously classified within fungi (Hawksworth et al., 1995), but most genera and species of entomopathogenic fungi belong to the Zygomycota and Deuteromycota (sexual forms: Ascomycota) (Samson et al., 1988). Fungal taxonomy,

especially at the higher levels of classification is continually being amended (Hibbett et al., 2007).

Overall, fungi have complex life cycle stages with asexual and sexual periods, fungal taxonomy at the higher level is normally based on sexual forms. Some of the entomopathogenic fungi lack a known sexual stage and they are only observed as the asexual forms, these fungi are classed in the Deuteromycetes known as imperfect fungi (Hajek, 2004).

Both *Beauveria bassiana* and *Metarhizium anisopliae* are fungi with asexual reproduction (anamorphs) and have no sexual stage. These fungi were formerly placed in the class Hyphomycetes of the phylum Deuteromycota (also known as fungi imperfecti). The Deuteromycota is a temporary classification for fungi with no known sexual, or teleomorph stage. In this system, all fungi that no have sexual stage were placed in Deuteromycoa. However, successive studies based on morphological and molecular attributes have demonstrated that some of these “imperfect fungi” are anamorphs (asexual forms) which belong to the Phylum Ascomycota (Order: Hypocreales: Family: Clavicipitaceae) (Roy & Cottrell, 2008).

It is now possible to place these fungi in their real position (Driver et al., 2000). *Cordyceps bassiana* is the teleomorph (the sexual form) of *B.bassiana* and *Metacordyceps taii* is the teleomorph of *M.anisopliae* var. *anisopliae*. Nowadays these fungi are placed in the phylum Ascomycota and classified as follows (Table 1.1).

Table 1.1. Outline classification of *Beauveria* and *Metarhizium* genera

Phylum	Class	Order	Family	Genus
Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	Beauveria Metarhizium

The genus *Beauveria* comprises 6 species as follows:

Beauveria bassiana, *B. brogniartii*, *B. amorphia*, *B. vermiconia*, *B. velata*, and *B. caledonicu*

Bischoff et al. (2009) indicated the species *M. anisopliae*, *M. guizhouense*, *M. pingshaense*, *M. acetidum* stat. nov., *M. lepidiotae* stat. nov and *M. majus* stat. nov in the genus *Metarhizium*. Authors have also described the new species *M. globosum* and *M. robertsii* and claimed that *M. taii* is a later synonym of *M. guizhouense*.

1.5.5. Morphology

***Beauveria bassiana*:** The surface of the colony is white to cream and fluffy to powdery. The fungal hyphae are tubular, narrow, septate fragile filaments; it is usually white becoming slightly yellowish over time. The hyphal width differs from 1.7 to 2.8 μm . Conidiogenous cells are nearly flask shaped with a narrow zigzag terminal extension (Larone, 2002); they are also proliferate sympodially. Conidia are single-celled and globose or ovate in shape, and usually germinate on a geniculate or at each bend point of zigzag rachis. The conidia range in size from 2-4 μm in diameter (Larone, 2002).

***Metarhizium anisopliae*:** The fungus is easily identified because it forms green chains of cylindrical conidia that densely compact on the infected host, causing green muscardine disease (Zimmerman, 1993). Fungal colonies are initially white or creamy mycelium, becoming shades of green/yellow to shades of dark green during sporulation. Conidiophores are simple or double- branched. The range of conidial size is from 4.8-7.2 μm in length, and from 1.6-3.5 μm in width (Gouli et al., 2005). Two varieties of *M. anisopliae* have been recorded that differ in conidia size. The conidia in *M. anisopliae* var. *majus* are approximately twice as large as *M. anisopliae* var. *anisopliae* (Tulloch, 1976).

1.5.6. Infection processes and biology

Entomopathogenic fungi differ from other insect pathogen groups in their ability to invade a host by penetrating its cuticle. The general life cycles of entomopathogenic fungi are considerably similar (Roy et al., 2006). Both *B. bassiana* and *M. anisopliae* exhibit dimorphic life cycles. If the insect host is absent, the fungi have an asexual, vegetative life (saprophytic

phase), however they have a pathogenic life cycle when the host insect is present.

Metarhizium anisopliae and *B. bassiana* are able to infect different developmental stages of their host such as eggs, larvae, pupae and adults (Gouli et al., 2005).

The infection process

The infection process involves the following stages:

(A) recognition and spore attachment to host, (B) germination and penetration of spore to integument (C) growth and proliferation within host, (D) re-emergence from the host cadaver followed by conidial production (Zacharuk, 1973). The pathogenesis process of both *B. bassiana* and *M. anisopliae* are generally similar (Gouli et al., 2005).

A: Attachment of a viable spore to the cuticle surface of a susceptible host is an essential stage for growth of the fungus and fungal infection. For most entomopathogenic fungi attachment of conidia on the insect host cuticle is a passive action with the aid of wind and water. Dry spores of *B. bassiana* and *M. anisopliae* have an outer layer composed of interwoven fascicles of hydrophobic rodlet layers. The attachment of dry spores is considered to be due to non-specific hydrophobic pressure enforced by the rodlets (Boucias et al., 1988). These rodlets are formed by hydrophobic proteins found in fungal aerial conidia (Kershaw & Talbot, 1998). These hydrophobic proteins were found in *B. bassiana* aerial conidia but not in blastospores (Bidochka et al., 1995).

B: Attaching to the cuticle surface, the spores absorb moisture and germinate producing germ tubes. Germination is influenced by availability of nutrients, oxygen, water, pH and temperature. Entomopathogenic fungi with restricted host range appear to need more specific requirements for germination than those which have a wide host range (St Leger et al., 1989). The germinated hyphae penetrate the insect's integument. Penetration involves both mechanical and enzymatic actions (Ferron 1981). The production of cuticle- degrading enzymes such as proteases, chitinases and lipases by fungi are important factors facilitating penetration (Boucias & Pendland, 1998; Charnley, 1984; Dean & Domnas, 1983; Samsináková et al., 1971). The inability to produce the enzymes in some strains may delay the infection processes (Bidochka & Khachatourians, 1990). Proteases are considered the major cuticle degrading enzymes, as their activity appears to precede the action of chitinases (Smith et al., 1981; St. Leger et al., 1989). Among the proteases found in entomopathogenic fungi, the chymoelastase PrI has been well known and its role in cuticle invasion has been

demonstrated (Castrillo et al., 2005; Goettel et al., 1989; St. Leger et al., 1989; Zibae & Bandani 2009).

C: The fungus enters and circulates in the haemolymph while proliferating by budding into the insect's body; it rapidly colonizes all tissues and organs and utilizes nutrient resources while producing toxins. This process ultimately leads to death of the infected host. Success in colonization is dependent upon the ability of the fungus to overcome the host immune system after entering the haemolymph (Gillespie et al., 2000). Infected insect hosts suffer depletion of nutrients or starvation and death of the host occurs during colonization of the haemocoel usually 3-6 days after infection (under optimal conditions). However, in cool conditions infected insect may survive until 3-4 weeks after initial infection (Gouli et al., 2005). However, the time that fungi need to kill the host is dependent on the time required for attachment, germination, penetration and growth of the fungi (Stimac et al., 1993). Successful infection depends on various factors including ambient conditions (temperature and humidity), characteristics of the fungi (virulence, germination rate) and insect host life stage and susceptibility (Samson et al., 1988; Watanabe, 1987). Entomopathogenic fungi can also produce toxins to overcome insect host defences, indeed these fungi kill their hosts by reduction of food consumption, invasion and destruction of tissue, and the release of the toxins (Tanada & Kaya, 1993).

D: Once the entomopathogenic fungus has colonized the host, hyphae will emerge from the cadaver and the fungus reverts back to the asexual cycle (the saprophytic stage). In suitable conditions of temperature and humidity the fungus is able to produce spores on surface of the dead host. Conidia of *Metarhizium* and *Beauveria* spp. are hydrophobic and are passively dispersed from infected cadavers by wind, rain splash or contact with another insect. In order for horizontal transmission of pathogens and then mycosis to occur in a pest population, conidia sporulated from cadavers must be able to disperse and sufficient humidity is necessary for germination of conidia.

Ambient conditions especially relative humidity and temperature, are important for growth and germination. Suitable humidity, available nutrients and surface topography are the factors affecting successful germination (Dillon & Charnley, 1990; St. Leger et al, 1994, 1991). For all entomopathogenic fungi, abiotic factors (e.g., temperature, humidity and ultraviolet radiation) have a lot of influence on insect infection. The optimum temperature range needed for mycosis is the subject of several studies. The optimum temperature for the

survival, growth, and pathogenicity of entomopathogenic fungi usually is between 20°C - 30°C (Clayton et al., 1983), however Gouli et al. (2005) suggested the range between 24 and 30°C as optimal temperature for fungal infection. Ibrahim et al. (1999) showed that 98% relative humidity (RH) is required for germination of *M. anisopliae*. However, Ferron (1977) found that *B. bassiana* can infect the insect host at ambient relative humidities less than the 92% required for germination and mycelial growth *in vitro*. The author also suggested that fungal germination on the cuticle of insect (after successful adhesion) may be less dependent on ambient humidity, because the microclimate of the insect cuticle is similar to that of their host plants.

Ultraviolet light is highly detrimental for germination and survival of the fungi in field condition (Edington et al., 2000; Ignoffo, 1992; Inglis et al., 1997; Moore et al., 1996). Conidia exposed to sunlight for 24 hours during a few days, lose between 50 and 100% of their virulence and viability (Gardner et al., 1977), however they can survive for several years in soil conditions protected from solar radiation with high relative humidity and low temperature (Gaugler et al., 1989; Roberts & Campbell, 1977).

1.6. Use for pest control

Entomopathogenic fungi are not used extensively for classical biological control (Hajek, 2004), only entomophthoralean fungi, known as obligate pathogens, can be used for classical biological control. *Beauveria bassiana* and *M. anisopliae* have been developed for inundative biological control. The spores of these fungi can be mass produced on a variety of inexpensive artificial media and have a relatively long shelf-life when they are stored in the cold with appropriate relative humidity.

Temperature and moisture equilibrium is important to fungal spore longevity in storage. Hong et al. (1997) investigated the effect of temperature and moisture on spore longevity; they reported that the longevity of conidia of *M. anisopliae* was greatest during storage at low relative humidity and temperature.

Entomopathogenic deuteromycetes such as *B. bassiana* and *M. anisopliae* may be formulated and applied in a way similar to chemical pesticides (Prior & Greathead, 1989).

Aerial conidia produced by solid-state fermentation or blastospores produced in liquid culture, from an initial storage slant are required to provide inoculum for the production media for scaled-up production of conidia or blastospores. Aerial conidia are currently the

best method (Holker, 2004), and superior to mycelia and blastospores because they are similar to the spores produced naturally on the cadavers of insects killed by mycosis (Deshpande, 1999; Feng et al., 1994; Wraight et al., 2001; Roberts & St. Leger, 2004). Conidia can be applied in several formulations, the maintenance of conidial viability in formulations during storage is crucial for obtaining successful insect control in the field.

1.6.1. Formulation of entomopathogenic fungi

Granular Formulations

Granular formulation includes dry mycelium or spores which are formulated as contact baits. Application of dry mycelium relies on sporulation in the field to produce the infective aerial conidia particularly where water and humidity are a limiting factor (Burgess et al., 1998; Knudsen et al., 1991; Krueger et al., 1992; Pereira & Roberts, 1991). The contact baits protect spores from sunlight and attract grasshoppers and locusts. Formulations that use maize-starch extrusion technology or formulations with wheat bran have shown promise in the control of *S. gregaria* and *Melanoplus sanguinipes* (Caudwell & Gatehouse, 1996; Inglis et al., 1996).

Sprayable Formulation

Sprayable formulations includes several liquid formulations described below:

(i) the use of oil-soluble sunscreen with oil-carrier (Hunt et al., 1994; Moore et al., 1993; Shah et al., 1998); (ii) the use of emulsions based on oil and water (Alves et al., 1998); (iii) the use of water-soluble or suspendable absorbers with water carriers (Cohen et al., 1991; Ignoffo et al., 1997; Shapiro 1989, 1992, Shapiro & Robertson, 1990; Shasha et al., 1998); and (iv) encapsulation with a water carrier (Behle, et al., 1997; Ignoffo et al., 1991; McGuire & Shasha, 1995; Shasha & Dunkle, 1989; Shasha & McGuire, 1989; Tamez-Guerra, et al., 1996).

The use of oil formulations for spray application requires the production of lipophilic conidia which can be suspended easily in oil. Despite the advantages of submerged conidia (blastospores) and the possibility of production of many mitosporic fungi in submerged liquid fermentation (Hegedus et al., 1990; Hildebrand & McCain, 1978; Jackson & Bothast, 1990; Jenkins & Prior, 1993; Khurana et al., 1993; Lascaris & Deacon, 1994; van Winkelhoff & McCoy, 1984), these types of spores are not able to be formulated in oil because they are hydrophilic.

Generally oil-based formulations appear to have three advantages versus water-based formulations: **(i)** It has been reported that *Metarhizium anisopliae* var. *acridum* formulated in oil is more effective at low humidity (Bateman et al., 1993; Prior et al., 1988); **(ii)** oil-based formulations may be used in regions where humidity is limited (Hedgecock et al., 1995; Hong et al., 1997, 1998; McClatchie et al., 1994); **(iii)** oil-based formulations are compatible with ultra- low volume application, which is important for reducing spray volume in remote desert areas where water is not easily available. In contrast water formulations can be used in greenhouses, or locations with high ambient humidity.

1.7. Aims and objectives

The hypotheses tested by the research presented in this thesis were as follows:

- 1) The grasshopper *Uvarovistia zebra* (Orthoptera: Tettigoniidae) can be infected by *Beauveria bassiana* DEBI 001 and *Metarhizium anisopliae* 715C.
- 2) Different nymphal instars and adults of the Sunn pest *Eurygaster integriceps* show different susceptibility to *B. bassiana* DEBI 001 and *M. anisopliae* 715C.
- 3) The fungi *B. bassiana* and *M. anisopliae* formulated in oil-bait could be more effective against *U. zebra* than those formulated in water-bait.
- 4) *Beauveria bassiana* and *M. anisopliae* stored in different storage media show different degeneration.
- 5) The virulence of *B. bassiana* DEBI 001 and *M. anisopliae* 715C can be affected by subculturing in artificial media or passing through the grasshopper *U. zebra*.

In order to test these hypotheses and fulfil the aims, experiments were conducted which have been reported in chapter 2-7 of this thesis.

The objectives of these were as follows:

Chapter 2. Efficacy of *Beauveria bassiana* and *Metarhizium anisopliae* on *Uvarovistia zebra* (Orthoptera: Tettigoniidae) via contact and ingestion

- To identify the pathogenicity of the *B. bassiana* and *M. anisopliae* strains to *U. zebra* using water-spore formulation via contact application.
- To assess the pathogenicity of the fungi to *U. zebra* via ingestion using lettuce as a bait inoculated with water based conidia formulation.
- To identify LC₅₀ and LT₅₀ of the fungi when they were applied by contact application.

Chapter 3. Effect of infection by *Beauveria bassiana* and *Metarhizium anisopliae* on the feeding of *Uvarovistia zebra* (Orthoptera: Tettigoniidae)

- To identify if the food consumption by infected insects is reduced before they die.
- To identify how long the fungi take to cause any reduction of feeding by infected insects.
- To compare the efficacy of the two fungi in reducing food consumption of *U. zebra*.

Chapter 4. Pathogenicity of the isolates *Beauveria bassiana* and *Metarhizium anisopliae* on different instars nymphs of the Sunn pest *Eurygaster integriceps* Puton (Heteroptera: Scutellaridae)

- To assess the effect of the isolates *B. bassiana* DEBI 001, and *M. anisopliae* isolate 715C against *E. integriceps*.
- To evaluate the relative susceptibility of different instar nymphs and adult to the fungi.

Chapter 5. Effect of oil and water bait formulations on the efficacy of *Beauveria bassiana* and *Metarhizium anisopliae* against *Uvarovistia zebra*

- To determine the efficacy of *B. bassiana* and *M. anisopliae* in two formulations (oil and water) and on a bait substrate (lettuce) against the tettigoniid grasshopper *Uvarovistia zebra*.
- To compare the pathogenic potential of the fungi when they were used as a bait with two formulations.

Chapter 6. The viability, mycelial growth and conidia production of *Beauveria bassiana* and *Metarhizium anisopliae* preserved in different storage media

- To quantitatively determine the viability of the fungi.
- To determine spore production (sporulation) and mycelia growth of the fungi.

Chapter 7. Virulence of *Beauveria bassiana* and *Metarhizium anisopliae* passaged through artificial media and an insect host *Uvarovistia zebra*

- To evaluate the effect of *in vitro* subculturing on the virulence of *Beauveria bassiana* DEBI 001 and *Metarhizium anisopliae* 715C.
- To evaluate the effect of passages through the grasshopper *U. zebra* on the virulence of *Beauveria bassiana* DEBI 001 and *Metarhizium anisopliae* 715C.

Chapter 2. Efficacy of *Beauveria bassiana* and *Metarhizium anisopliae* on *Uvarovistia zebra* (Orthoptera: Tettigoniidae) via contact and ingestion

Abstract

The pathogenicity of the fungi *Beauveria bassiana* and *Metarhizium anisopliae* against a locust *Uvarovistia zebra* was assessed. *Uvarovistia zebra* is a long horned grasshopper (Tettigoniidae) and this is the first time these entomopathogenic fungi have been used against a species from this family. The isolates *Metarhizium anisopliae* 715C and *Beauveria bassiana* DEBI 001 were separately tested in laboratory conditions against the insects using a topical spray and via ingestion with food. Field collected third-instar nymphs were treated with different spore concentrations. Both fungal isolates were pathogenic.

The highest concentrations of 1.5×10^8 spores/ml for *B. bassiana* and 2×10^7 spores/ml for *M. anisopliae* caused 100% and 53.3% mortality respectively when they were sprayed on the nymphs. When ingested by insects using lettuce bait inoculated with different spore concentrations the cumulative mortality caused by *M. anisopliae* at a concentration of 5×10^6 spores/ml was 43%, whereas *B. bassiana* at the same concentration caused 30% mortality although this difference was not significant. The strains of both fungi tested caused significant mortality of *U. zebra* by both contact and ingestion.

Key words: *Beauveria bassiana*, *Metarhizium anisopliae*, *Uvarovistia zebra*, Biological control, entomopathogenic fungi

2.1. Introduction

Over use of chemical insecticides against locusts and grasshoppers has had a negative impact on the environment (Lomer et al., 2001). As a consequence there has recently been renewed interest in the use of microbial agents, especially entomopathogenic fungi, as biological controls of some pests. Entomopathogenic fungi are excellent candidates for use in biological control because they do not need to be ingested to cause infection; they infect their hosts by contacting and then direct penetration of the cuticle (Wright et al., 2002). Entomopathogenic fungi are important key factors to regulate insect population in nature (Chamley, 1997). Numerous and diverse isolates of entomopathogenic fungi have been tested for their potential to control insects pests. In addition to different species and strains of entomopathogenic fungi, different formulations and application methods have also been evaluated (Feng et al., 2004a, 2004b; Langewald et al., 1997; Pu et al., 2005; Wraight et al., 2000; Wraight and Ramos, 2002). Faria & Wraight (2007), presented a comprehensive list of 171 mycopesticides products which 160 (93.6%) of those are considered to have pathogenicity on species of 10 insect orders. Entomopathogenic fungi are attracting attention as potential biological control agents (Clarkson & Charnley, 1996), for the many acridid and coleopteran pests which have no known viral or bacterial diseases and they are the only

practical microbial control of insects that feed by sucking plant sap such as Homoptera (Hajek & St. Leger, 1994; St.Leger et al., 1996).

Under intensive study for biological control, the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* display a broad host range and are able to target a number of diverse arthropod species. These are the most widely used entomopathogenic fungi and have been used for biological control of agricultural pests especially acridid grasshoppers and locusts in different parts of the world.

Bateman et al. (1996), screened the pathogenicity of 159 isolates of the genera *Metarhizium* and *Beauveria* against desert locust *Schistocerca gregaria* adults; they reported that approximately 50 isolates of the genus *Metarhizium* showed a comparable virulence with the standard strain of *Metarhizium* (IMI 330189) used in all screens.

Different isolates of *Metarhizium anisopliae* have been obtained from locusts and grasshoppers in Africa (Shah et al., 1997), Australia (Prior, 1997), Madagascar (Delgado et al., 1997). Isolates of *M. anisopliae* var. *acridum* have been evaluated and applied to control locusts (Acrididae) under the name of Green Muscle[®] in Africa (Douthwaite et al., 2000; Lomer et al., 2001) and Green Guard[®] in Australia (Milner & Hunter, 2001). Among the entomopathogenic fungi products listed by Faria and Wraight (2007), *Beauveria bassiana* constitute (33.9%) and *Metarhizium anisopliae* also constitute (33.9%) of total productions. Because of the wide host range and diversity in isolations these fungi are widely used as biological agents.

Different formulations and application methods have been tested to evaluate the efficacy of the entomopathogenic fungi. Faria & Wraight (2007), listed eleven different fungal formulation types employed to apply entomopathogenic fungi against pests. There are examples where fungal spores have been used to treat grasshoppers by contact action. Prior et al. (1995) applied an inoculation technique using a conidia suspension formulated in oil pipetted beneath the dorsal pronotum to assay isolates of *Metarhizium* spp against the desert locust *Schistocerca gregaria*.

Among the 5 fungal isolates of entomopathogenic fungi used against the grasshopper *Zonocerus variegatus* (L), the highest LT₅₀ of 2 days was observed for *B. bassiana* (Balogun & Fagade, 2004). Prior et al. (1988) reported that aerial conidia of *M. anisopliae* formulated in oil are more effective against grasshoppers than aqueous formulation.

Metarhizium anisopliae var. *acridum* was applied using an Ultralow-volume with a hand-held atomizer sprayer under field condition against the gregarious grasshopper *Rhammatocerus schistocercoides* (Rehn) and reduced populations by 66-80% depending on the dose applied (Faria et al., 2002). Direct and residual sprays of *M. anisopliae* var *acridum* using different formulations (oil-in-water and water-based) showed highly significant differences between formulations, applied to *Locusta migratoria* (L) in a laboratory investigation (Kassa et al., 2004).

Ultra Low Volume (ULV) spraying with oil formulations for locust and grasshopper control is a common method to apply the fungi especially where water supplies are difficult such as in the desert in Africa. However in other conditions, bait formulations could be effective (Lomer et al., 2001). Inoculation via ingestion using bait inoculated with either oil-spore mixtures or only spore solution is also used for testing the efficacy of entomopathogenic fungi. Inglis et al. (1996) tested the efficacy of *Beauveria bassiana* against grasshoppers *Melanoplus sanguinipes* (Fabricius) using two formulations (oil and water) and two bait substrates (lettuce and brain). The authors found that conidia formulated in oil were more effective than those formulated in water, but there was no significant difference between the lettuce and bran. A study by Consolo et al. (2003), demonstrated the pathogenicity of both *B. bassiana* and *M. anisopliae* to San Antonio beetle *Diabrotica speciosa* (Germar) by feeding of infected corn sprouts baits.

Uvarovistia zebra (Uvarov) (Orthoptera: Tettigoniidae) is an univoltine native grasshopper, distributed on the southern slopes of the Alborz Mountains in the north of Qazvin province in Iran (Latitude: N 36° .33' 45.3601", Longitude: E 50° .5' 12.0117"). In some years, following good conditions for population growth, they can invade and damage field crops, and rangeland grass. The nymphs of the first instar appear in early April (Keyhanian & Ghazavi, 1996).

These insects inhabit rangelands which must be protected as natural environments. To prevent the adverse impacts of chemical insecticides use, entomopathogenic fungi are considered as a potential alternative for control of this pest. Orthoptera constitute 10% of the insects targeted by the entomopathogenic fungal products presented by Faria & Wraight (2007). However evaluations of entomopathogenic fungi for control of grasshoppers and locusts generally have focused on Acrididae (short-horned grasshopper) species.

In this study, experiments were done to identify the potential of strains of *B. bassiana* and *M. anisopliae* for control of the long-horned grasshopper *Uvarovistia zebra* (Orthoptera: Tettigoniidae).

The objectives of these experiments were:

- To identify the pathogenicity of the *B. bassiana* and *M. anisopliae* strains to *U. zebra* using water-spore formulation via contact application.
- To assess the pathogenicity of the fungi to *U. zebra* via ingestion using lettuce as a bait inoculated with water based conidia formulation.
- To identify LC_{50} and LT_{50} of the fungi when they were applied by contact application.

2.2. Materials and methods

2.2.1. Source of insects

Second and third-instar nymphs of *Uvarovistia zebra* were collected from mountainous rangeland of the Alamout region in the north of Qazvin province in Iran.

The insects suffered high mortality and showed cannibalistic behaviour during transfer to the laboratory, particularly when many individuals were confined in a small space. In order to minimise this, a small number of insects (4-7) were placed into plastic bags and then the bags were placed on ice. The low temperature helped decrease insect activity and in this way, locusts were transferred to the lab unharmed. The insects were kept in groups of 15 in cages (45×38×38cm H×L×W) made of aluminium with wire mesh sides and placed in the laboratory. They were fed with fresh lettuce for five days in order to acclimate to the diet of lettuce and laboratory condition before beginning the experiments.

2.2.2. Source and culturing of fungi

Beauveria bassiana isolate DEBI 001, and *Metarhizium anisopliae* isolate 715C, were obtained from the "Iranian Research Institute of Plant Protection" in Tehran, Iran. The former was isolated by Dr Ghazavi from the soil of a cherry orchard in Fashand region in Tehran, Iran and the latter isolated by Dr Zare from an unknown species of locust. Both isolates were propagated and maintained on standard potato dextrose agar (PDA) (Merck).

Liquid medium Potato-dextrose- broth (PDB) was used for production of conidia required for experiments.

To produce the liquid medium 200g of peeled potatoes were ground and boiled in water, before being strained and poured into an Erlenmeyer flask where 20g dextrose was added to

it. The volume was made up to 1 l by adding distilled water. Liquid medium was autoclaved for 20 minutes at a temperature of 121 °C and pressure of one atmosphere, and cooled before being inoculated with fungal spores propagated on PDA. Spores were harvested from 2 – 3 week old surface culture by scraping and used to inoculate the liquid medium. The flask was held in a shaker (110 rotations a minute) for 5 days at 25 °C. After this time the blastospore concentrations were determined using a haemocytometer and were 1.5×10^8 and 2×10^7 spores/ml for *B. Bassiana* and *M. anisopliae* respectively. This suspension was the primary concentration of spores.

Four different concentrations were produced from each of the main suspensions of each fungus to use in the experiments. The formula $C_1V_1=C_2V_2$ was used for this purpose.

C1= the concentration in the main suspension.

C2= the target concentration required.

V1= the volume of the main suspension.

V2= the volume of C2 that must be achieved by dilution.

2.2.3. Initial experiments to prove the pathogenicity of fungi

In the first instance, the pathogenicity of both *B. bassiana* and *M. anisopliae* isolates to the locust *U. zebra* was tested. Five locusts were treated with suspensions consisting either of spores of *B. bassiana* or of *M. anisopliae* (1.5×10^8 and 2×10^7 spores/ml, respectively). The insects' bodies were cleaned with cotton wool soaked with ethanol (70%) before being inoculated. Each insect was sprayed with 1 ml of solution and kept in glass dishes covered with mesh cloth. The death of locusts was confirmed if they remained immobile after prodding with a glass rod. Seven days after applying the fungi, the dead locusts were put into sterile Petri dishes with high humidity and incubated at 25 ± 1 °C in order to retrieve fungal spores.

2.2.4. Bioassay

The bioassay experiments were arranged as a completely randomized design with five treatments (concentrations and control) and three replicates for each treatment.

2.2.4.1. Contact application of fungi

In two separate experiments spore suspensions of *Beauveria bassiana* isolate DEBI 001 and *Metarhizium anisopliae* isolate 715C (produced in liquid medium) were sprayed on third-instar nymphs of *U. zebra*. The bioassay involved treatments with four different spore

concentrations made from the primary suspension and water (control). *Beauveria bassiana* was used at 2×10^4 , 2×10^5 , 2×10^7 , 1.5×10^8 spores/ml and *M. anisopliae* at 2×10^4 , 2×10^5 , 2×10^6 , 2×10^7 using a hand held sprayer with a flow rate of 70 ml/min adjusted by appropriate nozzles (Kassa et al., 2004). Each replicate comprised 10 insect nymphs placed in one cage. The sprayer was calibrated so that 100ml of each concentration was used for 3 replicates (30 insects). Thus each insect was directly and individually sprayed with about 3.3ml of the appropriate concentration while they stood on filter paper. After 15 min the treated insects were transferred to the cages. To prevent cross contamination in the laboratory, spraying was done outside. At first the water (control) treatment was applied and then the other treatments working from low concentration to high. The conditions in the laboratory were adjusted to 15:9 L:D 75%-80 RH% and temperature between 25-30°C. Records of insect mortality started 24 hours after the treatments were applied and continued for 15 days. Every day the number of insects that had died in the previous 24 hours was noted. The number of insects surviving was also recorded daily and the control value was determined. Lethal concentrations (LC₅₀) of the fungi based on four conidial concentrations were calculated 15 days after treatment. Lethal time (LT₅₀) for each fungus was also calculated from the survival curve.

2.2.4.2. Ingestion of fungi

The insects were fed with fresh lettuce for five days to acclimatise them to both the diet and lab conditions before being assayed. Four different concentrations (5×10^3 , 5×10^4 , 5×10^5 , 5×10^6 spores/ml) made from the primary suspension of each isolate were used for this bioassay. Each replicate embraced 10 third - instar nymphs of *U. zebra*. A 7-mm-diameter disc of lettuce leaf inoculated with fungal spore suspension was used for each insect. The lettuce discs were immersed in the suspension of the appropriate concentration (or water control) for 5 seconds. The treated discs were left for 20 min at ambient temperature to dry and were then presented to insects in individual containers (Figure 2.1). The insects were allowed to feed on the discs and after 20 h, when the last insect had ingested all the bait, they were transferred to ventilated plastic containers (30×15×8 cm L×W×H). The containers were fitted with a perforated metal floor to reduce contact with frass (Inglis et al., 1996). Throughout the experiments, insects were maintained at 40-55 RH%, 15:9 L: D and temperature between 25-30°C. The insects were fed daily for 12 days with fresh (untreated) lettuce and every 48 h their mortality was recorded.



Fig 2.1. The nymphs were fed with inoculated lettuce discs in individual containers.

2.2.5. Confirmation of the fungal infection in locusts

Dead insects were taken out of the cages and cleaned with cotton wool soaked with 75% ethanol before being placed individually in sterilized Petri dishes on moist filter paper. Petri dishes were placed into an incubator at 27°C for 4-7 d to retrieve mycelia on the insect surface in order to confirm the fungal infection of dead insects. After 3-5 days, fungal mycelia were observed growing out of the bodies of insects that died from mycosis. Insects that did not produce mycelia were recorded as other mortality, but cumulative mortality (mortality caused by fungal infection plus other mortality) until day 12 of the experiment was analysed. Retrieved mycelia were cultured in PDA and were identified by Dr Zare, a mycologist working at the Iranian Plant and Diseases Research Institute in Tehran.

2.2.6. Statistical analysis

Cumulative mortality at the end of the experiment was analyzed by ANOVA, and treatment means compared using Tukey test. The effect of fungi were compared by t-test ($\alpha = 0.05$). The median survival time (MST) for each treatment was estimated using the Kaplan-Meier survival analysis (Kaplan & Meier, 1958). Multiple comparisons were used to determine significant differences between the treatments (Tukey test). Time for 50% mortality (LT_{50}) and concentration for 50% mortality (LC_{50}) were determined using the probit analysis program. SPSS 15.0 for windows was used for all analyses.

2.3. Results

The result of preliminary tests showed that both fungi were capable of infecting *U. zebra* by both contact and ingestion, with consequent death of the insect. Mycelia retrieved from dead insects were identified using morphological characteristics as the fungal species that had originally been applied to the insects (Fig 2.2 & 3).



Fig 2.2. *Uvarovistia zebra* with *Beuveria bassiana* mycelium



Fig 2.3. *Uvarovistia zebra* with *Metarhizium anisopliae* mycelium

2.3.1. Contact application of fungi

The highest concentrations of 1.5×10^8 spores/ml for *B. bassiana* and 2×10^7 spores/ml for *M. anisopliae* were most effective. The former caused 100% and the latter 53.3% mortality at the end of experiment and significant differences were found between these and all other concentrations of each fungus (*B. bassiana*: $F_{(4,10)} = 205.37$, $P < 0.001$; *M. anisopliae*: $F_{(4,10)} = 76.66$, $P < 0.001$). Percentage mortality at the end of experiment caused by different concentrations of each fungus has been shown graphically (Figure 2.4 A&B). At the same concentration (2×10^7 spores/ml) *B. bassiana* and *M. anisopliae* caused 66.6% and 53.3% mortality respectively and this difference is not statistically significant ($t_{(4)} = 1.79$; $P = 0.1467$). Maximum daily mortality caused by *B. bassiana* with 1.5×10^8 spores/ml was observed at day 7, however *M. anisopliae* with 2×10^7 spores/ml produced most mortality at day 11.

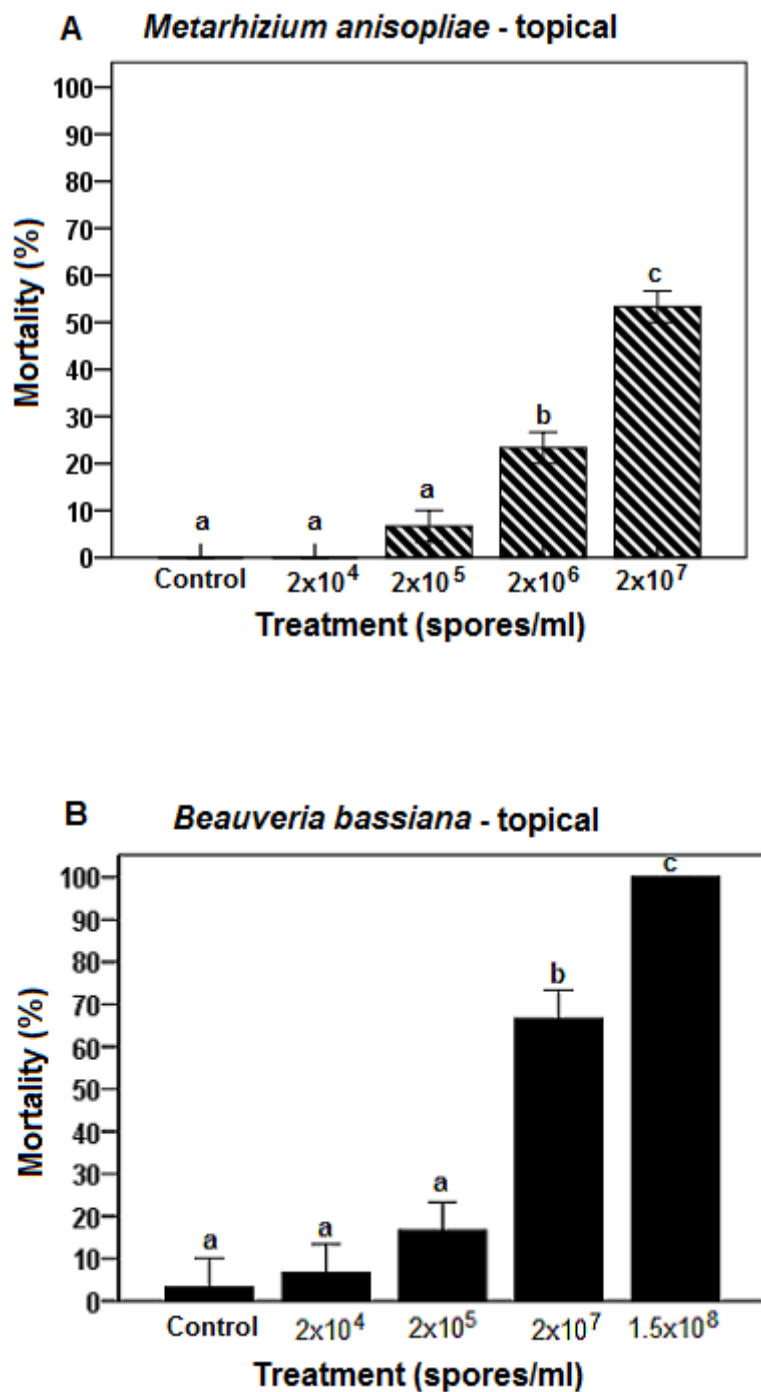


Fig 2.4. The mean (\pm SE) percent mortality ($n = 30$ nymphs within 3 replicates) of *U. zebra* 15 days after contact treatment with *M. anisopliae* (A) and *B. bassiana* (B) at different concentrations. For each fungus, mean \pm SE values with the same letter along the bars of different concentrations are not significantly different ($P > 0.05$ Tukey test). Insect survival during the experiment (15 days) has been shown separately for each fungus (Fig 2.5 A&B). All of the insects treated with the highest concentrations (1.5×10^8 spores/ml) of *B. bassiana* had died by the end of day 11.

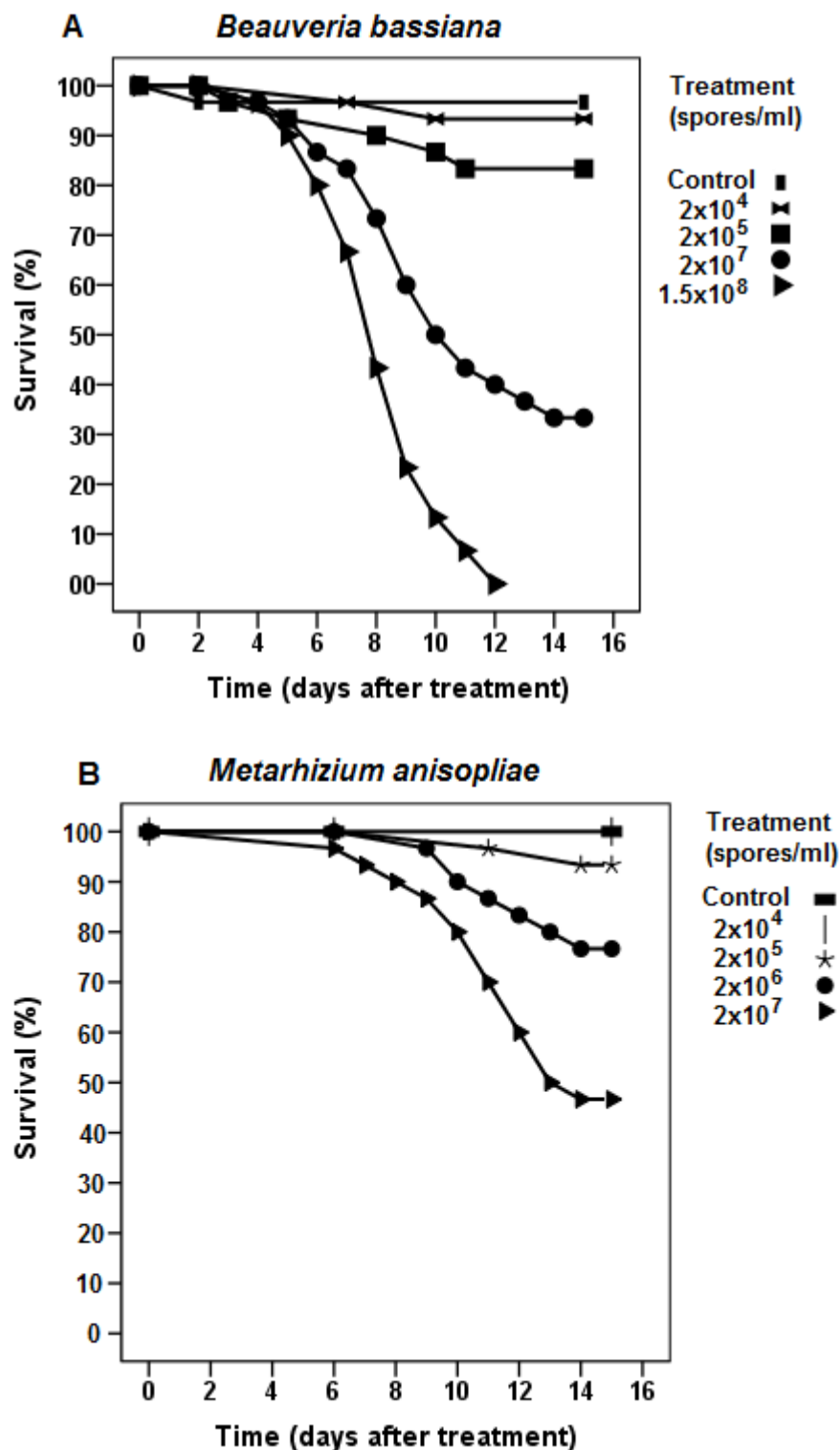


Fig 2.5. Survival of *U. zebra* after contact treatment with *M. anisopliae* (A) and *B. bassiana* (B) at different concentrations.

The LC₅₀s were estimated for the isolates of each fungus applied to third instar nymphs of *U. zebra* (Table 2.1). Results of the probit analysis indicate significant relationships ($P < 0.05$) between log dosage and probit mortality.

Table 2.1. Median lethal concentrations of *Beauveria bassiana* and *Metarhizium anisopliae* isolates for *Uvarovistia zebra*.

Isolate	Intercept	Slope	Variance	LC ₅₀ (conidia/ml)
<i>B. bassiana</i> DEBI 001	1.23	0.95	0.023	3.59×10^6
<i>M. anisopliae</i> 715C	1.83	0.953	0.04	1.46×10^7

The LT₅₀ value at 2×10^7 and 1.5×10^8 spores/ml for *B. bassiana* and 2×10^7 spores/ml for *M. anisopliae* were calculated. *Beauveria bassiana* killed 50% of the nymphs in 7.82 and 8.99 days at the 1.5×10^8 and 2×10^7 concentrations respectively. LT₅₀ for *Metarhizium anisopliae* at the highest concentration 2×10^7 was estimated 12.88 days.

2.3.2. Ingestion of fungi

The isolate of *M. anisopliae* was slightly more effective than *B. bassiana* when ingested. In both fungi there were significant (*B. bassiana*: $F_{(4,10)} = 14.12$, $P < 0.001$; *M. anisopliae*: $F_{(4,10)} = 42.00$, $P < 0.001$) differences between the highest concentration (5×10^6 spores/ml) and lower concentrations and the control (Figure 2.6 A&B). *Metarhizium anisopliae* at a concentration of 5×10^6 spores/ml caused 43.3% mortality, however *B. bassiana* at the same concentration caused 30% mortality although this difference was not significant ($t_{(4)} = 1.99$; $P = 0.1166$).

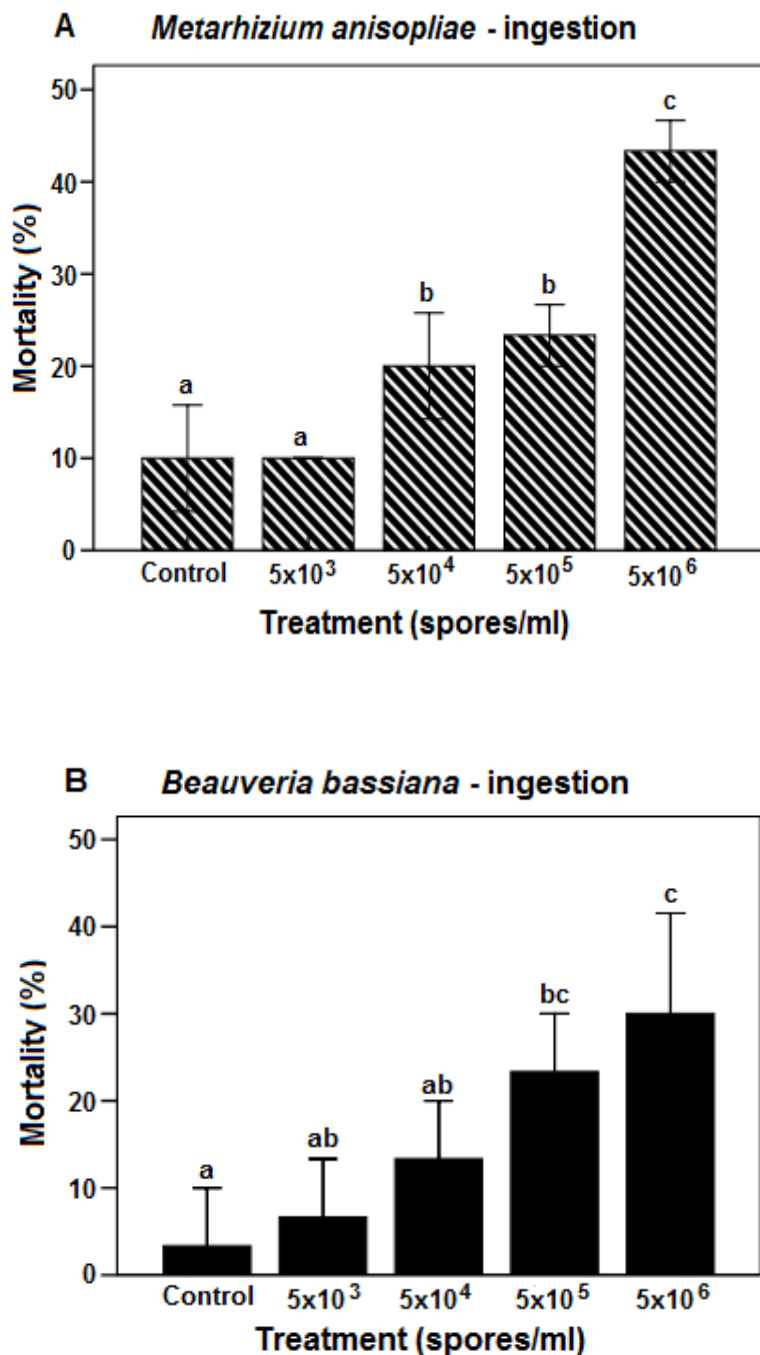


Fig 2.6. The mean (\pm SE) percent mortality (n = 30 nymphs within 3 replicates) 15days after ingestion treatment with *M. anisopliae* (A) and *B. bassiana* (B) at different concentrations. For each fungus, mean \pm SE values with the same letter along the bars of different concentrations are not significantly different ($P > 0.05$ Tukey test).

Maximum daily mortality caused by the highest concentration of *B. bassiana* and *M. anisopliae* were at 8 and 10 days after treatment respectively. Percent daily mortality of insects fed lettuce with the highest concentration (5×10^6 spores/ml) of *B. bassiana* and *M.*

anisopliae is shown in Figure 2.7. The maximum daily mortality (13.3%) caused by *B. bassiana* was observed on day 8, however, the maximum mortality (13.3%) caused by *M. anisopliae* with the same concentration was observed on day 10 after treatment.

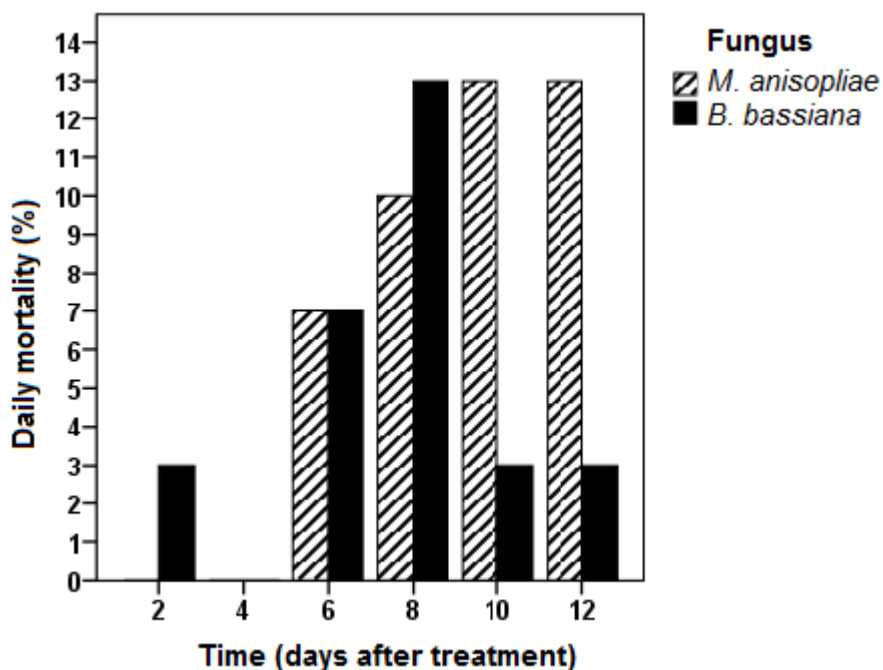


Fig 2.7. Daily mean percent mortality of *U. zebra* fed lettuce bait treated with 5×10^6 spores/ml of the fungi *B. bassiana* and *M. anisopliae*.

2.4. Discussion

This study demonstrated that the isolates used are capable of causing infection and mortality of *U. zebra* either via contact or ingestion. Both fungal isolates show potential as control agents for this insect.

With contact application the *B. bassiana* isolate at the same concentration caused more mortality than *M. anisopliae*. Whereas *M. anisopliae* caused more mortality than *Beauveria bassiana* when they were applied via ingestion, but these differences were not statistically significant.

Although the isolate of *M. anisopliae* caused more mortality than that of *B. bassiana* when they were ingested, *B. bassiana* took less time to infect and kill the insects and this faster action was also found with contact applications.

At the highest concentration used, the maximum daily mortality caused by *B. bassiana* occurred on days 8 and 7 after treatment using ingestion and contact application respectively. However, *M. anisopliae* produced maximum daily mortality on days 10 (with ingestion

application) and day11 (with contact application). The first insect mortality caused by the highest concentration of *B. bassiana* and *M. anisopliae* was observed at 3 and 6 days after treatment respectively. These times are similar to the results of others researchers. Bateman (1997) claimed that insect field mortality caused by pathogen rarely occurs earlier than 6 days after application. In a field trial the fungal product *Metarhizium anisopliae* var. *acridum* strain IMI 330189 developed by the LUBILOSA project, showed the first observable mortality of the acridid species at 7-10 days after application and the full effects were observed 14-18 days after application (Lomer et al., 1997). In this and most other trials the rate of 5×10^{12} spores ha^{-1} has been used to evaluate the pathogenicity of *Metarhizium* products such as Green Muscle (Lomer et al., 2001).

It is difficult to make a comparison with the rates used in the current work as a suitable formulation needs to be developed and the inoculum received by each insect for a given application rate per hectare needs to be known. In this study the LC_{50} of *B. bassiana* and *M. anisopliae* were estimated as 3.59×10^6 and 1.4×10^7 spores/ml respectively. It seems that, in spite of the LC_{50} being at a reasonable concentration the fungi took a long time to kill the insects; at the highest concentration the LT_{50} s of 7.82 and 12.88 days were estimated for *B. bassiana* and *M. anisopliae* respectively.

In a fungal isolate virulence bioassay, Balogun & Fagade, (2004) reported the LT_{50} s of 2 and 5 days for *Beauveria bassiana* and *Metarhizium* sp. respectively when they were applied to the grasshopper *Z. variegatus* using an oil formulation. As with the present study *B. bassiana* took less time than *M. anisopliae* to kill 50% of the insects.

Pathogenicity of entomopathogenic fungi via ingestion against the grasshoppers has been already reported, however most studies have been done on short horned grasshoppers using oil-bait formulation. Inglis et al. (1996) reported the mycosis of four isolates of *B. bassiana* with different spore concentrations formulated in oil on lettuce disks against the grasshopper *M. sanguinipes*. The current study demonstrated the pathogenicity of the fungi via ingestion when they were used against a long horned grasshopper using water-bait formulation. Inglis et al. (1996) found that conidia formulated in oil on lettuce discs were more effective against grasshopper nymphs than those formulated in water.

We can draw the following conclusions from this study.

This study has shown that tettigoniid locusts may be controlled by use of suitable isolates of either *Metarhizium anisopliae* or *Beauveria bassiana*. Both fungal agents can be applied by contact or by treating baits. In order to obtain the best control of the pests, a high concentration of the fungal spores (more than 10^7 spores/ml) must be used. In order to integrate these fungi into an effective management programme for *U. zebra* there needs to be further research on the different isolates and complementary research on the ecology and economics of the cropping system in the area where this species is a pest.

Chapter 3. Effect of infection by *Beauveria bassiana* and *Metarhizium anisopliae* on the feeding of *Uvarovistia zebra* (Orthoptera:Tettigoniidae)

Abstract

The effect of infection with the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* on food consumption by *Uvarovistia zebra* was investigated using different spore concentrations. Preliminary results showed that both *B. bassiana* and *M. anisopliae* had an effect on food consumption of the insects infected with the fungi. In both fungi a significant ($P < 0.05$) reduction of food consumption and faeces production by insects were observed between the highest spores concentration (5×10^6 spores/ml) and other treatments. Compared with control insects, the insects treated with 5×10^6 spores/ml of *B. bassiana* and *M. anisopliae* showed 60% and 63% reduction in mean food/insect respectively. The cumulative percent mortality of the insects treated with the highest concentration of *B. bassiana* and *M. anisopliae* were 57.7% and 55.5% respectively at the end of the experiments.

Key words: *Beauveria bassiana*, *Metarhizium anisopliae*, *Uvarovistia zebra*, entomopathogenic fungi, food consumption

3.1. Introduction

The length of time that entomopathogenic fungi take to kill the target insect is one of their perceived disadvantages. Insect pathogens unlike chemical insecticides do not have a rapid impact on pest insects and need several days to kill their hosts; during this period the insects may continue feeding and cause damage to crops (Fargues et al., 1994; Hajek, 1989). The inability of mycoinsecticides to kill the insect host rapidly is one of the major factors limiting the utility of these biological control agents for control of pests including locusts and grasshoppers (Bateman & Thomas, 1996).

However, infection with entomopathogenic fungi can produce some control of damage resulting from reduced food consumption (Thomas et al., 1997). Therefore, reduction in food consumption by locusts infected with entomopathogenic fungi reduces the progressive damage and a locust that has stopped feeding could not be considered a significant pest (Moore et al., 1992).

Significant reduction of feeding by the locust *Locustana pardalina* (Walker) (Acrididae), *Schistocerca piceifrons piceifrons* (Walker) (Acrididae) and *Shistocerca gregaria* (Forsk.) (Acrididae) have been demonstrated following infection by *Metarhizium anisopliae* var *acridum* (Arthurs & Thomas, 2000; Hernández-Velázquez et al., 2007; Moore et al., 1992; Seyoum et al., 1994).

Reduction of feeding by the grasshoppers *Rhammatocerus schistocercoides* (Rehn) (Acrididae), *Zonocerus variegatus* (L.) (Pyrgomorphidae) and *Hieroglyphus daganensis* (Krauss) (Acrididae) infected with *M. flavoviride* was observed 2-3 days after inoculation (Faria et al., 1999; Thomas et al., 1997, 1998).

Apart from locusts and grasshoppers, reduction in feeding by insects infected with the fungi *B. bassiana* and *M. anisopliae* has been also reported from other insect orders such as *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), *Megalurothrips sjostedti* (Trybom) (Thysanoptera: Thripidae), larvae of *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae), larvae of *Ocinara varians* (Lepidoptera: Bombycidae) and pea leafminer *Liriomyza huidobrensis* (Diptera: Agromyzidae), (Ekesi et al., 2000; Fargues et al., 1994; Hussain et al., 2009; Migiro et al., 2011., Tefera & Pringle, 2003).

In contrast, Cheung & Grula (1982) reported that no reduction in feeding by larvae of corn earworm (*Helicoverpa zea* Boddie.) infected with *B. bassiana* was observed before death.

Food consumption by infected insects is one of several factors, such as host mortality, that indicate the virulence of a fungal pathogen and need to be evaluated to measure the pathogenicity of the fungus. (Ekesi & Maniania, 2000; Fargues et al., 1991, 1994; Moore et al., 1992; Ondiaka et al., 2008; Seyoum et al., 1994; Thomas et al., 1997).

In this study, experiments were done to identify the effect of infection by the fungi on the feeding of third instar nymphs of *Uvarovistia zebra* (*Tettigoniidae*) (long horned grasshoppers) by both direct (food consumption) and indirect (faecal production) investigation.

The objectives of these experiments were:

- To identify if the food consumption by infected insects is reduced before they die.
- To identify how long the fungi take to cause any reduction of feeding by infected insects.
- To compare the efficacy of the two fungi in reducing food consumption of *U. zebra*.

3.2. Materials and methods

3.2.1. Source of insects and fungal isolates

Third-instar field collected nymphs of *Uvarovistia zebra* were used for the bioassay. The nymphs were collected from mountainous rangeland of the Alamout region in the north of Qazvin province in Iran. This insect is susceptible to mechanical damage and also shows cannibalistic behaviour during transferring to the laboratory. Collected nymphs were placed in small groups (4-7 nymphs) into plastic bags and placed on ice and then transferred to the

laboratory unharmed. The insects were kept in groups of 15 in cages (45×38×38cm H×L×W) made of aluminium with wire mesh sides in the laboratory. They were fed with fresh lettuce for five days in order to acclimate to the diet of lettuce and laboratory conditions before beginning the experiments.

The isolates *Beauveria bassiana* DEBI 001, and *Metarhizium anisopliae* 715C, obtained from the Iranian “Iranian Research Institute of Plant Protection” were also used in the experiments. The former was isolated by Dr Ghazavi from the soil of a cherry orchard in Fashand region in Tehran, Iran and the latter isolated by Dr Zare from an unknown species of locust.

3.2.2. Culturing of the fungi

Both isolates were maintained on standard potato dextrose agar (PDA) (Merck). Spores were harvested from 2-3 week old surface culture for each fungus by scraping and suspending the inoculum in 1 l liquid medium potato-dextrose-broth (PDB) which had been sterilized for 20 minutes at a temperature of 121 °C. The inoculated solution was shaken (110 rotations a minute) for 5 days at 25 °C to produce conidia. Then two suspensions (PDB) as the primary concentrations of both *B. bassiana* and *M. anisopliae* were prepared. Conidial concentrations were determined using a haemocytometer and were adjusted to 5×10^6 spore/ml for both *B. bassiana* and *M. anisopliae*. Four different concentrations (5×10^3 , 5×10^4 , 5×10^5 , 5×10^6 spore/ml) were then produced from the spore suspension of each fungus to use in the experiments.

3.2.3. Bioassay and experimental methods

The experiments were arranged as a completely randomized design with five treatments (four conidial concentrations 5×10^3 , 5×10^4 , 5×10^5 , 5×10^6 spores/ml and water as a control). Each treatment involved 45 third-instar nymphs of the locust divided in three replicates (Three cages). The experiments were done in a laboratory at 25-30 °C with 75%-80% relative humidity.

The conidial suspensions and control (distilled water) treatments were sprayed on the insects using a hand held sprayer with a flow rate of 70 ml/min adjusted by appropriate nozzles (Kassa *et al.* 2004). The sprayer was calibrated so that 160 ml of each concentration was used for 3 replicates (45 insects). Thus each insect was directly and individually sprayed with about 3.5 ml of the appropriate concentration while they stood on filter paper. After 15 min the treated insects were transferred to the cages. Control groups received the same rate of

water without conidia. The insects in each replicate were fed on diet of lettuce using 35 g fresh lettuce every 48 hour.

Feeding was assessed by measuring food consumption and monitoring faecal production. At the beginning of the experiment, a sample of lettuce was dried to determine its dry weight percentage. For this purpose, some different parts of mature and young leaf of the lettuce were broken into small pieces and mixed with each other. Then 7g fresh weight from the mixed lettuce was taken and placed into an oven adjusted to 95°C for about 17 hours (the sample was left in the oven until it reached a constant weight). Food consumption of insects for a 48 h period was measured before the insects were inoculated. Afterwards, every 48 h the uneaten lettuce from each cage was replaced with fresh lettuce and dried at 95°C until it reached a constant weight to get the dry weight percentage of the uneaten lettuce. The faeces produced from each cage were also collected every 48 h and dried at 95°C until reaching constant weight.

Mortality caused by each treatment was recorded every 48 h until day 12 of the experiment. The data obtained were analyzed by analysis of variance (ANOVA). Multiple comparisons were used to determine significant differences between means of infected and control locusts at $P < 0.05$ (Tukey test). Comparisons between the two fungi were made using the t-test.

3.3. Results

Food consumption, and faecal production by the insects treated with different concentrations of the fungi were analyzed. In both *B. bassiana* and *M. anisopliae*, the greatest reduction in feeding was observed by insects infected with the highest spore concentration (5×10^6 spores/ml) (Table 3.1). Reduction in feeding of the infected insects started 2 days after treatment, but a significant reduction in feeding was not observed until 3-4 days after treatment (Figure 3.1). A significant reduction of food consumption ($F_{(4, 10)} = 16.22$, $P < 0.05$) and faeces production ($F_{(4, 10)} = 14.57$, $P < 0.05$) were observed between the insects treated with the highest concentration (5×10^6 spores/ml) of *B. bassiana*, and other concentrations. There were also significant differences in feeding ($F_{(4, 10)} = 52.65$, $P < 0.05$) between insects treated with the highest concentration of *M. anisopliae* and other concentrations. Reduction in faeces production by the insects treated with *M. anisopliae* were observed as well, but a significant difference ($F_{(4, 10)} = 32.52$, $P < 0.05$) was observed between the highest concentration and three other concentrations of 5×10^3 , 5×10^4 spores/ml and control (Table 3.1).

No significant differences in reduction of food consumption ($t_{(4)} = 1.75$, $P = 0.1541$), faeces production ($t_{(4)} = 0.93$, $P = 0.4044$) and mortality ($t_{(4)} = 0.70$, $P = 0.5185$) were observed between the fungi at the same concentration of 5×10^6 spores/ml.

In comparison with control insects, the treated insects with 5×10^6 spores/ml of *B. bassiana* and *M. anisopliae* showed 60% and 63% reduction in mean food/insect respectively.

However, there was no significant difference between food consumption in the control and 5×10^3 spores/ml treatment of both fungi.

Mean food consumption every 48 h during the experiment by insects treated with the highest concentration (5×10^6 spores/ml) has been shown in Figure 3.1. The greatest reductions in feeding by insects treated with concentration 5×10^6 spores/ml of *M. anisopliae* and *B. bassiana* were recorded at 6 and 8 days after treatment respectively. Also, in comparison with the first 48 h, after 8 days the mean food consumption by insects was reduced by 52% and 56% when treated with *B. bassiana* and *M. anisopliae* respectively.

Table 3.1. Mean food consumption and faeces production (mg dry weight) measured every 48 h over 12 days, by 3rd instar *Uvarovistia zebra* treated with different concentrations of *B. bassiana* and *M. anisopliae*.

Treatment (spores/ml)	<i>Beauveria bassiana</i>		<i>Metarhizium anisopliae</i>	
	Food/insect (mg dry weight) Means \pm SE	Faeces/insect (mg dry weight) Means \pm SE	Food/insect (mg dry weight) Means \pm SE	Faeces/insect (mg dry weight) Means \pm SE
Control	83.41 \pm 5.71 ^{ab}	71.73 \pm 4.02 ^a	80.47 \pm 2.74 ^a	63.56 \pm 2.88 ^a
5 \times 10 ³	87.57 \pm 3.95 ^a	68.82 \pm 6.55 ^a	70.14 \pm 0.86 ^a	52.58 \pm 3.01 ^a
5 \times 10 ⁴	59.36 \pm 5.53 ^b	47.78 \pm 4.68 ^a	4.92 ^b	34.76 \pm 3.34 ^b
5 \times 10 ⁵	69.04 \pm 8.08 ^{ab}	55.40 \pm 6.68 ^a	57.30 \pm 2.02 ^b	30.73 \pm 4.20 ^{bc}
5 \times 10 ⁶	33.09 \pm 1.56 ^c	22.32 \pm 3.04 ^b	29.99 \pm 0.82 ^c	19.15 \pm 1.52 ^c

Mean followed by the same letter are not significantly different.

For each fungus (N= 90, n = 15)

Table 3.2 shows the percent mortality recorded every 48 h. The greatest mortality by insects infected with the concentration 5 \times 10⁶ spores/ml of *B. bassiana* and *M. anisopliae* were recorded at day 8 and 12 respectively. Table 3.2 also shows that the cumulative percent mortality of the locust treated with 5 \times 10⁶ spores/ml of *B. bassiana* and *M. anisopliae* reached 58% and 56% respectively at the end of the experiment. Significant differences in mortality were found between the highest concentration (5 \times 10⁶ spores/ml) and the other treatments of each fungus (*B. bassiana*: $F_{(4, 10)} = 129.80$, $P < 0.05$; *M. anisopliae*: $F_{(4, 10)} = 108.00$, $P < 0.05$) (Table 3.3).

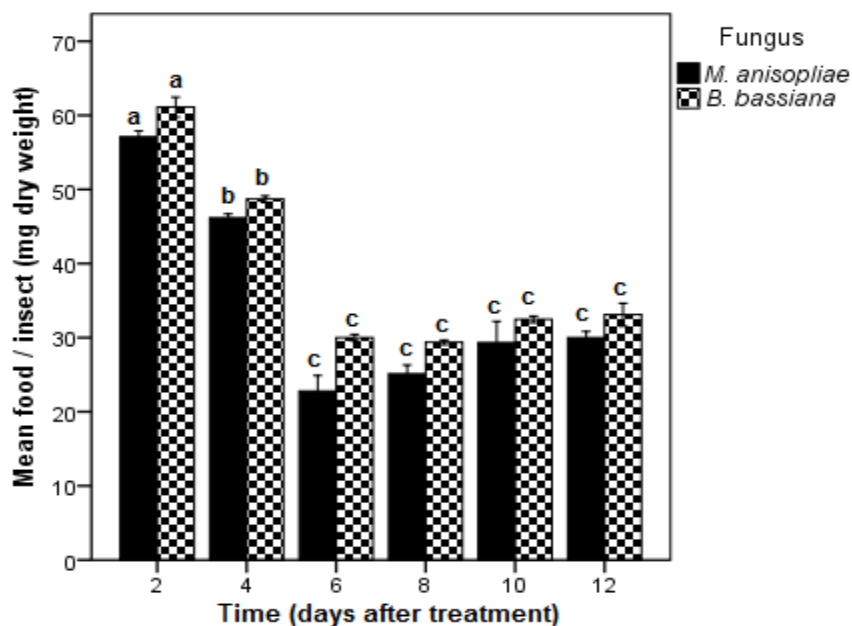


Fig 3.1. Mean (\pm SE) food consumption / insect ($n = 45$ nymphs within 3 replicates) by insects treated with concentration 5×10^6 spores/ml *B. bassiana* and *M. anisopliae*. Mean followed by the same letter shows that there are no significant differences in feeding between the days.

Table 3.2. Percent mortality of insects treated with highest concentration (5×10^6 spores/ml) recorded every 48 h after treatment.

Mortality of insects treated with 5×10^6 spores/ml shown as a percentage of the numbers starting the experiment.						
Days	N1	N2	<i>B.bassiana</i>	N1	N2	<i>M. anisopliae</i>
2	45	45	0 ^a	45	45	0 ^a
4	45	42	6.66 ^{ab}	45	44	2.22 ^a
6	42	39	6.66 ^{ab}	44	42	4.44 ^a
8	39	30	20.00 ^c	42	36	13.33 ^b
10	30	23	15.55 ^{bc}	36	30	13.33 ^b
12	23	19	8.88 ^{ab}	30	20	22.22 ^c
Cm			57.7%			55.5%

N1 = number of surviving insects at the start of the day.

N2 = number of surviving insects at the end of that 48 h period.

Cm = cumulative mortality

Mean followed by the same letter are not significantly different.

Table. 3.3. Mean mortality caused by each fungal concentration at the end of experiment.

	<i>B. bassiana</i>	<i>M. anisopliae</i>
Treatment (spores/ml)	Mean mortality	Mean mortality
Control	0 ^a	.33 ^a
5×10 ³	1.33 ^{ab}	1.67 ^a
5×10 ⁴	2.00 ^b	1.67 ^a
5×10 ⁵	7.00 ^c	6.33 ^b
5×10 ⁶	8.67 ^d	8.33 ^c

N= 45 insects within 3 replicates for each treatment

3.4. Discussion

Although *B. bassiana* and *M. anisopliae* unlike chemical insecticides do not have a speedy effect, the reduction in food consumption by the locusts can be considered as an advantage in pest control. As infection with entomopathogenic fungi leads to a reduction in feeding 2-3 days after treatment it can contribute to the overall effects of the slow acting fungus. The present study showed that a reduction of feeding in nymphs of *U. zebra* infected with the highest concentration (5×10^6 spores/ml) started 2 days after inoculation (Table 3.1), but a significant reduction in feeding comparing the same conidial concentration with control was observed 3-4 days after treatment for both fungi. The results obtained from the present study agree with findings by Moore et al. (1992) and Hernández-Velázquez et al (2007) demonstrating a significant reduction in the desert locust and *Schistocerca piceifrons piceifrons* feeding 3 days after application of *M. anisopliae* var *acridum* respectively.

Significant mortality in locusts was observed 6 and 8 days after infection with *B. bassiana* and *M. anisopliae* respectively. This demonstrates that food consumption of third instar nymphs of *U. zebra* infected with *B. bassiana* DEBI 001 and *M. anisopliae* 715C was reduced before they die due to the fungal infection.

There was no statistical difference in mortality of insects treated with the fungi at the same concentration (5×10^6 spores/ml), but *Beauveria bassiana* caused more mortality than *M. anisopliae*. In contrast, at the same concentration, reduction in feeding by the insects treated with *M. anisopliae* was more than those treated with *B. bassiana*, but again with no statistical difference.

As can be seen in Table 3.1, the solutions (treatments) containing less than 5×10^6 spores/ml did not have a notable effect reducing food consumption by insects. Reduction in feeding due to fungal infection may affect body fat accumulation at sexual maturity and consequently reproductive potential of infected, but surviving insects. Effect of infection by *B. bassiana* and *M. anisopliae* on the oviposition of locust and other insect hosts have been investigated in some studies (Arthurs & Thomas, 2000; Migiro et al., 2011; Ondiaka et al., 2008). Accordingly, reduction in feeding of insects infected by entomopathogenic fungi can affect their population density in the next generation.

In conclusion, reduction in feeding can be observed in *U. zebra* infected by the fungi; consequently, the insect damage decrease. However, in the case of very high population, reduction of food consumption resulting by the fungi may not be consequent to decrease of the damage caused by locusts.

Achieving high population of the grasshopper *Rhammatocerus schistocercoides*, the reduction of food following application of *M. flavoviride* would not be sufficient to prevent economic damage (Faria et al., 1999).

Chapter 4. Pathogenicity of the isolates *Beauveria bassiana* and *Metarhizium anisopliae* on different instars nymphs of the Sunn pest *Eurygaster integriceps* Puton (Heteroptera: Scutellaridae)

Abstract

The aim of this study was to assess the pathogenicity of isolates of *Beauveria bassiana* DEBI 001 and *Metarhizium anisopliae* 715C applied to different nymphal instars and adults of Sunn pest, *Eurygaster integriceps*. Insects were immersed in conidial suspension (5×10^7 spores/ml) for 5 s. Mortality was recorded daily for 14 days while insects were maintained on fresh wheat ears and stems. Both fungi had pathogenic effects on *E. integriceps*. *Beauveria bassiana* was more effective than *M. anisopliae* with significant differences between the fungi observed in all nymphal instars and adults. Fourth and fifth-instar nymphs were significantly more susceptible than adult and third-instar nymphs when the insects were infected by *M. anisopliae*. However, no significant difference was observed in the susceptibility of fourth, fifth-instar nymphs and adults when the insects were infected by *B. bassiana*. The fifth-instar nymph was the most susceptible stage with 60% and 46% mortality caused by *B. bassiana* and *M. anisopliae* respectively.

Key words: *Beauveria bassiana*, *Metarhizium anisopliae*, Su pest, *Eurygaster integriceps*, instars nymphs, pathogenicity

4.1. Introduction

Sunn pest *Eurygaster integriceps* Puton is the key insect pest of wheat and barley in Iran and in a wide area of the Near and Middle East, West Asia and North Africa, it also is found in Eastern and South Europe (Kazzazi et al., 2005; Radjabi, 2000).

Sunn pest feeds on both vegetative stages of the host plants and maturing grain. Overwintered adults attack the leaves and stems of young, succulent wheat and barley, causing them to wither and die prior to ear formation. Nymphs and new-generation adults feed on grains. Feeding insects inject a toxic enzyme into the grains which destroys the gluten and reduces the baking quality of the flour (Hariri et al., 2000; Javaheri, 1995). Grain attacked by Sunn pest also shows reduced germination (Critchley, 1998; Cseresnyes, 1972; Genc et al., 2008; Koksel et al., 2009; Paulian and Popov, 1980).

A comprehensive understanding of Sunn pest's biology is necessary for management of its population. There have been many studies of Sunn pest's life history in different parts of Iran (e.g. Afshar, 1933; Davatchi, 1954; Salavatian, 1991), the most detailed of which was provided by Radjabi (2000). Sunn pest is a univoltine insect with two different phases in the life cycle. The first phase (growth and development) is spent in wheat fields during the spring, and the second phase (late adult and diapause) is spent in obligatory diapauses in grassland at high altitudes during the summer, autumn and winter (Radjabi, 2000). However, the life cycle of this insect can vary with geographical location.

The most important damaging stage in the life cycle of Sunn pest is the nymphal (after the third instar) and adult period when the insects feed most, before migration.

High crop losses caused by Sunn pest can be seen over the 15 million hectares affected by these insects annually (Critchley, 1998; Jahavery, 1995; Miller & Morse, 1996; Moore & Edgington, 2006). At present, chemical control is the main method of crop protection using spray applications of the organophosphorus insecticide, fenitrothion (O,O-dimethyl O-4-nitro-m-tolyl phosphorothioate) and the pyrethroid insecticide, deltamethrin ((*S*)- α -cyano-3-phenoxybenzyl (1*R*)-*cis*-3-(2,2-dibromovinyl)-2,2dimethylcyclopropanecarboxylate). Other insecticides such as fenthion (O,O-dimethyl O-4-methylthio-m-tolyl phosphorothioate) and trichlorfon (dimethyl (*RS*)-2,2,2-trichloro-1-hydroxyethylphosphonate) may also be used (Mossalinejad et al., 2002). In Iran, since the 1970's, the organophosphorus insecticide fenitrothion was used extensively against Sunn pest (Alizade et al., 2010). In 2005, about 1.8 million hectares in Iran alone was treated against Sunn pest (Moein Namini, 2006). The yearly cost of chemical insecticides used for control of Sunn pest in Eastern Europe and west and central Asia, is estimated at US\$40 million (El Bouhssini et al., 2002). Apart from the high cost of chemical control, human health, pest resistance and environmental contamination are important issues. Consequently the present reliance on chemical control must be replaced with integrated pest management (IPM) approaches including the applications of more environmentally friendly biological control agents against Sunn pest. Natural biocontrol is a key factor regulating the Sunn pest population (Critchley, 1998; Iranipour et al., 2010a; Kivan & Kilic, 2002, 2004a, b; Kutuk et al., 2010; Miller & Morse, 1996; Radjabi, 2000, 2007) so the conservation of biological control agents, and / or the production and use of them directly, could be effective ways to reduce the use of chemical insecticides and its negative consequents.

Developing mycoinsecticide products based particularly on *Beauveria bassiana* is one possibility to control the Sunn pest. Comparing some fungal isolates, high virulence of several isolates of *B. bassiana* and one of *M. anisopliae* against Sunn pest have been reported (Parker et al., 2003; Talaei & Kharazi-Pakdel, 2002).

These authors also reported that over 50% of the 221 fungal isolates obtained from collections of Sunn pest in different countries were *B. bassiana* or *Beauveria* sp. Several fungal isolates have shown potential to control the Sunn pest when they were tested in laboratory conditions and during preliminary field work (El-Bouhssini et al., 2002).

Talaei et al. (2009) investigated the susceptibility of different parts of Sunn pest adult body (antennae, tarsi, ventral abdomen, pronotum and total body) to *B. bassiana*. The authors reported a significant difference in mortality among the treatments, ranging from lowest (16.6%) for the pronotum treatment, to highest (48.9%) for the total body.

Abdulahi et al. (2010) characterized eleven isolates of *B. bassiana* isolated from Sunn pest and cultured under different temperature regimes. The authors found differences in virulence of the isolates causing 86-100% mortality to adult Sunn pest.

A study was done by Zibae & Bandani, (2009) to investigate the physiological mechanisms of *B. bassiana* infecting Sunn pest. Both subtilisin-like (Pr1) and trypsin-like (Pr2) cuticle degrading proteases were produced by *B. bassiana* and the former had better activity in degrading insect cuticle than the latter (Zibae & Bandani, 2009a).

Mortality in field-treated Sunn pest sprayed with *B. bassiana*, ranged from 47.6% for insects exposed only to the fungus spray, 74.2% for unsprayed insects exposed to treated wheat and 86.4% for insects receiving direct spray and being exposed to treated wheat (Edgington et al., 2007).

A study by Zibae & Bandani, (2009b) confirmed the insecticidal potential of five different type biopesticides and insect growth regulators (*B. bassiana* secondary metabolites, *Artemisia annua* extract, Buprofezin, pyriproxyfen and metoxyphenozone) on adult of Sunn pest. The authors reported that the highest percentage mortality (100%) was observed in *B. bassiana*, *A. annua* extract, and Buprofezin treatments.

The objectives of this study were

- To assess the effect of the isolates *B. bassiana* DEBI 001, and *M. anisopliae* isolate 715C against *E. integriceps*.
- To evaluate the relative susceptibility of different instar nymphs and adult to the fungi.

4.2. Materials and methods

4.2.1. Source of insects

The Sunn pest nymphs (third, fourth and fifth instar nymphs) and adults were collected from wheat fields in Qazvin, Iran using a sweep net. Collected insects were grouped according to their nymphal instar, based on their size, colour and morphology, and placed in ventilated plastic containers (30×15×8 cm L×W×H) covered with fabric mesh. The insects were maintained at $27 \pm 2^\circ\text{C}$ with 70 - 75% RH and 16:8 L:D on wheat stems and ears which were replaced every day with fresh ones. Insects were kept in these conditions for 3 days to acclimate to the laboratory conditions before beginning the bioassay.

4.2.2. Source, culturing and providing of fungal suspension

The isolates of *Beauveria bassiana* DEBI 001 and *Metarhizium anisopliae* 715C obtained from the “Iranian Research Institute of Plant Protection” in Tehran, Iran were cultured on Potatoes dextrose agar (PDA) plates and incubated at $25 \pm 1^\circ\text{C}$. Conidia of the isolates were gently scraped from the surface of two weeks old cultures and suspended into a tube containing distilled water and 0.02% Tween 80[®] solution (MERCK, Germany). The suspension was stirred and filtered through a single layer of linen to remove culture debris and mycelia. Conidial concentrations were estimated using a haemocytometer with a light microscope and were 8×10^7 and 5×10^7 spores/ml for *B. Bassiana* and *M. anisopliae* respectively. The concentrations in the primary solution were adjusted to 5×10^7 spores/ml by dilution with distilled water and Tween and then used in the experiments. Viability of the spores for both isolates was also determined on PDA plates before being used in the experiments. Spores with a viability of more than 90% (after 24 h) were used for the bioassay.

4.2.3. Bioassay

Susceptibility to the isolates *B. bassiana* and *M. anisopliae* of the different instars nymphs and adult of the Sunn pest was evaluated. The bioassay experiments were arranged as a completely randomized design with four treatments (third, fourth, fifth nymphal instars and adults) repeated four times (replicates). Each replicate comprised 10 insects placed in one of the mesh covered, plastic containers mentioned above. The experiments were done for both fungal isolates separately under the same laboratory conditions and using the same conidial concentration.

To evaluate the pathogenicity of the entomopathogenic fungi nymphs and adults were inoculated using a conidia concentration of 5×10^7 spores/ml containing 0.02% Tween 80® solution. The insects were treated by immersing them in the appropriate conidial suspension for 5 s and placing them on filter paper for 15 min before transferring to the plastic containers described above. The same numbers of insects were treated with distilled water containing 0.02% Tween 80® as the control for each treatment.

After 24 hours, mortality was recorded daily for 12 days while insects were maintained on fresh wheat stems and ears. Dead insects were removed and incubated at $26 \pm 1^\circ\text{C}$ to confirm the presence of fungal mycelia. Analysing data for comparison of the treatments, the mortalities were converted to percentages and adjusted for control mortality, using Abbott's formula. However original numbers of surviving and dead insects were used for survival analysing without adjusting for control mortality.

4.2.4. Statistical analysis

Data on percent mortality of Sunn pest at the end of experiment were transformed (arcsine) and analyzed using analysis of variance. Means were compared by the Tukey-test and considered to be statistically different at the 5% significance level. Student's t-test was used to compare the pathogenicity of the two fungi. SPSS statistical package 18.0 was used for analyses. The median survival time (MST) for each treatment was estimated using the Kaplan-Meier survival analysis.

4.3. Results

Both fungal isolates showed pathogenicity against Sunn pest with consequent death of the insects. Mycelia retrieved from dead insects confirmed that the insects died of mycosis (Figure 4.1).



Fig. 4.1. *Eurygaster integriceps* with *B. bassiana* mycelium.

Comparison of susceptibility among treatments showed no significant difference in percent mortality at the end of experiments between fourth, fifth instar nymphs and adults infected by *B. bassiana* ($F_{(3,12)} = 5.56, P > 0.05$) although third instar nymphs were less susceptible. Similarly there was no significant difference in the mortality of fourth and fifth instar nymphs infected by *M. anisopliae* ($F_{(3,12)} = 9.15, P > 0.05$) and both were more susceptible than third instars and adults (Figure 4.2 A& B).

Comparing the efficacy of the fungi, *B. bassiana* was more effective than *M. anisopliae* against all nymphal instars and adults and these differences were statistically significant (third instar: $t_{(6)} = 2.98, P < 0.05$; fourth instar: $t_{(6)} = 2.59, P < 0.05$; fifth instar: $t_{(6)} = 2.92, P < 0.05$; adult: $t_{(6)} = 4.42, P < 0.01$). Maximum daily mortality for all treatments treated by *B. bassiana* and *M. anisopliae* were observed 7 and 8 days after treatment respectively.

Percent survival of insects in each day during the experiment has been shown graphically (Figure 4.3 A&B).

The median survival time (MST) for adult, fourth and fifth-instar nymphs treated by *B. bassiana* which showed over 50% mortality were 11, 12 and 10 days after treatment respectively. The highest mortality and lowest MST was observed for fifth-instar nymph treated with *B. bassiana*. Mean and median survival times for both fungi have been shown in table 4.1.

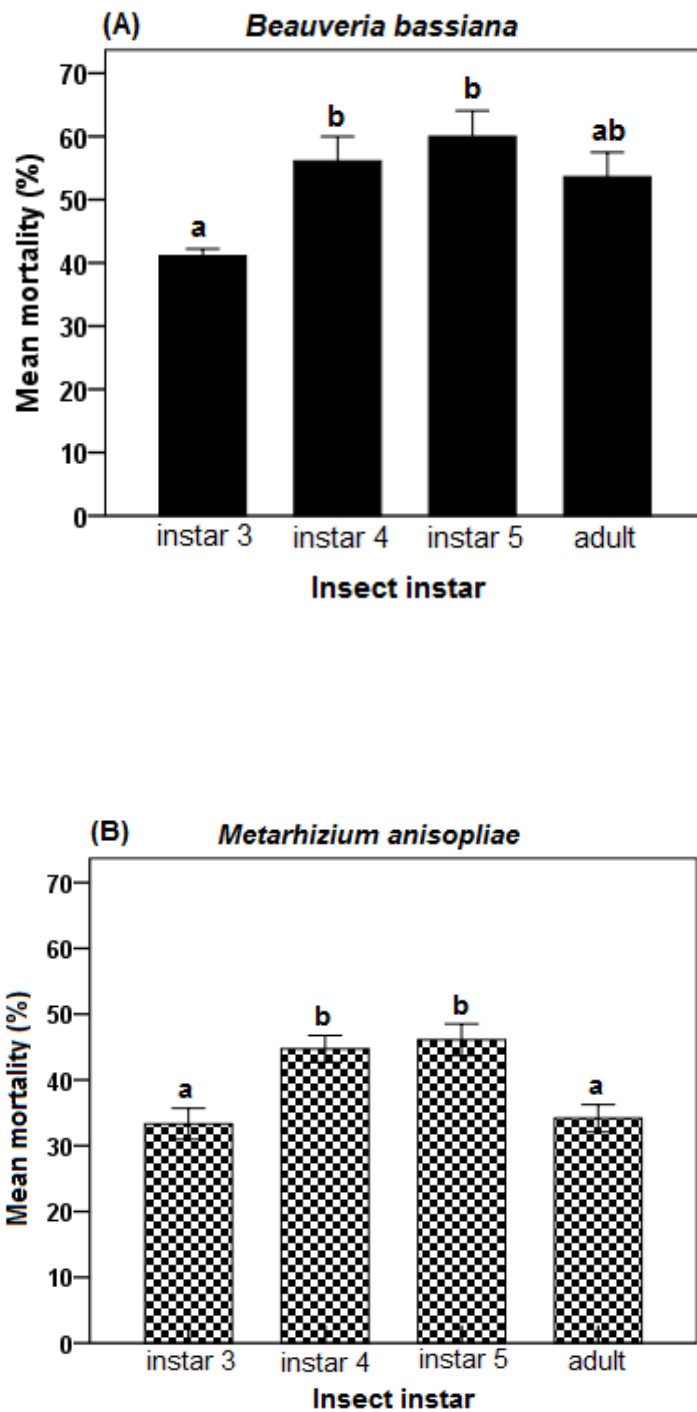


Fig 4.2. Mean percent mortality of different nymphal instars and adults of Sunn pest treated with *B. bassiana* (A) and *M. anisopliae* (B) (5×10^7 spores/ml). Bars with the same superscript letter are not significantly different ($P > 0.05$ Tukey test).

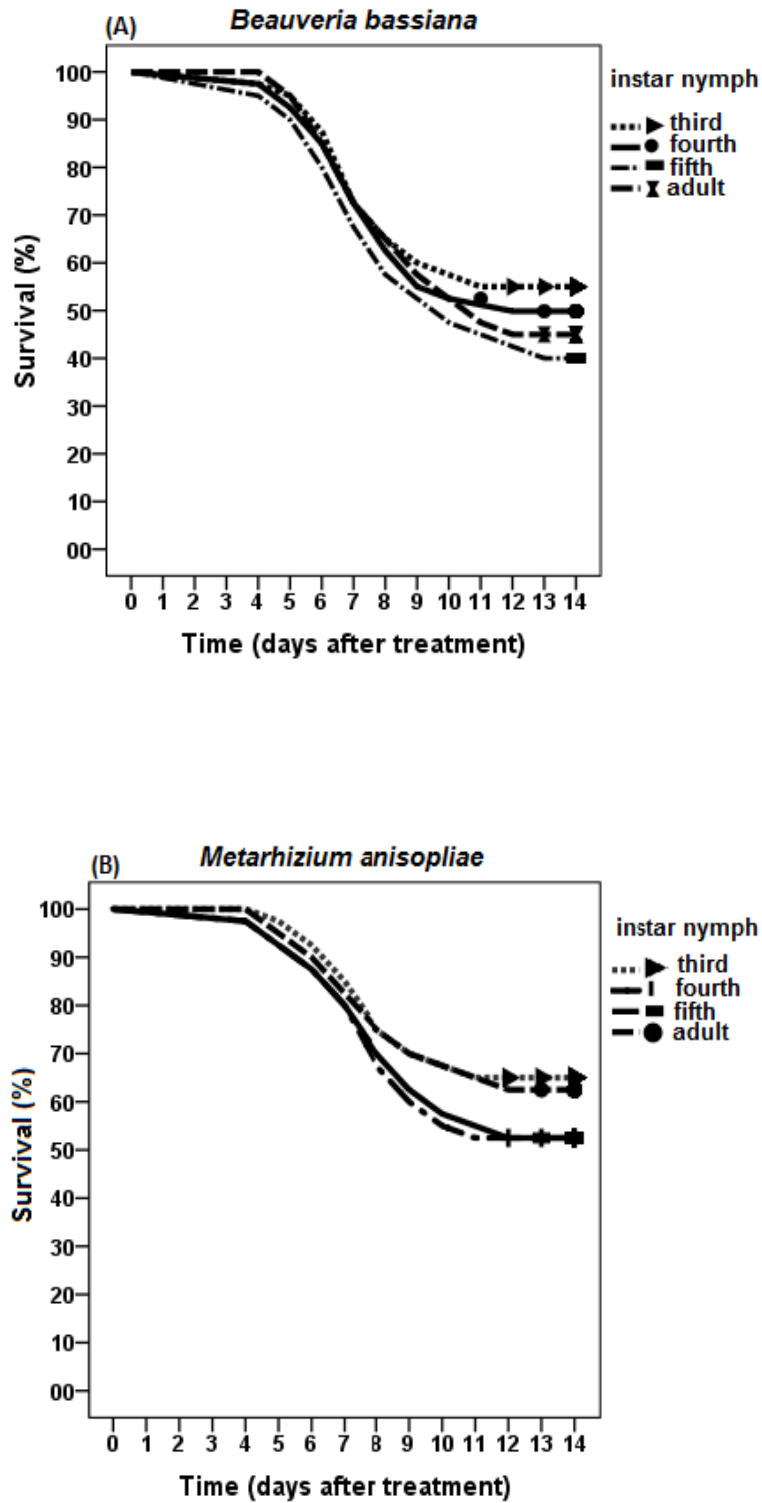


Fig.4.3. Percent survival of different instar nymphs of Sunn pest following inoculation with 5×10^7 spores/ml of *B. bassiana* (A) and *M. anisopliae* (B).

Table 4.1. Mean and median (\pm SE) survival times (in days) of the different life stages of Sunn pest treated with *B. bassiana* and *M. anisopliae* using a conidia concentration of 5×10^7 spores/ml.

Nymphal instar	Mean	Median	Mean	Median
	<i>B. bassiana</i>		<i>M. anisopliae</i>	
third instar	11.00 \pm 0.55	-----	11.82 \pm 0.49	-----
fourth instar	10.69 \pm 0.56	12 \pm 0.00	10.97 \pm 0.54	-----
fifth instar	10.17 \pm 0.56	10 \pm 1.89	11.07 \pm 0.53	-----
Adult	10.65 \pm 0.53	11 \pm 1.89	11.70 \pm 0.50	-----

N = 40 within 4 replicates

4.4. Discussion

Among the entomopathogenic fungi isolates of *B. bassiana* have often been evaluated against Sunn pest and it is the species most commonly associated with *E. integriceps*.

However, numerous studies indicate that Sunn pest is susceptible to infection by diverse species of entomopathogenic fungi (Edgington & Moore, 2005; ICARDA Annual Report, 2003; Moore & Edgington, 2006; Parker et al., 2003; Skinner et al., 2004).

The present study showed that *Beauveria bassiana* DEBI 00 was more pathogenic to all stages tested than *Metarhizium anisopliae* 715C. The former caused 60% mortality to the most susceptible nymphal instar (fifth-instar nymphs), whereas the latter only caused 46% mortality to the same instar by the end of experiment. This may be because neither of the fungi was isolated from *E. integriceps*.

In comparison with other studies using strains isolated from Sunn pest, less mortality was observed in the present study. Tanada & Kaya (1993) suggested that the strains of fungi isolated from specific hosts are more virulent for that host than strains isolated from another host. None of the isolates used in the present study were isolated from Sunn pest. *Metarhizium anisopliae* 715C was isolated from a grasshopper and *B. bassiana* DEBI 001 was isolated from soil.

As can be seen in Figures 4.2(B), fifth instar nymphs were most susceptible to infection by *M. anisopliae*, however third instar nymphs and adults were the least susceptible to the fungus. In regard to the nymphs and adults infected by *B. bassiana* (Figure 4.2 A), third-instar nymphs once again were least susceptible.

Adult insects were less susceptible than fourth and fifth instar nymphs and this agrees with observation of Haji Allahverdi Pour et al. (2008) that demonstrated fifth instar nymphs of Sunn pest were more susceptible to *B. bassiana* than adults.

Relative susceptibility of different development stages of a host depends on the host species and on the fungal isolate (Ferron, 1985). For example adults of *Blattella germanica* (L.) (Blattodea: Blattellidae) were more susceptible to *M. anisopliae* infection than nymphs (Lopes and Alves, 2011). According to Romaña & Fargues (1992) the older larvae of *Melolontha melolontha* (L.) (Coleoptera: Scarabaeidae) were clearly more susceptible to *Beauveria brongniartii* than the younger larval instars. In contrast the susceptibility of *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) larvae to *B. bassiana* decreased with age (Fargues, 1991).

The differential susceptibility observed in insects infected by entomopathogenic fungi, could be related to moulting by the nymphal stages, which is relevant when the pathogen is inoculated immediately prior to ecdysis or when the time interval between sequential moults is short (Ekesi and Maniania, 2000; Vestergaard et al., 1995; Vey and Fargues, 1977). However, according to the results of the present study high doses and long periods are required for the isolates to cause satisfactory levels of mortality.

Chapter 5. Effect of oil and water bait formulations on the efficacy of *Beauveria bassiana* and *Metarhizium anisopliae* against *Uvarovistia zebra*

Abstract

The effects of two bait formulations (oil with spores on lettuce discs and water with spores on lettuce discs) of *B. bassiana* and *M. anisopliae* on the grasshopper *Uvarovistia zebra* were assessed. Third-instar nymphs of *U. zebra* were fed on lettuce inoculated with different spore concentrations formulated either in oil or water. Both oil and water formulations had lethal effects on insect nymphs. For both fungi, the spores formulated in oil on lettuce discs were more effective against insect nymphs than those formulated in water. At the highest concentration (5×10^6 spores per lettuce disc), *B. bassiana* caused 55% and 35% mortality using oil and water formulations respectively. *Metarhizium anisopliae* caused 61% and 40% mortality of insects treated with the same spore concentration formulated in oil and water respectively.

Key words: *Metarhizium anisopliae*, *Beauveria bassiana*, *Uvarovistia zebra*, bait formulation

5.1. Introduction

Numerous and diverse isolates of both *B. bassiana* and *M. anisopliae* have been tested for potential control of locusts and grasshoppers using different methods and formulations (Alves et al., 1998; Douthwaite et al., 2001; Driver et al., 2000; Hunt et al., 1994; Hunter, 2005; Ignoffo et al., 1997; Moore et al., 1993; Shah et al., 1998; Shapiro 1989, 1992, Shapiro & Robertson, 1990). Some microbial control agents, such as *Nosema locustae* Canning, nematodes, entomopoxviruses and fungi have been formulated in baits for use against locusts and grasshoppers due to the requirement for ingestion or protection against adverse environmental conditions (Caudwell, 1993). Although, entomopathogenic fungi do not need to be ingested to cause infection they are highly susceptible to low humidity, high temperature, UV radiation and other adverse environmental conditions. Formulations such as baits can protect the fungal spores from these environmental conditions. Bait formulation also encourage insect feeding and prevent the loss of spores.

The quantity of mycopesticide required to control grasshoppers can be reduced using bait formulations (Ewen & Mukerji, 1987; Johnson & Henry, 1987; Mukerji et al., 1981). Apart from control of pests in the field, bait formulations are often used in bioassays as the best method to test the efficacy of entomopathogenic fungi as they avoid interactions with the characteristics of insects which make topical application difficult. These characteristics include the hydrophobic cuticle, mobility, small size of early-instar nymphs or larvae and their susceptibility to mechanical damage during handling (Inglis et al., 1996). Consequently baits formulated as either oil+conidia or water+conidia are often used for bioassay, and

oil+conidia formulations are more frequently used. Topically applied oil formulations can be toxic to insects (Goettel & Johnson, 1992), however the oil+conidia method minimises the toxic effects caused by oil (Inglis et al., 1996). Entz et al. (2008), applied oil formulations of various isolates of *M. anisopliae* to infect three grasshoppers species *Melanoplus sanguinipes* (Fabricius), *M. bivittatus* (Say) and *M. packardii* (Scudder) using ingestion of lettuce-leaf wafers (0.7 cm diameter), to identify the lethal effect and LT_{50} of the isolates.

The formulation of entomopathogenic fungi as baits against tettigoniid grasshoppers has not been studied previously. To develop such baits an understanding of the efficacy of bait formulations on the target insect is necessary. Therefore the objectives of this study were:

- To determine the efficacy of *B. bassiana* and *M. anisopliae* in two formulations (oil and water) and on a bait substrate (lettuce) against the tettigoniid grasshopper *Uvarovistia zebra*.
- To compare the pathogenic potential of the fungi when they were used as a bait with two formulations.

5.2. Materials and methods

5.2.1. Insects and fungal isolates

Second and third- instar field collected nymphs of *U. zebra* were placed in groups of 10 in ventilated plastic containers (each 30×15×8 cm L×W×H) fitted with a perforated metal floor to reduce contact with frass. The nymphs were fed with fresh lettuce for five days in order to acclimate to the diet of lettuce and laboratory conditions before beginning the experiments. The isolates *Beauveria bassiana* DEBI 001, and *Metarhizium anisopliae* 715C, obtained from the “Iranian Research Institute of Plant Protection” were cultured on PDA to use in the experiments. The former was isolated by Dr Ghazavi from the soil of a cherry orchard in Fashand region in Tehran, Iran and the latter isolated by Dr Zare from an unknown species of locust.

Spores were gently scraped from 7-10 day old surface cultures using a spatula and suspended in sterile distilled water or oilseed rape (canola) obtained from Agricultural Organisation of Qazvin province. Both suspensions were stirred and filtered through a single layer of linen to remove culture debris and mycelia.

Concentrations of conidia were then estimated using a haemocytometer and conidial densities were adjusted so that 70 μ l contained 5×10^3 , 5×10^4 , 5×10^5 and 5×10^6 spores.

5.2.2. Bioassay

The bioassay experiments were arranged as a completely randomized design with five treatments (four conidia concentrations and control) and four replicates for each treatment. Each treatment comprised 40 insects placed equally in the replicates.

Nymphs (third-instar) were maintained on a diet of lettuce in a controlled environment chamber under 15:9 L:D photoperiod, 35- 55% RH% and 25-30°C temperature regime.

The baits (lettuce discs) were inoculated separately with the spore formulations at the concentrations above. In order to inoculate the baits, 70µl of each suspension was pipetted onto separate 10- mm- diameter lettuce discs. The control treatments consisted of oil and water alone. Treated lettuce discs were left for 15-20 min at ambient temperature to dry and were then presented to insects in individual containers. The insects were maintained in the containers individually and allowed to feed on the discs for 20-24 h; consequently each insect ingested a defined dose of spores. When the last insect had ingested all the bait, they were transferred to plastic containers (30×15×8 cm L×W×H) each containing 10 insects. Insects that did not consume the treated lettuce discs were removed from the experiment. After 24 h, insects mortality was recorded every 48 h and the experiments continued for 14 days while the insects were fed daily with untreated fresh lettuce. Abbot's formula was applied to correct for the percentage mortality in the control (Butt & Goettel, 2000).

Dead insects were removed from the containers, cleaned with cotton wool soaked with 75% ethanol and placed in 9 cm diameter Petri dishes on a sterilized paper and incubated at 29°C, 95% RH. Dead insects that produced mycelia of the fungi after incubation were considered to have died from mycosis. Insect mortality at the end of experiments was analyzed by ANOVA, and treatments were compared using Tukey test. The effect of fungi were compared by t-test.

5.3. Results

Preliminary results showed that the fungal spores on lettuce with either oil or water had a pathogenic effect on *U. zebra*. Comparison of formulations showed a significant (*B. bassiana*: $F_{(9,30)} = 48.03$, $P < 0.05$ and *M. anisopliae*: $F_{(9,30)} = 48.93$, $P < 0.05$) increase in insect mortality when spores of the fungi were applied in oil as opposed to water (Figure 5.1 A & B).

At the highest concentration (5×10^6 spores/insect) using oil, 61.5% and 55% mortality were observed at the end of experiment by *M. anisopliae* and *B. bassiana* respectively. However at the same spores concentration formulated in water the fungi cause 40 and 35% mortality. Significant differences in insect mortality were also observed among the different spore concentrations in both fungi and formulations (*B. bassiana*; water formulation: $F_{(4,15)} = 27.90$, $P < 0.05$ and *B. bassiana*; oil formulation: $F_{(4,15)} = 74.75$, $P < 0.05$) and (*M. anisopliae*; water formulation: $F_{(4,15)} = 27.91$, $P < 0.05$ and *M. anisopliae*; oil formulation: $F_{(4,15)} = 82.09$, $P < 0.05$) (Tables 5.1 & 5.2).

Comparison of the fungal isolates at the highest spore concentration (5×10^6 conidia/insect) showed that although *M. anisopliae* was more effective in both formulations these differences were not statically significant (Spores in water: $t_{(6)} = 1.00$; $P = 0.3559$; Spores in oil: $t_{(6)} = 1.25$; $P = 0.2579$).

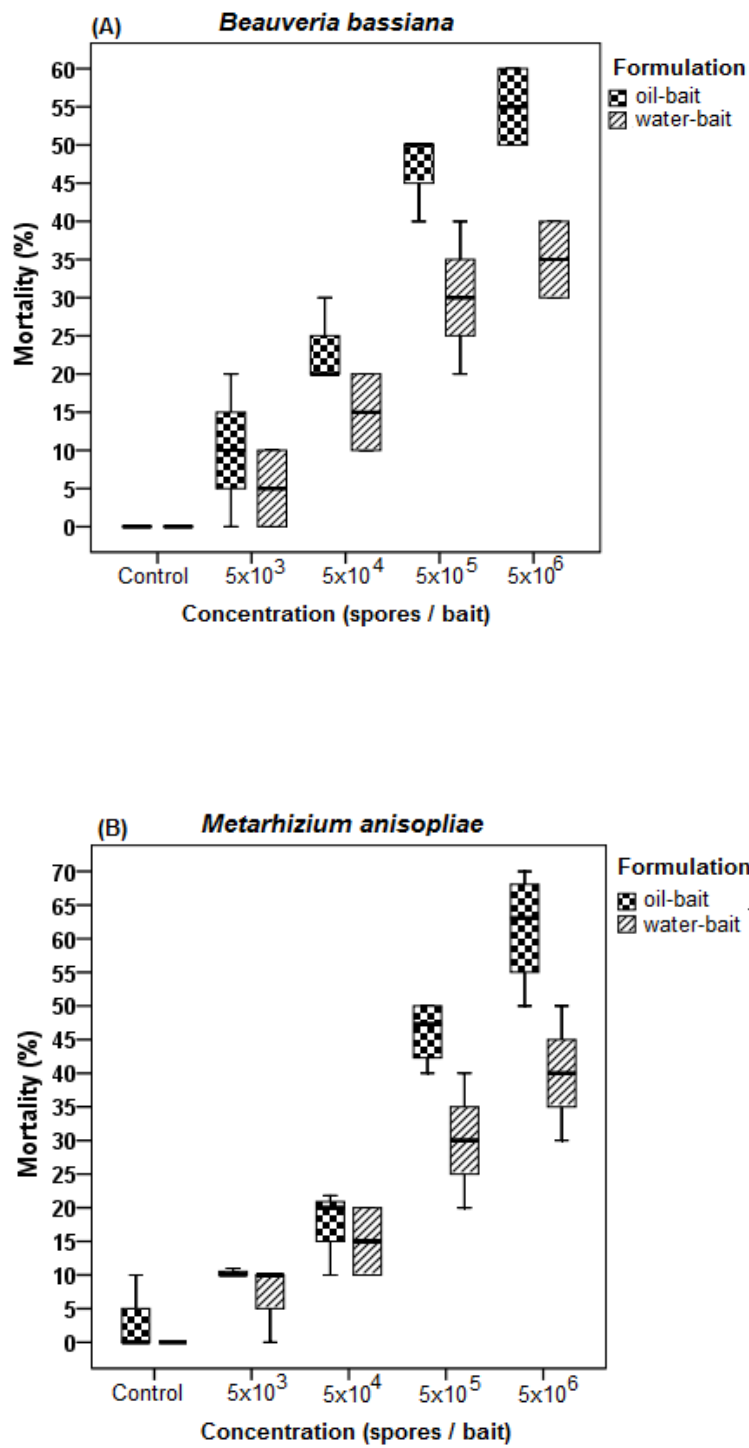


Fig 5.1. Mean (\pm SE) percent mortality (n=40 insects within 4 replicates) of *U. zebra* treated with two different bait formulations of *B. bassiana* (A) and *M. anisopliae* (B) at different concentrations.

Table 5.1. The mean (\pm SE) percent mortality of *U. zebra* fed on lettuce discs treated with *B. bassiana* formulated in oil or water.

Treatment (spores/insect)	<i>B. bassiana</i> Mean \pm SE mortality (%)	
	Spores- oil-lettuce	Spores-water-lettuce
Control	0 \pm 0 ^a	0 \pm 0 ^a
5 \times 10 ³	10 \pm 4.08 ^a	5 \pm 2.88 ^{ab}
5 \times 10 ⁴	22.5 \pm 2.5 ^b	15 \pm 2.88 ^b
5 \times 10 ⁵	47.5 \pm 2.5 ^c	30 \pm 4.08 ^c
5 \times 10 ⁶	55 \pm 2.88 ^c	35 \pm 2.88 ^c

N = 400 insects

n = 40 nymphs within 4 replicates

Means within a column, followed by the same letter are not significantly different at the $P = 0.05$ level.**Table 5.2.** The mean (\pm SE) percent mortality of *U. zebra* fed on lettuce discs treated with *M. anisopliae* formulated in oil or water.

Treatment (spores/insect)	<i>M. anisopliae</i> Mean \pm SE mortality (%)	
	Spores-oil-lettuce	Spores-water-lettuce
Control	2.50 \pm 2.50 ^a	0 \pm 0 ^a
5 \times 10 ³	10.26 \pm 0.24 ^{ab}	7.5 \pm 2.5 ^{ab}
5 \times 10 ⁴	17.95 \pm 2.68 ^b	15 \pm 2.88 ^b
5 \times 10 ⁵	46.15 \pm 2.41 ^c	30 \pm 4.08 ^c
5 \times 10 ⁶	61.53 \pm 4.36 ^d	40 \pm 0 ^c

N = 400 insects

n = 40 nymphs within 4 replicates

Means within a column, followed by the same letter are not significantly different at the $P = 0.05$ level.

5.4. Discussion

Grasshopper *U. zebra* nymphs ingesting bait substrate (lettuce) inoculated with spores in oil or water are susceptible to infection. The formulation of oil-based spores was more effective than the water-based formulation.

Greater efficacy of entomopathogenic fungi spores formulated in oil against acridid grasshoppers and locusts has been demonstrated before. Inglis et al. (1996) found that the formulation of *B. bassiana* (10^5 conidia) spores in oil was more effective (95% mortality) against the grasshopper *Melanoplus sanguinipes* (Fabricius) than those in water (55% mortality). The present study found similar results. Of course, the rate of mycosis and

mortality found in the present study differs naturally from other studies because of the differences in fungal isolates, target insect and spore concentrations used in the studies.

The reasons why spores in oil are more effective than those in water have been described by various authors. Johnson & Goettel (1993) suggested that oil may improve the adhesion of the conidia to the bait, consequently, dislodgement of inocula from the bait substrate is reduced during application. Interestingly the superiority of formulations in oil relative to water were observed in either bait or spray applications. There are some possible advantages in the use of oil, rather than water as a carrier such as infection at lower humidities (Bateman et al., 1993), greater viability (Prior et al., 1988), decreased sensitivity to UV radiation (Inglis et al., 1995; Moore et al., 1993) and stimulated germination (Winder & Van Dyke, 1990).

This study has shown the superiority of oil formulation to water formulation, but the efficiency of this formulation should be tested in field conditions. Formulation of insecticides will be influenced by climatic conditions particularly relative humidity. The utility of formulations also depends upon the species of target insect. Behavioural nutrition, motion activity and biology of the target insect particularly emergence times of the nymphal instars are the determinants of a formulation's success in the field.

In early to middle of spring, when the grasses are abundant, the grasshoppers may have no desire to eat the baits, whether bait is formulated with spores in oil or in water. However, the distribution of plentiful baits at the right time, may cause more sporulation and will provide infection of the insects contacting the baits. In oil formulation the spores coated with oil will more likely survive. These spores also are more capable of entering into the host, because in the presence of oil the lipids in the insect cuticle are replaced with an aqueous phase (Locke, 1984). Use of suitable spores is an important factor to make an effective formulation. Although production of submerged conidia in liquid medium offers several advantages to aerial culture (Jenkins & Goettel, 1997), this type of spore cannot be easily formulated in oil (Jenkins & Thomas, 1997). On the other hand, submerged conidia are more infective in a water-based formulation, which may be related to their faster germination rates (Al-Aidroos & Roberts, 1978; Al-Aidroos & Seifert, 1980; Charnley, 1984; Dillon & Charnely, 1985; Hassan et al, 1983).

Overall, apart from the technical aspects and climate conditions, the use of the formulation must consider costs associated with spore production, and application.

Chapter 6. The viability, mycelial growth and conidia production of *Beauveria bassiana* and *Metarhizium anisopliae* preserved in different storage media

Abstract

The aim of this work was to assess the viability of isolates of *Beauveria bassiana* DEBI 001 and *Metarhizium anisopliae* 715C after 6 months storage in different media; PDA, sand and distilled water. The isolates were stored at $4 \pm 1^\circ\text{C}$ for 6 months. Spore germination, spore production and mycelial growth of the isolates were estimated before and after being stored. A significant decrease in germination was observed after 6 months storage in all media. There were no significant differences in conidia production before and after storage for *B. bassiana* preserved in PDA and sand and for *M. anisopliae* preserved in PDA. The sand and water media also significantly reduced colony growth.

Keywords: Viability, mycelia growth, storage, *Beauveria bassiana*, *Metarhizium anisopliae*

6.1. Introduction

Despite recent advances in the use of entomopathogenic fungi for insect pest control, there are some disadvantages which cause limitation to the use of these fungi. In general, entomopathogenic fungi are inclined to lose their viability and change in colour, growth and morphology when they are preserved or successively sub-cultured in artificial media. The success of entomopathogenic fungi as biocontrol agents depends upon the maintenance of viability by conidia during storage (McClatchie et al., 1994) and germination rate, aggressiveness of the fungus and host specificity (Samson et al., 1988).

The continued viability of entomopathogenic fungal collections is very important to research on fungi assayed for potential biocontrol of insects. Thus, methods of preservation are required to ensure the viability, morphological, physiological, and genetic stability of the fungal cultures over time. According to Gallo et al. (2008), the methods developed for the preservation of fungal isolates are divided in two groups, according to the continued or suspend metabolism of the fungus.

The storage methods include sterile water, sub-cultures in agar, cool storage at temperatures ranging from 5 to 8°C and deep freezing at -20°C or lower. Cool and deep freezing storage have demonstrated success in preserving fungal isolates over long periods. Culture on potato- dextrose agar medium at -70°C has given satisfactory preservation for periods ranging from 6 months to 13 years (Pasarell & McGinnis, 1992).

There have been various studies to describe the phenomena of degeneration, phenotypic instability, conidial viability and virulence attenuation of entomopathogenic fungi when cultured in artificial media (Aizawa, 1971; Fargues & Robert, 1983; Ibrahim et al., 2002; Kawakami, 1960; Morrow et al., 1989; Nagaich, 1973; Ryan et al., 2002; Schaerffenberg,

1964). Some studies reported that successive cultures of entomopathogenic fungi in artificial media cause phenotypic alteration (changes in colour, growth, and morphology) and degeneration that leads to decay in sporulation, metabolites, and virulence of the fungi (Butt et al., 2006; Ibrahim et al., 2002; Nagaich, 1973; Ryan et al., 2002).

The development of mycoinsecticides is based on fungal quality reliant to a great extent on viability, and there are many methods for measuring fungal viability (Goettel & Inglis, 1997). The viability of the entomopathogenic fungi should be evaluated before application since it is an important measure of the quality of the product (Alves et al., 1996). Viability of conidia is normally assessed as the germination capacity of the conidia whereas the virulence of entomopathogenic fungi has been correlated with the velocity of conidia germination (Hassan & Charnley, 1989).

Most studies have analyzed the effect of storage temperatures on the viability of stored fungi. In this study, experiments were done to study the influence of three types of storage media on *B. bassiana* DEBI 001 and *M. anisopliae* 715C preserved for 6 month in the same constant temperature.

The objectives of the study were:

- To quantitatively determine the viability of the fungi.
- To determine spore production (sporulation) and mycelia growth of the fungi.

6.2. Materials and methods

6.2.1. Isolates and storage media

Seven day old cultures of the isolates *Beauveria bassiana* DEBI 001 and *Metarhizium anisopliae* 715C cultured in PDA media were obtained from the “Iranian Research Institute of Plant Protection” in Tehran, Iran to use for experiments. The former was isolated by Dr Ghazavi from the soil of a cherry orchard in Fashand region in Tehran, Iran and the latter isolated by Dr Zare from an unknown species of locust.

The isolates were stored in PDA, sand or distilled water. For this, the conidia were scraped from the surface of Petri dishes (containing the 7 day old culture) using a scalpel blade and they were placed into distilled water. A separate suspension was made for each isolate with a concentration of 5×10^7 spores/ml using sterile distilled water. Three Petri dishes containing PDA medium, three screw-cap vials (27×57 mm diameter × length) containing around 18-20 ml distilled water and three similar vials containing approximately 20-25 grams

sand were used for preservation of each fungus. To preserve the isolates, 0.5 ml of the conidial suspension prepared from the original sample was poured in each storage formulation and kept at $4 \pm 1^\circ\text{C}$. Conidia germination, mycelial growth and sporulation of the fungi were determined as described below before being stored.

6.2.2. Viability assay

6.2.2.1. Assay of viability before preservation

To determine viability before storage, 0.1ml of the conidial suspension of each fungus containing approximately 10^6 spores/ml were spread onto four plates (as replicates) containing a thin layer of potato-dextrose agar (PDA) medium. The plates were incubated at $25 \pm 1^\circ\text{C}$ and the viability of cultures was estimated by microscopically assessing the percentage conidial germination after 24 and 48 hours. The germination rate of each fungus was determined by counting 100 conidia per plate (replicate) selected at random. Conidia were considered to have germinated if the observed germ tube was longer than the conidial diameter (Luz & Fargues 1997) or the length of germ tube was at least half of the conidial length (Vu et al., 2007). The latter criterion was used in the present study to assay the conidial germination.

6.2.2.2. Assay of viability after preservation

The viability of the fungi was also assayed after storage. First the densities of conidia in samples taken from the three different storage media were estimated. For this 1g of each stored formulation of PDA and sand were taken and mixed to make 10 ml with distilled water. Each suspension was stirred using a shaker to break up conidia aggregates and then the suspension was filtered through two layers of muslin to remove mycelia, agar fragments and sand. The stored formulation of water was vigorously stirred to ensure the spores were completely mixed. Afterwards 1 ml from this suspension was taken and made up to 10 ml with distilled water. For each suspension, conidia concentration was determined using a haemocytometer and the resultant conidial suspension adjusted to 5×10^6 spores/ml, using sterile distilled water. Afterwards, 0.1ml of the suspension from each fungus / formulation combination, containing approximately 5×10^5 spores/ml were spread onto four plates (four replicates) containing PDA medium. The plates were incubated at $25 \pm 1^\circ\text{C}$ and germination of cultures was estimated as described in section 6.2.2.1.

6.2.3. Assay of mycelial growth and conidia production

Sporulation of the isolates preserved in different storage media was determined by estimating the density of spores. Each stored isolate was cultured on plates containing PDA medium with four replicates for each storage medium. Conidia concentrations were estimated 5 days (for *B. bassiana*) and 8 days (for *M. anisopliae*) after incubation when the mycelia reached full diameter. Conidia were gently scraped from the surface of each plate into 10 ml distilled water using a razor blade and then the concentrations were estimated using a haemocytometer.

Mycelial growth of both isolates was also investigated before storage and after storage estimating the diameter of the colonies at 72 hours intervals. In order to estimate the mycelial growth the plates containing PDA were inoculated with conidia obtained from each storage formulation (treatments). For this, four plates (four replicates) were considered for each treatment and each plate was inoculated with 50 µl of suspension contained 100 spores placed in the centre of ten cm Petri dishes.

6.2.4. Experimental method and statistical analysis

Experiments were carried out in laboratory conditions and arranged as a completely randomized design. There were four replicates of each treatment.

Viability of the isolates, mycelial growth and conidial production determined before and after storage were compared to each other.

The data were analyzed by ANOVA, and treatment means compared using the Tukey test.

6.3. Results

Germination of both fungal isolates stored in the three storage media declined after 6 months storage. Significant differences (*B. bassiana*: $F_{(3,12)} = 321.19$, $P < 0.01$ and *M. anisopliae*: $F_{(3,12)} = 192.19$, $P < 0.01$) in conidia germination were observed before and after storage in all storage media (Table 6.1 & 6.2). However, there were no significant differences ($P > 0.05$) among the storage media in germination after the storage period.

Results showed that conidia production of *B. bassiana* before storage was significantly ($F_{(3, 12)} = 8.62$, $P < 0.05$) greater than those preserved in water and sand. However there was no significant difference ($P > 0.05$) between conidia production of *B. bassiana* before and after storage when it was stored in PDA (Table 6.3).

With *M. anisopliae* there was no significant difference ($P > 0.05$) between the conidia production before and after storage when fungus was stored in PDA. However there were

significant reductions ($F_{(3, 12)} = 6.9, P < 0.05$) in the number of spores produced after storage using media of sand and water.(Table 6.4).

Data for mycelial growth of *B. bassiana* and *M. anisopliae* has been shown in Table 6.5. The analysis of variance ($F_{(3, 12)} = 25.45, P < 0.05$) showed a significant effect of the sand and water media reducing the mycelial growth of *B. bassiana*. However there was no significant difference ($P > 0.05$) between the mycelial growth before and after storage using PDA medium.

The growth of *M. anisopliae* before storage was also significantly greater ($F_{(3, 12)} = 20.91, P < 0.05$) than after storage in water and sand, but no significant difference was observed before and after storage when the fungus was stored in PDA.

Table 6.1. The mean (± 1 SE) percent germination of *B. bassiana* before and after preservation in different storage media.

Stored formulation	Germination (%) after storage	n	Germination (%) before storage	n
PDA	75.93 \pm 0.41 ^a	4	97.5 \pm 0.45 ^b	4
Sand	77.81 \pm 0.47 ^a	4		
Water	75.87 \pm 0.88 ^a	4		

Means followed by same superscript letter are not significantly different at $P < 0.05$.

Table 6.2. The mean (± 1 SE) percent germination of *M. anisopliae* before and after preservation for 6 months in different storage media.

Stored formulation	Germination (%) after storage	n	Germination (%) before storage	n
PDA	77.18 \pm 0.27 ^a	4	97.56 \pm 0.64 ^b	4
Sand	79.18 \pm 0.9 ^a	4		
Water	77.62 \pm 0.82 ^a	4		

Means followed by same superscript letter are not significantly different at $P < 0.05$.

Table 6.3. The mean (± 1 SE) conidia produced by *B. bassiana* before and after 6 months storage.

Stored formulation	Sporulation after storage (spores/ml $\times 10^6$)	Sporulation before storage (spores/ml $\times 10^6$)
Water	16.5 ^a	43.2 ^c
Sand	21.2 ^{ab}	
PDA	35 ^{bc}	

Means followed by same superscript letter are not significantly different at $P < 0.05$
N= 4 (repeated for times)

Table 6.4. The mean (± 1 SE) conidia produced by *M. anisopliae* before and after 6 months storage.

Stored formulation	Sporulation after storage (spores/ml $\times 10^6$)	Sporulation before storage (spores/ml $\times 10^6$)
Sand	14.6 ^a	33.2 ^b
Water	14.7 ^a	
PDA	32.2 ^b	

Means followed by same superscript letter are not significantly different at $P < 0.05$
N= 4 (repeated for times)

Table 6.5. Mycelial growth of *B. bassiana* and *M. anisopliae* before and after storage

Colony diameter (mm) of <i>B. bassiana</i>			Colony diameter (mm) of <i>M. anisopliae</i>				
Before storage	After 6 months storage		Before storage	After 6 months storage			
	Storage formulation			Storage formulation			
	PDA	Sand	Water	PDA	Sand	Water	
66.4 ^b	65 ^b	50.4 ^a	48.6 ^a	60.7 ^b	58.7 ^b	50 ^a	48.8 ^a

Treatments followed by same superscript letters are not significantly different at $P < 0.05$

6.4. Discussion

For a working mycoinsecticide, producing abundant conidia in an artificial culture media and maintaining its viability during storage are essential. The isolates which have acceptable pathogenicity and can be applied as a mycoinsecticide may be stored for a while. The present study showed a significant decline in conidia germination of both isolates preserved in the storage media, as the viability of the fungi was reduced 18-20% after 6 month storage.

A germination decrease after storage using artificial media may be expected, but it can depend on diverse factors, such as incompatibility with formulants (Alves et al., 1998; Faria et al., 2009; Mohanet al., 2007), temperatures either below or above the optimal range following inoculation on media (Devi et al., 2005; Luz & Fargues, 1997), and long-term storage at above freezing temperatures (Alves et al., 1996; Magalhães & Boucias, 2004; McClatchie et al., 1994; Moore et al., 1995).

Conidia production was less influenced by storage, as there were no significant differences in spore production by *B. bassiana* before and after storage using PDA and sand. However the sporulation of fungus stored in water was significantly reduced. This result supports Borman et al. (2006) stating, although sterile water has been proven to be an easy method for preservation of most fungi, it can lead to a loss in the sporulation rates of fungi, as well as to changes in morphological characteristics.

In colony growth assay, PDA had no significant effect compared with the colony produced before storage. In conclusion, the preservation of conidial viability is influenced by storage and ambient conditions. Apart from the storage formulation other factors such as temperature, humidity, and light also impact on the stability of conidia of entomopathogenic fungi during production and storage (Clerk & Madelin, 1965).

Although, a significant difference in conidial germination and sporulation were observed in the preserved fungi, the macroscopic observation showed that none of the preservation methods induced macro-morphological alterations.

Degeneration of entomopathogenic fungi due to storing in artificial media have been reported in wide range of fungi, however why the fungi degenerate is unclear (Butt et al., 2006). It has been reported that entomopathogenic fungi preserved on nutrient rich media are more susceptible to degenerate (Butt et al., 2006). It could be caused by repression of cuticle-degrading enzyme Pr1 in the nutrient rich media (Butt et al., 1996; Wang et al., 2002). Among the media used in this study, PDA had more effect on the fungal germination, as a greater reduction in germination of both fungi was observed when they were stored in PDA. However PDA media had less effect on sporulation and mycelia growth of the fungi. The current study also showed that in all storage media, sporulation of *M. anisopliae* was more (not statistically) reduced than *B. bassiana*, however *B. bassiana* was more affected in germination decrease in comparison to *M. anisopliae*. These differences are probably due to the genetic variability between isolates.

Each of the preservation methods and storage media can be used for a certain goal, they also have some advantages and disadvantages. Storage in sterile water is an easy and inexpensive method used for most fungi, but it might lead to changes in morphological characteristics and sporulation rates of fungi (Borman et al., 2006). Subculturing in agar is a simple and usual method used to preserve fungal isolates. However this method is time-consuming when a large number of species are being stored, there is a high possibility of contamination, and physiological and genetic changes might occur (Homolka et al., 2007).

It has been demonstrated that deep freezing storage is an acceptable method for preserving fungal isolates long-term. Oliveira et al. (2011) suggested that glycerol-freeze is the most capable preservation method for long-term storage.

Preservation methods using drying, silica gel, lyophilization, liquid nitrogen or cryogenic freezer beads at -70°C are used to suspend the fungal metabolism, lyophilization and cryopreservation can reduce the possibility of mutation (Borman et al., 2006). However, these methods need special and expensive equipment (Nakasone et al., 2004).

Most of studies investigating the preservation methods and storage media, analyzed the effect of storage temperatures on the fungal viability. In all these studies both refrigerating

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and freezing temperatures were suggested as an acceptable method for storing the fungal conidia for 24 and 80 months respectively (Sandhu et al., 1993).

Chapter 7. Virulence of *Beauveria bassiana* and *Metarhizium anisopliae* passaged through artificial media and an insect host *Uvarovistia zebra*

Abstract

The effects of repeated subculturing of *Beauveria bassiana* and *Metarhizium anisopliae* *in vitro* and passages through insects on their virulence against *Uvarovistia zebra* were investigated. The virulence of both fungi reduced after four subcultures in Potato Dextrose Agar, but this reduction was not quite significant for *B. bassiana*. Attenuated fungi obtained from the fourth subculturing were passaged through *U. zebra*. Following passage there was a small, but non-significant increase in the virulence of the fungi.

Key words: Virulence, *Beauveria bassiana*, *Metarhizium anisopliae*, artificial media

7.1. Introduction

Entomopathogenic fungal species particularly *B. bassiana* and *M. anisopliae* are being evaluated and developed for use as an alternative control for insect pests. For use as biological control agents their virulence is important, as success of any of these fungi depends significantly on the fungal virulence. Virulence of entomopathogenic fungi can be affected by repeated subculturing in artificial media or passage through insect hosts. Constancy of fungal virulence following successive subculturing on artificial media is a desirable trait for production of biocontrol agents (Brownbridge et al., 2001; Vandenberg & Cantone, 2004). Attenuation or enhancement of virulence of entomopathogenic fungi following repeated subculturing in artificial media or passage through insect hosts have been previously reported (Fargues & Robert, 1983; Kawakami, 1960; Schaerffenberg, 1964).

Some studies have reported that successive cultures in artificial media cause attenuation in fungal virulence. In contrast, some studies reported that no decline was observed in virulence of fungi subcultured in artificial media. Single passage of entomopathogenic fungi through a suitable host can restore or increase the virulence, however some workers reported that virulence can only be increased after two or more successive passages (Butt et al., 2006). The effect of repeated *in vitro* subculturing and *vivo* passaging on viability, morphological characteristics and virulence, varies within entomopathogenic fungi strains. There are many studies assessing the effect of repeated *in vitro* subculturing and *in vivo* passaging on the virulence of entomopathogenic fungi, and several different observations have been reported. Some positive and negative results obtained from different studies have been shown in table 7.1.

Table 7.1. The results of some studies assessing the effect of repeated *in vitro* culturing (on artificial media such as PDA or SDA) and *in vivo* passaging of entomopathogenic fungi.

Type of assay	Fungus	Repeats	Result	References
<i>in vitro</i>	<i>B. bassiana</i>	16	decreased virulence against <i>Leptinotarsa decemlineata</i>	Schaerffenberg, (1964)
	<i>B. bassiana</i>	-----	no decrease in virulence against <i>Bemisia argentifolii</i>	Brownbridge et al., (2003)
	<i>B. bassiana</i>	2	significant reduction in virulence	Quesada-Moraga & Vey, (2003)
	<i>B. bassiana</i>	15	no decline in virulence	Brownbridge et al., (2003)
	<i>Verticillium lecanii</i>	2 or 3	Significant reduction in virulence	Nagaich, (1973)
	<i>Aschersonia aleyrodis</i>	19	lost virulence against <i>Trialeurodes vaporariorum</i>	Fransen et al., (1987)
	<i>Paecilomyces fumosoroseus</i>	30	no change in virulence against <i>Diuraphis noxia</i> or <i>Plutella xylostella</i>	Vandenberg & Cantone, (2004)
	<i>M. anisopliae</i>	12	no decrease in virulence against <i>Tenebrio molitor</i>	Ansari & Butt, (2011)
<i>In vivo</i>	<i>B. bassiana</i>	-----	became more virulent against <i>Alphitobius diaperinus</i>	Steinkraus et al., (1991)
	<i>B. bassiana</i>	2	increase in virulence	Quesada-Moraga & Vey, (2003)
	<i>M. anisopliae</i>		virulence was restored	Shah et al., (2005)
	<i>M. anisopliae</i>	1	increase in virulence against <i>Rhipicephalus microplus</i>	Guedes-Frazzon et al., (2000)
	<i>Culicinomyces clavissporus</i>		increase in virulence against <i>Aedes aegypti</i>	Cooper & Sweeney, (1986)
	<i>P. fumosoroseus</i>	15	decreased virulence against <i>Diuraphis noxia</i>	Vandenberg & Cantone, (2004)
	<i>P. fumosoroseus</i>	15	no change virulence against <i>D. noxia</i> and <i>P. xylostella</i>	Vandenberg & Cantone, (2004)
	<i>Nomuraea rilleyi</i>	12	no significant decrease or increase in virulence	Ignoffo et al., (1982)
	<i>Aspergillus flavus</i>	-----	no changes in virulence, increase in number of Conidia, decrease in the day fungal growth	Scully & Bidochka, (2005)

The objectives of this study were:

- To evaluate the effect of *in vitro* subculturing on the virulence of *Beauveria bassiana* DEBI 001 and *Metarhizium anisopliae* 715C.
- To evaluate the effect of passages through the grasshopper *U. zebra* on the virulence of *Beauveria bassiana* DEBI 001 and *Metarhizium anisopliae* 715C.

7.2. Materials and methods

7.2.1. Source of fungi and insects

The isolates *Beauveria bassiana* DEBI 001 and *Metarhizium anisopliae* 715C were obtained from the “Iranian Research Institute of Plant Protection” were used for the experiments. The former was isolated by Dr Ghazavi from the soil of a cherry orchard in Fashand region in Tehran, Iran and the latter isolated by Dr Zare from an unknown species of locust. Second and third-instar nymphs of the grasshopper *Uvarovistia zebra* collected from mountainous rangeland of the Alamout region in the north of Qazvin, Iran were used for the bioassay.

7.2.2. Conidial subculturing on artificial media

For subculturing and repeated *in vitro* spore transfers, spores were harvested from the surface of the 10 days old culture by scraping with a loop and subcultured to fresh Potato Dextrose Agar (PDA). Cultured fungi were incubated at 28°C with 70-80% relative humidity (RH) until sporulation was evident (approximately 6 days). This multi-spore *in vitro* transfer was repeated up to 4 times. Before subculturing, a suspension of harvested spores was prepared to use for bioassay and inoculate other insects. Spore concentration of the prepared suspensions was estimated using a haemocytometer and were 8×10^6 and 5×10^6 spores/ml for *B. bassiana* and *M. anisopliae* respectively. The concentrations in primary suspension were adjusted to 5×10^6 spores/ml used for experiments.

7.2.3. Conidial passaging through insect

For *in vivo* passage in the insect host, spores of the fourth *in vitro* subcultures were recovered from Petri dishes and suspended in sterile distilled water. Spore concentration of the suspension was adjusted to 5×10^6 spores/ml for both fungi. Third instar nymphs of *Uvarovistia zebra* were dipped in the spores suspension (5×10^6 spores/ml) containing 0.02% Tween 80® solution for 5 s and placed individually in a small plastic container. Inoculated insects were maintained on a lettuce diet until mortality in some of them occurred. Dead

insects were cleaned with cotton wool soaked with 70% ethanol and incubated at 28°C with 80-90% relative humidity to stimulate sporulation of the fungi. The spores from cadavers were harvested by scraping with a pointed needle and suspended in sterile distilled water to inoculate other insects. This insect passage was repeated 2 times and virulence of the fungi was evaluated by mortality in a bioassay.

7.2.4. Bioassay

The virulence of the fungi was assayed (i) with the original isolates before subculturing on artificial media (ii) after subculturing on artificial media (iii) after passage *in vivo* (passage in the insect). The same spore concentration of 5×10^6 spores/ml containing 0.02% Tween 80® solution was used for both fungi in each bioassay.

For each bioassay, forty, third-instar nymphs of *U. zebra* were dipped in the conidial suspension for 5 s and kept in group of 10 in 4 plastic containers (each 30×15×8 cm L×W×H). Nymphal mortality was recorded daily until 15 days after inoculation. The virulence of the fungi was measured using mortality monitored at day 15 after application of the fungi. Four replicates were used in each bioassay and 10 third instar nymphs were allocated per replicate. Treatment comparisons were performed using the t-test.

7.3. Results

The effects of the fungi *B. bassiana* and *M. anisopliae* subcultured four times in Potato Dextrose Agar (PDA) have been shown in Fig 1 A & B. The fungal virulence measured by insect mortality reduced 13% and 17% after subculturing for *B. bassiana* and *M. anisopliae* respectively. This reduction in virulence was not quite statistically significant for *B. bassiana* ($t_{(6)} = 1.98$, $P = 0.0942$), whereas reduction in virulence of *M. anisopliae* was significant ($t_{(6)} = 3.65$; $P < 0.01$).

The fungal virulence attenuated due to subculturing in artificial media increased slightly after two times passage though the insect host, but these increases were not statistically significant (*B. bassiana*: $t_{(6)} = 0.926$, $P = 0.3905$; *M. anisopliae*: $t_{(6)} = 0.397$, $P = 0.7049$) (Fig 2 A & B).

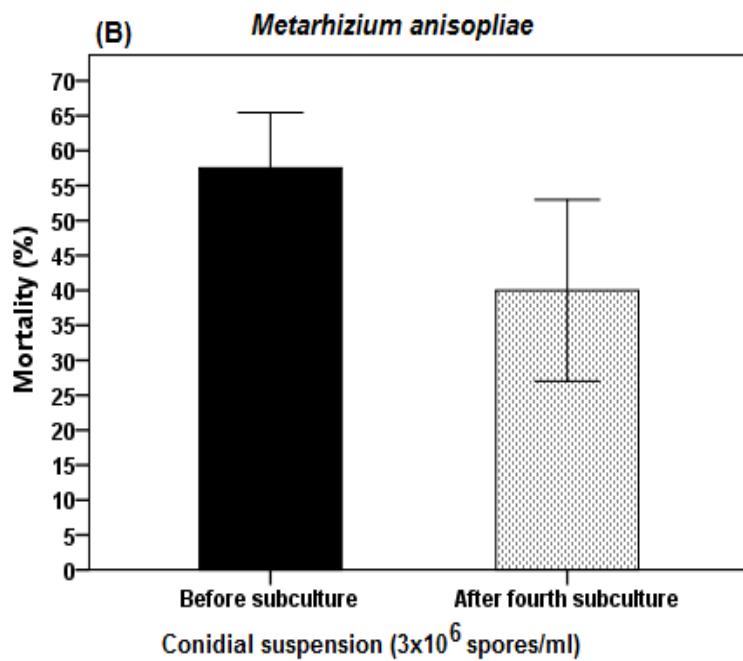
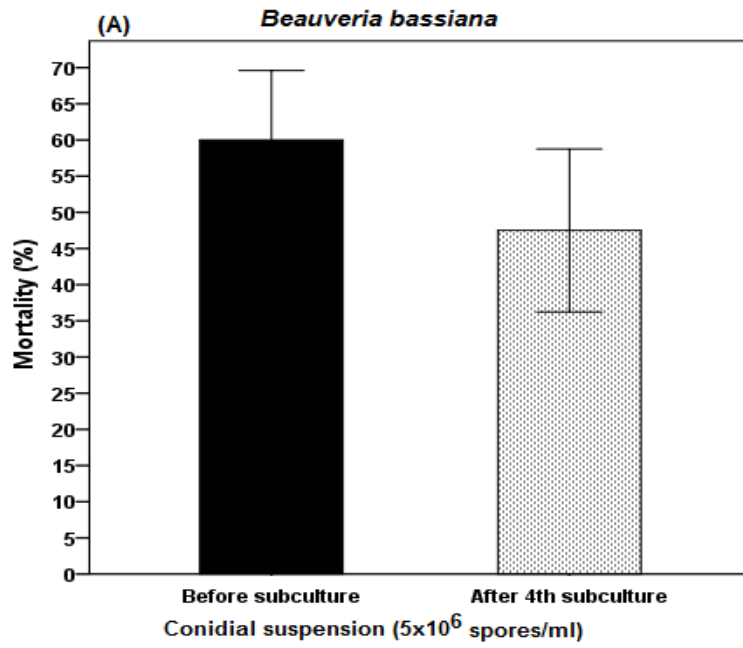


Fig 7.1. Mean percentage mortality caused by (A) *B. bassiana* and (B) *M. anisopliae* before and after successive subcultures.

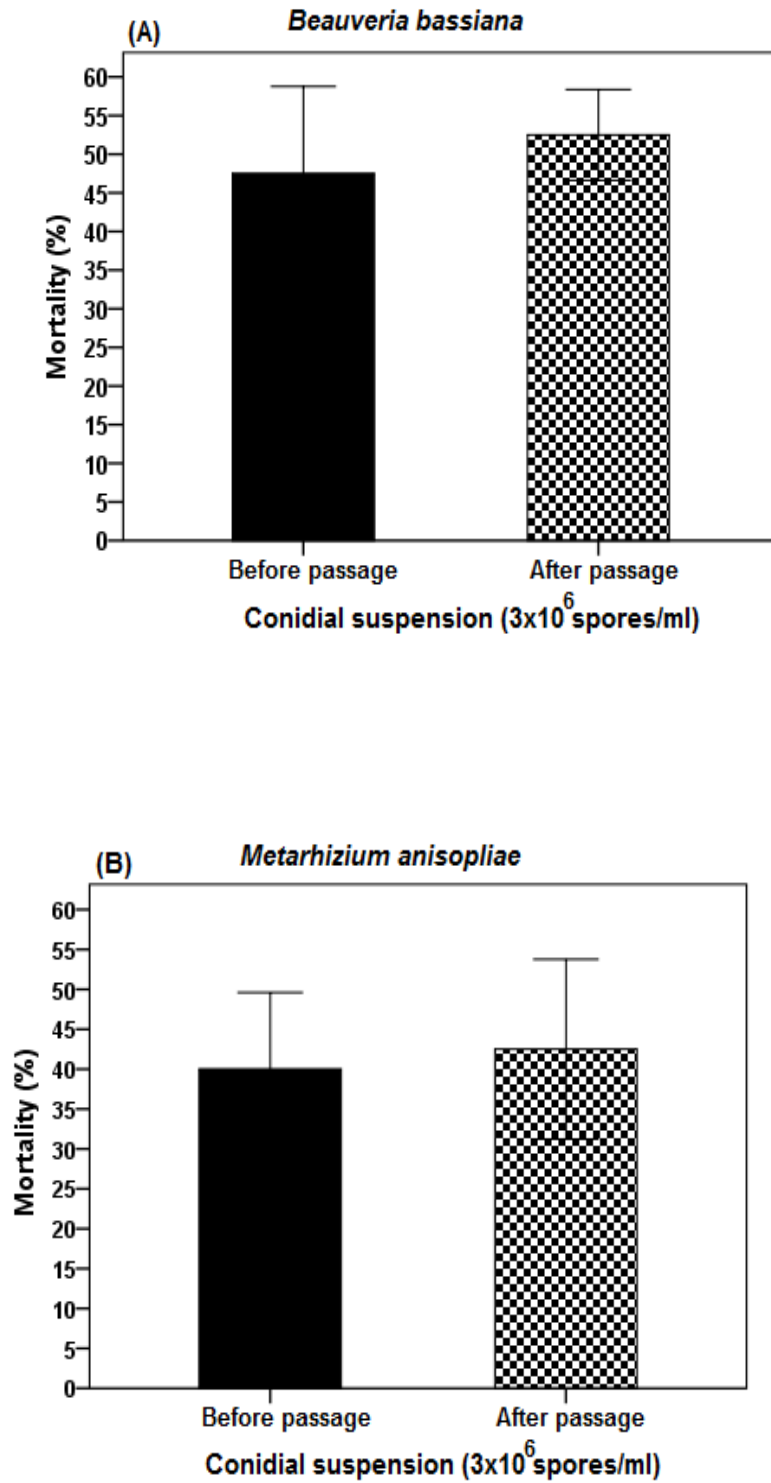


Fig 7.2. Mean percentage mortality caused by (A) *B. bassiana* and (B) *M. anisopliae* before and after passage through *U. zebra*.

7.4. Discussion

In the present study the virulence of both fungi reduced after four subcultures, but this reduction was not significant for *B. bassiana*. However, the trend for reduction in virulence of *B. bassiana* was similar and it seems that virulence of both fungi can be affected by successive subculture in artificial media. In general, most other studies reported that virulence of entomopathogenic fungi decline in *in vitro* subcultures. However changes in virulence of the fungi subcultured *in vitro* can be influenced by some factors such as nutrition and fungal strains. As can be seen the virulence of *B. bassiana* was less attenuated than *M. anisopliae* in the same lab conditions applied for this study. The effect of nutrition on virulence of entomopathogenic fungi has been reported as well. The ratio of C:N in artificial media has influence on fungal virulence, as the virulent conidia has relatively low endogenous C:N ratios. Shah et al. (2005) reported that *M. anisopliae* lost its virulence against *Galleria mellonella* and *Tenebrio molitor* (L) (Coleoptera: Tenebrionidae) after subculturing on a media with high C:N ratio. Virulence of entomopathogenic fungi is enhanced by starvation conditions (whether *in vitro* or *in vivo*), because the cuticle-degrading enzymes protease Pr1 which is an important virulence determinant, becomes depressed under starvation conditions (Butt et al., 1998; Wang et al., 2002) and then elevated level of the enzyme increase the virulence of fungi (Shah et al., 2005).

In general, artificial media provide nutrient conditions that are ideal for rapid propagation of the fungi. Entomopathogenic fungi will degenerate (changing morphology and losing virulence) when they are successively cultured on nutrient rich media (Butt et al., 2006).

In the current study attenuation of the fungal virulence was observed when they were serially subcultured on PDA media. Although, a high level of virulence attenuation due to repeated subcultures was not observed in this study, it seems that if the number of subcultures was more (more than four times) or the fungi had to be preserved for a longer period in a nutrient medium, the fungal virulence might be more attenuated.

In order to produce entomopathogenic fungi commercially, mass production has to use artificial media, however the successive production on artificial media lead to a loss of the fungal virulence (Wraight et al., 2001).

Investigating the effect of subculturing *in vitro* on virulence of the fungi is necessary before they are to be used for commercial production. Actually, finding the best method

and suitable artificial media to minimise the fungal virulence attenuation is one of the most important steps in the mass production process.

Strains of the fungi have different susceptibility to losing their virulence, some of them lose their virulence in a single subculture (Butt et al., 2006), however some strains need to be successively subcultured (10-12 times) to lose their virulence significantly (Hajek et al., 1990; Morrow et al., 1989). Butt et al. (2006) noted that virulence of entomopathogenic fungi may be temporarily restored in some media but they are eventually inclined to lose their virulence after *in vitro* subculturing. Though the extent of the effect of repeated *in vitro* subculturing on fungal virulence is influenced by various factors, but very little information is available about why a fungus becomes attenuated (Hutwimmer et al., 2008; Shah et al., 2005). However, the reasons for attenuation of entomopathogenic fungi subcultured *in vitro* have been explained in some studies. Butt et al. (2006) mentioned there are several components (adhesion, germination, penetration, colonisation and conidia and conidiophores differentiation) of the invasive and developmental processes of fungi that may be affected in attenuated fungi. Reduction in fungal virulence may be partly caused by the conidia lacking the cuticle-degrading enzymes (Nahar et al., 2008).

Several studies suggested that virulence was correlated, at least in part, with chitinase activity (El-Sayed et al., 1989; El-Sayed et al., 1993; St. Leger et al., 1996). Morrow et al. (1989) reported a difference in the adhesion among the virulent and attenuated conidia of *Nomuraea rileyi* on the surface of the host cuticle.

Serial passage experiments have been done with various entomopathogenic fungi passed through the insect host. While some of these experiments showed an increase in virulence of entomopathogenic fungi during serial *in vivo* passage (Hartmann & Wasti 1974; Hayden et al., 1992; Schaerffenberg, 1964; Wasti & Hartmann, 1975), others did not (Hall, 1980; Ignoffo et al., 1982).

In the present study the fungi were passed two times through the grasshopper *U. zebra*. The slightly enhancements in virulence of both fungi observed after passaging were not statistically different from the fungal virulence before passaging.

There are several reasons for an increase in virulence with passage through a suitable host. According to Adames et al. (2011) repeated passages through a suitable host may make better conidia adhesion and a faster rate of germination causing a faster invasion to the host. Overall, conidia produced on insect cadavers have higher levels of virulence than

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conidia produced *in vitro* (Shah et al., 2005; St. Leger et al., 1991), however conidia production via passaging through insect hosts is unlikely to be an economical method.

Chapter 8. General discussion

Although the list of research needed on entomopathogenic fungi and their use against insect pests is long, there are specific areas which deserve high priority. Evaluations of entomopathogenic fungi for control of grasshoppers and locusts generally have focused on Acrididae (short-horned grasshopper) species. This study examined pathogenicity of the isolates *B. bassiana* DEBI 001 and *M. anisopliae* 715C for first time on the tettigoniid (long-horned grasshopper) *Uvarovistia zebra*. These fungi were also assayed against different nymphal instars and adults of the Sunn pest *Eurygaster integriceps* in Qazvin plain, Iran. It should be remembered that there are various biotypes of *E. integriceps* in different climate conditions that show different behaviour.

The grasshopper *Uvarovistia zebra* is not currently considered as an important pest, however populations of this insect have increased over the last decade and high populations can invade and damage field crops and rangeland grass. In some years farmers have started to use chemical insecticides (bait formulation) in the rangelands to prevent the possibly invasion of this insects to the fields.

Eurygaster integriceps is the most economically important pest of wheat and barley in Iran. Generally, this pest is controlled by chemical insecticides using different methods of application such as ultra-low volume (ULV), crop-dusting and hand held sprayers.

The first step in the development of the entomopathogenic fungi strains *B. bassiana* DEBI 001 and *M. anisopliae* 715C for use against *U. zebra* and *E. integriceps* was evaluating the pathogenicity of these fungi. Isolates which have acceptable pathogenicity and can be applied as a mycoinsecticide may be stored for a while. According to Butt et al. (2006) strains of the fungi have different susceptibility to losing their virulence, some of them lose their virulence in a single subculture. Therefore, finding the susceptibility of these strains to degeneration and losing their virulence when they are stored or successively subcultured in artificial media was the second step.

8.1. Summary of major findings

The experiments and bioassays reported in Chapters 2, 3, 4, 5 and 7, were done in Iran, Qazvin, and the experiment reported in Chapter 6 was conducted in Newcastle University.

The results of this thesis provided important information on different aspects of the fungal strains used in this study. The efficacy of these indigenous fungi against the insects *U. zebra* and *E. integriceps* and their susceptibility to successive subcultures in artificial media are summarized as below:

- Experiments conducted in chapter 2 & 3, showed that both *B. bassiana* DEBI 001 and *M. anisopliae* 715C were capable of infecting the grasshopper *Uvarovistia zebra* by both contact and ingestion. Results also showed that these fungi had an effect on food consumption of the grasshopper.
- Results obtained from the experiment conducted in chapter 5 demonstrated that spores of both fungi, formulated in oil on lettuce discs were more effective against *U. zebra* nymphs than those formulated in water.
- Results of chapter 4 showed that both fungi had pathogenic effects on the Sunn pest *E. integriceps*. *Beauveria bassiana* was more effective than *M. anisopliae* with significant differences between the fungi observed in all nymphal instars and adults.
- Experiments conducted in chapter 6 showed a significant decrease in germination of the fungi after 6 months storage in artificial storage media. The sand and water media also significantly reduced colony growth.
- Results of the experiments conducted in chapter 7 showed that there was no significant enhancement in virulence of the fungi passed two times through the insect host.

8.2. Construction

The highest concentrations of 1.5×10^8 spores/ml for *B. bassiana* and 2×10^7 spores/ml for *M. anisopliae* caused 100% and 53.3% mortality respectively when they were sprayed on the nymphs of *U. zebra*. At the same concentration (5×10^7 spores/ml) *B. bassiana* and *M. anisopliae* caused 60% and 46% mortality respectively when they were applied on *E. integriceps*. It seems that these fungi were more capable of infecting *U. zebra* than *E. integriceps*. Pathogenic activity and virulence of the fungal isolates are the most important factors which should be considered. To achieve desirable results in practical use high mortality with moderate spore concentrations of the fungal isolates need to be obtained. Field assessments on *Metarhizium* sp. have been generally conducted at a spore concentration of

100 g or 5×10^{12} spores ha^{-1} therefore the product cost is considered to be more than equivalent chemical pesticides (Lomer et al., 2001). Due to the high cost of mycoinsecticide products, the most virulent fungal isolate should be selected for mass production and use for pest control.

8.3. Recommendations for future work

The next steps for continuation of this study are as follow:

8.3.1. Finding more virulent fungal isolates

Research should be continued to identify more virulent strains of the fungi. Several methods are available for collecting insect pathogens. To obtain virulent isolates the following steps should be followed:

(i) screening to determine the most virulent isolates among the Iranian culture collections (ii) screening to determine the most virulent isolates among exotic isolates (iii) obtaining new strains from the insects *U. zebra* and *E. integriceps*.

8.3.2. Studying characteristics of virulent isolates

Apart from virulence other characteristics of selected isolates such as conidial viability, storage qualities, and environmental tolerance need to be assessed. Therefore, continued expansion of research on fungal characteristics is needed so that the factors limiting mycosis development under field conditions will be better understood.

8.3.3. Field assessment

The pathogenicity of the fungi selected via screening the virulent isolates should be assessed in field conditions to be certain that they are effective against the insects in field conditions.

8.3.4. Mass production, formulation, application and long term preservation

The selected entomopathogenic fungi isolates will need to be preserved for a long time; however their virulence and characteristics may be affected by the media during preservation. Further studies need to be done in order to find the best artificial media and conditions for long-term preservation of the fungi without losing virulence. Research is also needed to find the best way for mass production of the fungal strains.

The type of product formulation depends upon the insect host feeding behavior and field conditions. Therefore, different formulations such as powders, granules or liquid forms should be the aim of mass production.

8.3.5. Integrating the use of fungi with other control tactics

The use of selected fungal isolates as a component of Integrated Pest Management (IPM) programmes is also considered as a requirement of future work.

8.4. The future role of entomopathogenic fungi in pest control in Iran

Due to the climatic diversity in Iran, a wide range of agricultural crops are produced in this country, and a large diversity of pest species can be observed. Biological control of pests in Iran began around eighty years ago when *Rodolia cardinalis* was imported by Jalal Afshar for control of *Icerya purchasi* in citrus orchards. Between 1946 and 1966 the Sunn pest egg parasitoid *Trissolcus* wasps were bred in insectaries and released in wheat fields, but unfortunately this program stopped in 1967. Although, extensive research on biological control of pests has been done in Iran, practical applications in the farms have not been very successful.

Due to the fast and obvious effect of chemical pesticides, farmers prefer to use them for control of pests. Although, according to the “Agricultural Research Education and Extension Organization of Iran” (2010) use of chemical pesticides has dropped 30% in the last five years, however the total amount of agrochemical pesticides (insecticides, nematicides, fungicides, herbicides and rodenticides) used in Iran is estimated at 17-25 million litres annum, which is more than the optimum requirement (Molazadeh, 2010). It seems that farmers’ lack of knowledge about the appropriate spray time and use is the main problem exacerbating pesticide use in Iran (Molazadeh, 2010). Given the diversity of biological control agents in Iran, extending biological control by conservation or mass rearing of beneficial insects and biopesticide production is the most appropriate method for improving pest control in Iran. Integrated Pest Management (IPM) and biological control have already been suggested as alternative approaches for pest control in Iran (Asgarinya, 2010; Molazadeh, 2010; Iranian Plant Protection Organization, 2010).

Among the biological control agents, the entomopathogenic fungi have characteristics that give them a privileged position for biological control programs. Due to the superiority of entomopathogenic fungi summarized below, they can take a leading role in the future of pest control in Iran.

- They infect their hosts by direct contact through the insect cuticle and can be mass produced (Prior & Greathead, 1989) and preserved for the long-term.
- They can be prepared in different formulations which are appropriate for different climate conditions and pest species.
- Unlike predators and parasitoids, entomopathogenic fungi can be sprayed like chemical pesticides. They can also be used by ultra-low volume application technique (Bateman, 1992).
- Some entomopathogenic fungi such as *B. bassiana* and *M. anisopliae* have a wide host range of insect pests.
- These fungi are capable of causing epizootics among insect pests in appropriate condition (high humidity and optimum temperature) (Clayton et al., 1985; Tanada & Kaya, 1993).

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