SLU
Sveriges lantbruksuniversitet

The potential of entomopathogenic fungal isolates as an environmentally friendly management option against *Acanthoscelides Obtectus*

Julia Grund, and Lina Hirsch on fieldtrip with classmates from Addis-Abeba University

Julia Grund and Lina Hirsch

Självständigt arbete vid LTJ-fakulteten, SLU, 15 hp, Miljömanagementprogrammet

SLU-Swedish University of Agricultural Sciences
Faculty of Landscape Planning, Horticulture and Agricultural Science – LTJ-Fakulteten
Department: Plant Protection Biology

Alnarp, 2010

Title: The potential of entomopathogenic fungal isolates as an environmentally friendly management option against Acanthoscelides obtectus.
Author´s Names: Julia Grund and Lina Hirsch

Supervisor: Ylva Hillbur, SLU, Faculty of Landscape Planning, Horticulture and Agricultural Science – LTJ-Fakulteten
Assistant Supervisor: Emiru Seyoum, Addis-Abeba University
Examiner: Birgitta Rämert, SLU, Faculty of Landscape Planning, Horticulture and Agricultural Science – LTJ-Fakulteten

Credits: 15 hp
Level: C (G2E)
Course title: Examensarbete inom kursblock Miljömanagement - Degree Project for Environmental Management
Course code: EX0371
Programme/education: Miljömanagement (kursblock) - Environmental Management
Name of serie: Självständigt arbete vid LTJ-fakulteten, SLU

Place of publication: Alnarp
Year of publication: 2010
Picture Cover: Julia Grund, and Lina Hirsch on fieldtrip with classmates from Addis-Abeba University

Online publication: http://stud.epsilon.slu.se

Key Words: *Acanthoscelides obtectus, Beauveria bassiana, Metarhizium anisopliae,* enthomopathogenic fungi, biological control agents, storage pests, pests in field

SLU

Sveriges lantbruksuniversitet

Swedish University of Agricultural Sciences
Faculty of Landscape Planning, Horticulture and Agricultural Science – LTJ-Fakulteten
Department: Horticulture

Acknowledgements

This paper has not only been an educational experience, but also the outcome of an inspirational trip to Ethiopia. When it was time to prepare our bachelor project in environmental management we both knew that we wanted to write about biological plant protection. Through our course administrator and teacher, Tora Råberg, we got in contact with Ylva Hillbur, who is involved in the Linnaeus Palme student exchange program between our University (SLU, Alnarp) and the University of Addis-Abeba. Soon after that we travelled to Ethiopia to study pests and their management for three months and also to perform the experiments described in this study. We want to thank Tora Råberg for her encouragement and for telling us about the Linnaeus Palme student exchange program. We also thank Dr. Ylva Hillbur for facilitating the project and making this study and our journey to Ethiopia possible. During our meetings she always inspired us to continuously improve our paper. Jan-Eric Englund has also helped us with the statistics, for which we sincerely thank him. We also want to thank our examiner, Bigitta Rämert for constructive feed-back.

Our thanks go also to our assistant supervisor, Dr. Emiru Seyoum, for his hospitality and guidance through all the challenges and throughout the experimental work, as well as to Tibebe Dejene, Ashenafi Kassaye, Mengistu Dawit and Abebe Asale Guttu, for always having time for our questions and for guiding us around in the lab. We thank our teacher, Dr. Emana, for taking us to an inspiring field trip where we could see the bean bruchids in storage. During our stay in Ethiopia we got to see a different culture and a different school system and made friends and contacts for the future. This has not only enriched our education but also given us wonderful memories for life.

For their continuous help and support during the journey that writing this paper has been we want to thank: Fredrik Tigerström, Eric Grund with family and the Hirsch family.

Abstract

The bean bruchid, *Acanthoscelides obtectus* is a major threat to bean production. In Ethiopia, where this study was performed, the damage by bruchids on stored beans has been reported to reach up to 38%. To use the isolates *Beauveria bassiana* and *Metarhizium anisopliae* in the management of *A. obtectus* can be an environmentally safe alternative for controlling the pest. In the present work the aim was to examine if the four chosen fungal isolates, *B. bassiana* DLCO 43 and *M. anisopliae* DLCO 91, 76, 28, would provide an effective way of managing *A. obtectus*. To test the impact of each of the fungal isolates on *A. obtectus* mortality, insects were placed in a Petri dish and sprayed with the spore solutions in three concentrations; $1x10^5$, $1x10^6$ and $1x10^7$ conidia/ml. All the fungal isolates had a high level of mycosis, ranging from 85-97%, meaning that a majority of the dead insects died of fungal infection. The control group had 0% in the mycosis test. The results show that all the fungal isolates, but especially the *Metarhizium* isolates, are effective against *A. obtectus* and achieve a high mortality.

Keywoards: *Acanthoscelides obtectus, Beauveria bassiana, Metarhizium anisopliae,* enthomopathogenic fungi, biological control agents, storage pests, pests in field

Sammanfattning

Acanthoscelides obtectus är ett av de allvarligaste hoten mot produktionen av bönor. I Etiopien, där denna studie har utförts, har de skador som *A. obtectus* gör på lagrade bönor rapporterats nå upp till 38%. Att använda svampisolaten *Beauveria bassiana* och *Metarhizium anisopliae* for att bekämpa *A. obtectus* kan vara ett effektivt och miljövänligt alternativ. I det aktuella arbetet var syftet att undersöka om de fyra svampisolaten, *B. bassiana* DLCO 43 och *M. anisopliae* DLCO 91, 76, 28, skulle vara ett effektivt sätt att bekämpa *A. obtectus*. För att testa effekten av varje svampisolat på *A. obtectus* dödlighet, placerades insekterna i Petriskålar och sprayades med sporlösningar i tre koncentrationer, $1x10^5$, $1x10^6$ and $1x10^7$ konidier/ml. Alla svampisolaten hade en hög nivå av mykos, från 85 till 97%, vilket innebär att en majoritet av de döda insekter dog av svampinfektion. Kontrollgruppen hade 0% i mykostestet. Resultaten visar att samtliga svampisolat, särskilt *Metarhizium*-isolaten, är effektiva mot *Acanthoscelides obtectus* och uppnår en hög dödlighet.

Table of contents

Introduction

BACKGROUND

The worldwide grain harvest increased approximately 1% annually between 1990 and 1997 while the average population growth rate in third world countries was 1.6% over this period (Dhaliwal and Koul, 2007). In addition, arable land is decreasing every year; about 2 billion hectares of arable land have been degraded due to population pressure (Dhaliwal and Koul, 2007). These facts make it important to obtain high yields and minimal losses in order to provide enough food for the world's increasing population. In eastern Africa (including Ethiopia), beans play a significant role as food supply for people of all income categories (Muir and White, 2000) and for poor people throughout the world they are especially important since they contain a large amount of protein. The increasing national and international trade in legume seed leads to an increasing risk for introduction of new pest species (Keals et al., 2002). Pests such as bean bruchids are problematic particularly in areas with a high density of bean production (Muir and White, 2000). In Ethiopia, where this study was performed, the damage by bruchids on stored beans has been reported to reach up to 38%, according to the International Center for Tropical Agriculture (Jones, 1999). Because they are highly adaptive and have a high reproductive rate the bean bruchid is difficult to control (Keals et al., 2002; Metcalf and Flint, 1979). One of the most commonly considered control methods is the use of chemical pesticides (Ghidiu, 2005). The problems with chemical pesticides are e.g that traceable amounts can be found in the crops they are used on (Andersson et al., 2006), they can contaminate water sources and pest insects have developed resistance against many of the pesticides used (Bellinger, 1996; Messmer and Dahl, 2009). It is therefore of great importance that new technologies and methods for pest management, such as biological control, quickly reaches the farmers and that sustainable policies are made and followed (Wortmann et al., 1998).

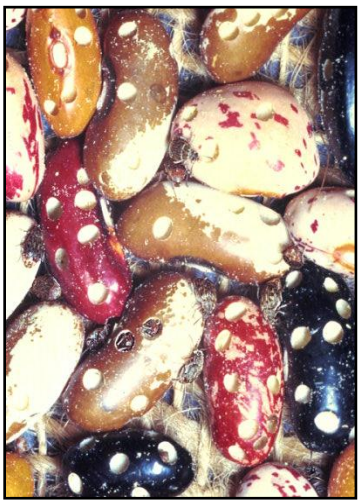

Figure 1. The damage of the Bean bruchid (INRA, 2010)

Pesticides and their adverse effects

The rapid acceleration of the use of chemical pesticides in agricultural production has in many cases lead to increased production, but it has also had several adverse effects: deteriorating the environment in different ways such as contaminating water sources and bottom sediments.

Pests have developed resistance against many pesticides and the pesticides can impact non-target organisms negatively, such as animals and humans (Amuwitagama, 2004). The Swedish National Food Administration revealed that one third of the samples from the categories cereals, fruits and vegetables contained traceable amounts of at least two pesticides. Samples from countries outside the EU contained residues more often than those from the EU (Andersson et al., 2006). The effects of chemicals on the environment are of public concern and the interest for environmentally sustainable agricultural products is increasing. Consequently, greater restrictions on pesticide use have been implemented and these have forced the industry to develop new sustainable alternative methods (Butt, 2001).

As mentioned, pesticides have many disadvantages such as harming non-target organisms (Messmer and Dahl, 2009). However there are alternatives to pesticides, one example is biological control. A recent definition of biological control is: *"The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging then it would otherwise be"* (Eilenberg and Hokkanen, 2006). In biological control there are four different strategies; classical biological control, inoculation biological control, inundation biological control and conservation biological control. In this study the strategy used is inoculation biological control. It can be defined as *"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 permanently"* (Eilenberg et al., 2001).

Biodiversity as represented by naturally occurring enemies of pests, such as bacteria, fungi, insects and viruses, is an important element in biological control systems for pest management (Butt, 2001). Broad spectrum chemical pesticides affect biodiversity negatively and kill a wide range of organisms, including many natural enemies which might otherwise prevent a pest from becoming severe (Pettersson and Åkesson, 2003). This is one reason why biodiversity is beneficial. In addition, many insects provide us with other important ecosystem services, e.g. pollination. Biodiversity has many definitions, a widely used one, accepted by the United Nations Convention on Biological Diversity (which is a convention including most of the countries in the world) (Wordiq, 2010), defines biodiversity as: "*The variability among living organisms from all sources, including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part: this includes diversity within species, between species and of ecosystems"* (Wordiq, 2010; Centrum för biologisk mångfald, 2010). However, biodiversity and most ecosystem services have little direct market value, if any, and their decline does therefore not send any warning signals to the economy. Ecosystem services can be defined as; "*Final ecosystem services are components of nature, directly*

enjoyed, consumed, or used to yield human well-being." (Boyd and Banzhaf, 2007). The fact that most ecosystem services are free often results in them being carelessly used or destroyed (Hails, 2008) even though it is costly and often not even possible to replace them (Ekelund, personal communication; Hails, 2008). Replacing chemical pesticides with entomopathogenic fungi would therefore have advantages. Like other biological control agents (BCA's) they do not poison the water around the fields with dangerous residues nor enter food-chains or impact biodiversity significantly as chemical pesticides might do (Butt, 2001; Lacey et al., 2001). Entomopathogenic fungi are also useful where pests have developed resistance against chemical pesticides (Butt, 2001).

THE PROBLEM

Pests on stored foods cause more economic losses than ones impacting earlier stages, since the products they feed on have already been processed in many steps: they have been grown, harvested and sometimes undergone further treatment and handling before being stored (El-Kashlan et al., 1995). The major pests on stored foods, such as beans, are mainly from the families Coleoptera and Lepidoptera. The common bean bruchid, in particular, causes extensive economic losses since it is a pest both in the field and in storage (Koona and Bouda, 2006; Keals et al., 2002).

Figure 2. The Bean bruchid with it's characteristic reddish legs and antennas (©INRA, 2010)

Figure 3. The Bean bruchid when emerged from the safe bean pod (© The State of Queensland, Department of Employment, Economic Development and Innovation, 2010)

It is of great importance for farmers to be able to store their beans without losses in order to sell them during the months when prices are highest. Losses in stored beans are correlated with the length of the storage time (Jones, 1999). Furthermore secondary-rotting by micro-organisms can occur since bean bruchid larval stage completion creates favourable conditions for fungi and bacteria due to elevated temperature and relative humidity (RH) (Jones, 1999). It is therefore socially and economically important to find sustainable methods for the management of insect pests, in this case the bean bruchids (Amuwitagama, 2004).

THE SYSTEM

The insect

The bean bruchid, *Acanthoscelides obtectus*, (Coleoptera: Bruchidae), is one of the most damaging agricultural pests in the seed beetle family, *Bruchidae* (Koona and Bouda, 2006). All legume species are victims of seed-beetles, both in the field and in storage (Keals et al., 2002). Bean bruchids originate from the United States, but they are highly adaptive and have spread geographically throughout the world. Their vast distribution is mainly the result of human transportation of beans between countries (Keals et al., 2002; Metcalf and Flint, 1979).

Figure 4. The Bean bruchid inside a bean with the operculum (©INRA, 2010)

The bean bruchid is a small, brown, slightly striped insect, covered with short hairs. The adult is only about 3-5 mm long. It has dark mottling and the legs and antennae are slightly reddish. The wing cases are short and do not quite reach the tip of the abdomen (Randall, 1998; Metcalf and Flint, 1979; INRA, 1997).

Figure 5. The wing cases are short and do not quite reach the tip of the abdomen

The bruchid eggs have a very short embryonic development that can vary from 3 to 30 days, depending on the surrounding climate. The eggs are attached to the peapod stem or within any natural cracks and look like whitish, ellipsoidal stripes. When they continue into the next development stage they become small, white, fat larvae that feed inside the pods. This stage can last for two weeks to six months, depending on the climate - most commonly it takes two to eight weeks (Metcalf and Flint, 1979; INRA, 1997). Before the bruchid pupates inside the bean it cuts a small operculum, a hard flap used as a kind of door, on the surface of the bean. This operculum keeps the bruchid safe inside the bean and later the fully developed insect exits the pod through this opening (Figure 4). The time for pupation can vary between 12 and 25 days, and as soon as the temperature is right, the fully developed bruchid becomes active and exits the pod (INRA, 1997). The larval feeding inside the bean or peapods leads to a reduction in crop yield, quality and bean viability for future sowing (Keals et al., 2002). One of the reasons why the bean bruchid is such a damaging pest is its high reproductive rate, which under beneficial conditions can result in multiple generations of offspring, especially in storage where the climate is ideal and food available. Under such conditions bruchids have the

possibility to multiply continuously and can complete up to seven generations per year (Metcalf and Flint, 1979).

Entomopathogenic fungi

Entomophthorales have been used in many successful control programmes for use in integrated pest management (Kannan et al; 2008; Kassa, 2003; Makaka and Caston, 2008: Chen, 2005; Lord, 2005, Scholte et al., 2004). They are placed in the class Zygomycetes and are pathogenic to insects and mites. Entomopathogenic fungi probably kill the host insect by physiological starvation when they have consumed all the insects' nutritional reserves. Entomophthorales are considered to be suitable for use as biological pesticides since they have a relatively narrow host range. The fungal isolate does not kill many different species and does not have a significantly negative impact on biodiversity or natural enemies (Butt, 2001; Kannan et al., 2008).

Enthomopathogenic fungi can affect and invade insects when they are in an infective spore stage. Conidia of hypomycetes fungi adhere to the cuticle by a non-specific adhesion mechanism which is mediated by the hydrophobicity of the cell wall (Kannan et al., 2008). When the spores germinate on the host's cuticle a germ tube is formed that penetrates the cuticle and invades the hemocoel of the insect. The fungus kills the insect by physically invading its body and also by producing toxins (Kannan et al., 2008; Krutmuanga and Mekchayb, 2005). The fungus multiplies within the insect, and if the conditions are favorable it can grow out of the insect, form conidiophores or analogous structures and sporulate (Kannan et al., 2008).

Fungal infection is seldom immediate, which can be one of its weaknesses as a biological control agent (Butt, 2001). If one can accept that it takes a little longer time than chemical pesticides, this method is an environmentally friendly alternative. Fungal isolates can withstand adverse conditions as resting spores in the dormant stage, which enables them to survive through periods when hosts are not present (Butt, 2001; Lacey, 1997).

Fungal isolates have a history of more than 100 years of safe use as pest control agents. An Italian scientist, Agostino Bassi (1773-1856), studied diseases in silkworms and discovered fungal isolates and their potential in insect control. He spent more than 30 years researching this field and gave one of the fungal isolates used in this study its name; *Beauveria bassiana*. Today, after many years of study, there still are no reports of significant adverse effects of the use of *B. bassiana* (Längle, 2006). The two fungal isolates of this study, *B. bassiana* and

Metarhizium anisopliae, are characterized by their high virulence and rapid germination and sporulation, which makes them interesting as BCAs (Al-Deghairi, 2008).

Beauveria bassiana

The fungal isolate *Beauveria bassiana* (Hypocreales: Cordycipitaceae) is found naturally in soils and is an entomophatogenic fungi, parasitizing insects and killing or disabling them (Butt, 2001) It has as mentioned, a rapid germination and sporulation, with a high virulence and good discharge of conidia which makes it an efficient control agent. *B. bassiana,* is cheap to produce in large quantities and easy to store (Al-Deghari, 2008). Studies have been done investigating the potential of *B. bassiana* as control agent of different soil borne insects, such as the May beetle (*Phyllophaga spp.*) and the Argentine stem bruchid (*Listronotus bonariensis*). But, as evolution has its way, many of these insects have developed tolerance against *B. bassiana* and it is now primarily tested against foliar feeding insects (UCONN IPM, 2010). The largest programme using fungi for pest management has been made by the People's Republic of China where at least 1,000,000 ha of pine forest is treated every three years with *B. bassiana* against the Pine moth, *Dendrolimus pini L*. (Butt, 2001; Lord, 2005).

Figure 6. *B. bassiana* with its white mould, called *Muscadine* disease (©Hidden forest, 2010)

Figure 7. *B. bassiana, Muscadine* disease (Hidden forest, 2010)

When spores from *B. bassiana* come into contact with the insect's cuticle, they cause a disease called *Muscadine* disease, so-called because of the characteristic white mould. The fungus penetrates through the cuticle and grows inside the insect. *B. bassiana* and other fungal pathogens only need to come into contact with their host insect to cause infection, unlike bacterial and viral pathogens that have to be consumed by their host insect (UCONN IPM, 2010). Well inside the insect, the fungus produces toxic compounds such as bassianolide, beauvericin, and oosporein. These toxic compounds serve different functions and have different effects such as antibiotics against other microorganisms, membrane damage and enzyme malfunction (Butt, 2001). The fungus now feeds on the insect's nutrients,

which eventually leads to the insect's death. When the insect is dead, the fungal isolate *B. bassiana* grows hyphaes that emerge and cover the insects with a white layer of downy mould and start to produce and release millions of new spores (Butt, 2001; UCONN IPM, 2010).

Metarhizium anisopliae

Figure 9. Insect infested with *Metarizium* (©Fermentek, 2010)

Metarhizium is an entomopathogenic fungi that kills the host insect by physically invading its body and consuming the insects nutritional reserves and also producing toxins (Kannan et al., 2008). The optimum temperature for *M. anisopliae* is 28ºC and at this temperature many insects have a high activity level and will be more likely to pick up the conidia than at a lower temperature. At high temperatures the rate at which the conidia stick to the insects may increase (Makaka and Caston 2008).

Figure 8. Insect infected with *Metarhizium* (© The State of Queensland, Department of Employment, Economic Development and Innovation, 2010)

Many experiments where *Metarhizium* has been evaluated as a BCA on a pest insect show that when the pest is infected by the fungi, its feeding activities are reduced before it dies. Such behavioral effects potentially contribute to reducing the damage that the pest causes to crops (Makaka and Caston, 2008). In another study where two isolates of *M. anisopliae* was used against black maize beetles, it was observed that the beetles often appeared on the soil surface, as opposed to in the soil, after being infected by the fungi. This behavior is beneficial to the spreading of the conidia since they can get into contact with other insects or be carried away by wind more easily when on the surface (Makaka and Caston, 2008).

In many experiments good results have been achieved using different *Metarhizium* isolates to control various insect pests. Kannan and coworkers studied the effect of conidia of *M. anisopliae* on the malarial vector *Anopheles stephensi* (2008). To further improve the efficiency of the solution, conidia formulated in oil suspensions are generally more effective than water formulations (Kannan et al., 2008, Makaka and Caston, 2008). An increase in the concentration of spores also increases the mortality (Kannan et al., 2008; Makaka and Caston, 2008). The fungal isolate also affects the result (Makaka and Caston, 2008; Nguyen Thi Loc et al., 2004). In fact, even the same isolate from various sources can vary in toxin production and virulence and therefore achieve different results (Nguyen Thi Loc et al., 2004).

Aim

The aim of this work was to increase the knowledge and inspire to further studies on the use of fungal isolates to manage bean bruchids and thereby decrease the use of chemicals that may harm the environment.

General objective

To evaluate the potential of four native entomopathogenic fungi as an environmentally friendly management option against, *Acanthoscelides obtectus.*

Specific objective

To examine the level of virulence of native fungal isolates against the target insect pest, *Acanthoscelides obtectus.*

To determine conidial concentration that is optimal to achieve significant lethal effect on *A obtectus* adults, under laboratory conditions.

To study the mortality rate followed by the infection of the fungal isolates, on the target, *A. obtectus*.

Materials and methods

The insect

Bean bruchids, *Acanthoscelides obtectus*, were obtained from a rearing at Addis Ababa University. They were kept in darkness at 27°C with a relative humidity (RH) in the range 40-50% until hatched. The insects used for the experiment were both females and males, of undetermined age.

The fungal isolates

Four fungal isolates, native to Ethiopia, were used in the experiment; *Metarhizium anisopliae* DLCO 91, 76, 28 and *Beauveria bassiana* DLCO 43. The fungal isolates were obtained from Addis Ababa University Insect Science Research laboratory and new cultures were regularly collected and renewed. In our laboratory our technician continuously made new cultures of these fungal isolates which were kept in Petri dishes, sealed with Parafilm™ , in an incubator. Spores from the fungal isolates were spread on a Sabouraud Dextrose Agar (SDA) media in Petri dishes using a sterile loop. To further prevent contamination, the spores were spread as rapidly as possible under a laminar flow hood. The Petri dishes were then sealed with Parafilm™ and kept at room temperature under natural light conditions in the laminar air flow hood for 24 hours. The Petri dishes with the fungal isolates were then moved to an incubator, with a temperature of 26°C in constant darkness and a RH of 40 % to obtain optimal growing conditions.

Production of spore solution and counting of spores

A sterile, aqueous Tween solution was prepared with 0.5% Tween 80. Spores from the fungal isolates were suspended in the solution. To homogenize the suspension the solution was blended with a vortex mixer for two minutes (Lacey, 1997).

To establish the concentration of the conidia in the solution, they were counted by using a haemocytometer under a compound microscope. The conidial suspension was further diluted with 0.5% Tween solution, until it reached a concentration with a countable number of spores. After having established the concentration of conidia, suspensions were diluted with distilled water to the concentrations $1x10^5$, $1x10^6$ and $1x10^7$ conidia/ml.

Viability test

A viability test was done before spraying the spore solutions on the insects to evaluate the resting spores' activity over a given time (Lacey, 1997). This was done to establish if the

spore solution was viable enough to be used for all replicates required over three consecutive days.

Four Petri dishes were autoclaved and prepared with autoclaved SDA. Lines were drawn with a waterproof marker on the bottom of each Petri dish to divide it into four, equal sections. Each Petri dish was marked with the name of the isolate, and the sections were numbered from 1 to 4, indicating the day of the viability test. Tween solutions containing an indefinite amount of spores from each of the fungal isolates were prepared. Using a fin pipette, ten 5 µl drops were applied under a laminar flow hood in section 1. A lid was placed on the Petri dish and it was sealed with Parafilm™ and placed at room temperature and ambient RH. After approximately five hours, when the applied fungal solution was dry, the Petri dish was turned upside down. This was done in order to prevent condensed water (formed when the agar decreased in temperature) from dripping down on to the agar and possibly causing contamination. The procedure was repeated for four consecutive days, placing the drops in section 2 on day 2, 3 on day 3 and 4 on day 4. The Petri dishes were observed every 24 hours and the viability, expressed as the percentage of drops with fungal solution that showed growth, was noted.

Mortality of fungal isolates

To test the impact of each of the fungal isolates on *A. obtectus* mortality, 10 insects were placed in a Petri dish with a filter paper and sprayed with the spore solutions. The four fungal isolates were tested in the three concentrations; $1x10^5$, $1x10^6$ and $1x10^7$ conidia/ml. To see how many insects that would die without being infested with fungi, a control group was sprayed with only Tween solution. The effect was evaluated on a daily basis during 15 days, by observing the mortality. All treatments were replicated three times and replicates were made on consecutive days.

Mycosis test

A mycosis test was made to see how many of the dead insects actually died from fungal infestation. Three Petri dishes were prepared, two with water and one with 70 % ethanol. The dead insects were dipped one by one, first in water, then in ethanol and then in water again in order to kill fungus on the surface of the insect. Each Petri dish contained insects from the three concentrations and the different fungal isolates were kept separately. The process was repeated for all the replicates. If fungi subsequently started to grow on the insect, the fungal

isolates had penetrated the insect's cuticle, meaning that the insect had died from fungal infection.

RESULTS

From day 1-3 the control had higher mortality than the concentrations; $1x10^5$, $1x10^6$ and $1x10^7$conidia/ml of the fungal isolate *Metarhizium anisopliae* DLCO 91 (figure 10 a). The lowest concentration and the control group had fairly equal mortality throughout the test and the middle concentration had slightly higher mortality. From day 4 the highest concentration had higher mortality than the control group although the difference decreased during the last days. The difference between the control group and all three concentrations was most pronounced at day 7. Due to the high standard deviation in all the tests in figure 10, no statistically reliable results can be shown, but there is a visible tendency that the mortality increases with a higher dose.

For the three concentrations; $1x10^5$, $1x10^6$ and $1x10^7$conidia/ml of the fungal isolate *M. anisopliae* DLCO 76, the control had higher mortality day 1-3 (figure 10b). The lowest and middle concentrations had fairly equal mortality throughout the test, and had slightly higher mortality than the control group. From day 4 the highest concentration had higher mortality than the control group although the difference between all the concentrations decreased the last days. The difference between the control group and all three concentrations was most pronounced at day 6-7.

All the three concentrations; $1x10^5$, $1x10^6$ and $1x10^7$conidia/ml of the fungal isolate *M. anisopliae* DLCO 28, had higher mortality than the control group from day 4 (figure 10c). The difference between all the concentrations and the control decreased the last days. The highest and middle concentrations had fairly equal mortality throughout the test, and all three concentrations had higher mortality than the control group. The difference between the control group and all concentrations was most pronounced at day 6-7.

The two concentrations; $1x10^5$, $1x10^6$ conidia/ml of the fungal isolate *Beauveria bassiana* DLCO 43, had approximately equal mortality as the control group (figure 10d). The highest concentration; $1x10^7$ had lower mortality than the control group until day 5 and then higher mortality.

The highest concentrations; $1x10^7$ conidia/ml, of the fungal isolates; DLCO 91, 76 and 28, had equal mortality throughout the test (figure 11). They had higher mortality than the control group from day 3 and most pronounced from day 4-8. The highest concentration of the fungal isolate DLCO 43 had lower mortality than the control group until day 5 and then higher mortality. DLCO 43 had lower mortality than the other fungal isolates during the majority of the test.

The control group shows 0% in the mycosis test, meaning that 0% of the dead insects died out of fungal infection (Figure 12). All the fungal isolates showed a high level of mycosis, ranging from 85-97%, meaning that a majority of the dead insects died out of fungal infection.

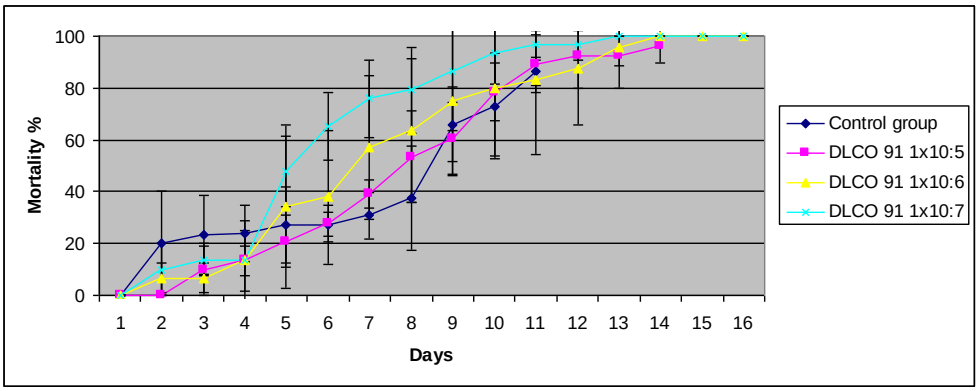

Figure 10 a) Mortality in % of *Acanthoscelides obtectus* infested with DLCO 91 and a control group sprayed with Tween solution (blank). The fungal isolates were tested in the concentrations $1x10^5$, $1x10^6$ and $1x10^7$ conidia/ml. The experimental period was 15 days for the fungal isolates and 10 days for the control groups. Each replicate consisted of 10 insects, N=3.

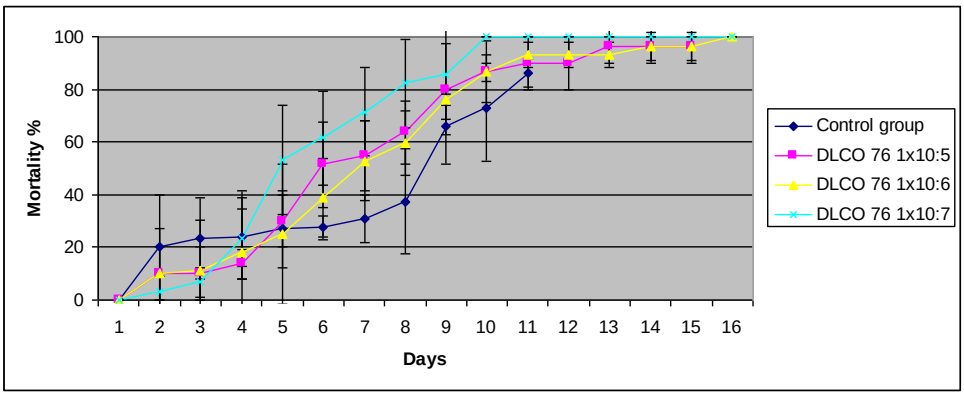

Figure 10b) Mortality in % of *Acanthoscelides obtectus* infested with DLCO 76 and a control group sprayed with Tween solution (blank). The fungal isolates were tested in the concentrations $1x10^5$, $1x10^6$ and $1x10^7$ conidia/ml. The experimental period was 15 days for the fungal isolates and 10 days for the control groups. Each replicate consisted of 10 insects, N=3.

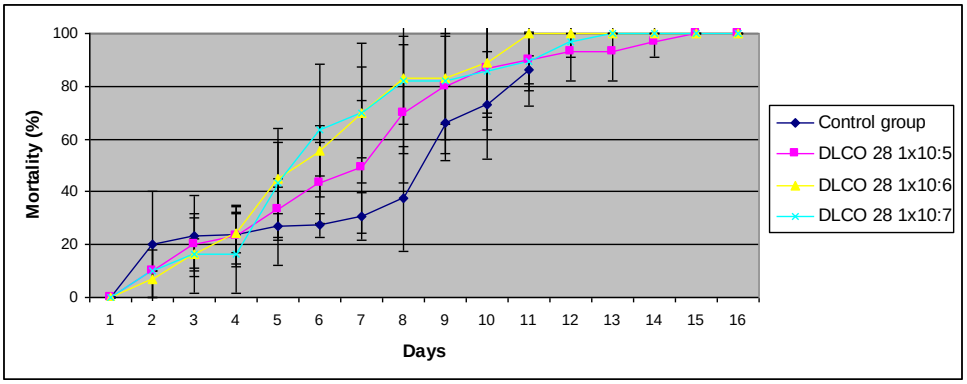

Figure 10c) Mortality in % of *Acanthoscelides obtectus* infested with DLCO 28 and a control group sprayed with Tween solution (blank). The fungal isolates were tested in the concentrations $1x10^5$, $1x10^6$ and $1x10^7$ conidia/ml. The

experimental period was 15 days for the fungal isolates and 10 days for the control groups. Each replicate consisted of 10 insects, N=3

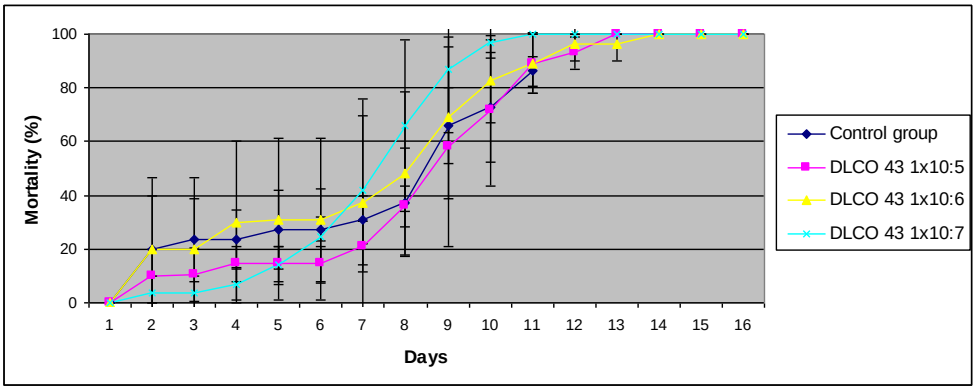

Figure 10d) Mortality in % of *Acanthoscelides obtectus* infested with DLCO 43 and a control group sprayed with Tween solution (blank). The fungal isolates were tested in the concentrations $1x10^5$, $1x10^6$ and $1x10^7$ conidia/ml. The experimental period was 15 days for the fungal isolates and 10 days for the control groups. Each replicate consisted of 10 insects, N=3.

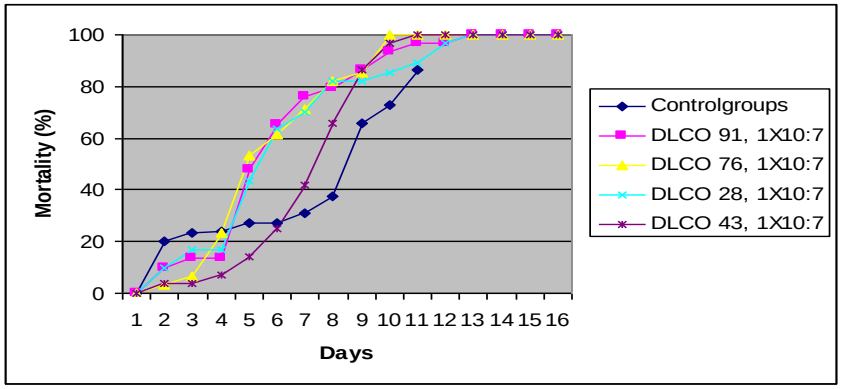

Figure 11. Mortality in % of *Acanthoscelides obtectus* infested with fungal isolates in the highest concentration; $1x10^7$ conidia/ml, compared with the control groups. The fungal isolates used were; DLCO 91, DLCO 76, DLCO 28, and DLCO 43. The experimental period was 15 days for the fungal isolates and 10 days for the control groups. Each replicate consisted of 10 insects, N=3.

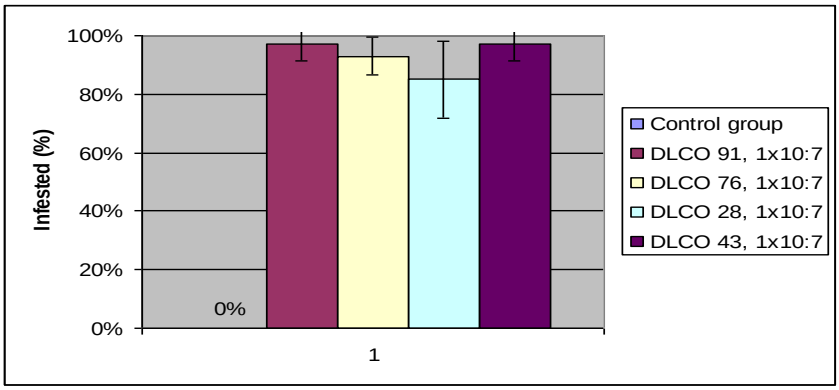

Figure 12. Mycosis test; % of the dead insects that have been infested and killed by the highest concentrations of the four different fungal isolates, compared with the control group. The experimental period was 15 days for the fungal isolates and 10 days for the control groups. Each replicate consisted of 10 insects, N=3.

Discussion

The aim of this study was to examine if fungal isolates would provide an effective way of managing the bean bruchid, *A. obtectus*. The fungal isolates; *M. anisopliae*, DLCO 91, 76 and 28 and *B. bassiana* DLCO 43, were tested and all of them were pathogenic to *A. obtecus* and generated a high mortality. The highest concentrations ($1x10^7$ conidia/ml) of the fungal isolates *M. anisopliae*, DLCO 91, 76 and 28, had equal mortality throughout the test and had higher mortality than the control group from day three. The mycosis test showed that these isolates infected and killed the majority of the insects and they are thus promising to test as biological control agents against the bean bruchid. The highest concentration of the fungal isolate *B. bassiana* DLCO 43 also showed a high mycosis but had lower mortality than the other fungal isolates during the majority of the test and appears to be a less efficient BCA candidate than the other isolates. All the fungal isolates showed a high level of mycosis, ranging from 85-97% (compared to 0% in the control group) meaning that a majority of the dead insects died of fungal infection. The results show that all the fungal isolates, but particularly *Metarhizium*, are affective against *A. obtectus* and achieve a high mortality. Other studies support the result that the isolates have a high lethal effect, though the experiments were performed on other insects (Kannan et al; 2008; Kassa, 2003; Makaka and Caston, 2008: Chen, 2005).

The insects

The fact that there was a high mortality in the control groups resulted in difficulties with determining if the insects died of natural causes or of the fungal isolate. However, in the mycosis test the majority of the insects in all the fungal treatments died of fungal infection whereas none of the insects in the control group were infected. The high mortality in the control groups may be explained by the fact that the insects used in the experiments were of unknown age. The insects were collected from jars in the laboratory with insects in different stages of the lifecycle. In the fungal treatments it is thus possible that the insects not showing mycosis died of old age before they were infected by the fungi. It is also conceivable that older insects may be more susceptible to fungal infection.

Conclusion

Currently the understanding of how effective the fungal isolates in this study are on *A. obtectus* is very limited due to few performed experiments, but many reports are available showing that the isolates cause a high mortality on other insects (Kannan et al; 2008; Kassa,

2003; Makaka and Caston, 2008). In this study it is shown that all the fungal isolates are affective against *A. obtectus* and achieve a high mortality. An increase in the concentration of spores generally also increases the mortality (Kannan et al., 2008; Makaka and Caston, 2008) and might generate a faster result. Future studies should thus include experiments with higher fungal concentrations.

In Ethiopia, where this study was performed, beans play an important role as food supply (Muir and White, 2000) and the bean bruchid is a major threat to bean production. In addition, climate change may result in a warmer climate, which would increase the area where *A. obtectus can* survive and increase the global spread of the bean bruchid. Similar tendencies has been seen for other insects, e.g. in the northwards expansion of the Flea beetle (*Longitarsus dorsalis),* which appears to be the result of increasing temperatures and the expansion of one of it's host plants (Beenen and Roques, 2010). It is therefore of importance to find a way to manage the bean bruchids, before their spread increases, without harming the environment or the people working with the crop.

Recommendations for future studies:

When planning to perform an experiment, we recommend observing the insects and how they behave to prepare how to handle them and prevent complications during the study. This is especially important if there is limited time to perform the study, like in this case. Adults of *A. obtectus* are only about 3-5 mm long and thus challenging to handle in experiments, especially to keep them from escaping from the test cages. The bean bruchids also have a tendency to appear dead which made it difficult to make a correct counting of the daily mortality. To avoid mistaking a live insect from a dead insect, one can carefully blow or gently poke it with a sterile instrument to activate it.

Also make sure that there is enough time to repeat the experiment if contamination or other problems occur. It can be interesting to evaluate which spore concentration and solution that is optimal to use for the fungal isolates in this study on *A. obtectus.* Generally, conidia formulated in oil suspensions are more effective than water formulations and an increase in the concentration of spores generally also increases the mortality (Kannan et al; 2008; Makaka and Caston, 2008). Interesting subjects for future studies can be to test the fungal isolates in storage and to test different ways of using them, such as spraying or applying them in different traps. Different life stages of the insect and the effects on female/male insects might also affect the result and can be a subject of future studies. An interesting comparison can be how high mortality the spore solution generates compared to commonly used chemical

pesticides and how long after the spraying the fungal isolates will have a lethal effect on the pest.

Conceptualization

The efficiency of the fungal isolates might be optimized by using them in a preventive way and regularly check if the crops are attacked by pests to spot the attack before it is to extensive or using a combination of BCAs such as pheromone traps infested with fungal isolates. Also fungal isolates can withstand adverse conditions as resting spores that enables it to survive through periods when hosts are not present (Butt, 2001; Lacy, 1997). Once applied, it is present and can be activated when pests attack the crop and act in a preventive way. When using fungi as a BCA, knowledge about the insects' behavior can be useful in order to enhance efficiency and to increase the spread of the fungi. In an experiment with *Metarhizium anisopliae* (Hypocreales: Cordycipitaceae), on the malaria vector, *Anopheles gambiae s.s*, it was shown that the insects were not only infected through direct contact with the fungus-impregnated material, but also indirectly through contact with other infected insects (Scholte et al., 2004). There are many possible ways to use the fungi and finding the optimal way of inoculating it depends on where it will be used, e.g. in storage, in field or in a greenhouse.

As demonstrated, the fungal isolates in this study provide an effective way of controlling *A. obtecus*. In general, fungi are also cheap to produce in big quantities and easy to store (Al-Deghari, 2008). There is a big potential of an increased use of entomophatogenic fungi as BCAs in the future and it is therefore important to investigate its optimal use on different pests and to optimize the spore concentration for different purposes.

References

Amuwitagama, I., (2004) Analysis of pest management methods used for Rice stem borer *(Scirpophaga incertulas)* in Sri Lanka based on the concept of Sustainable Development, Lund University, November 2002.

Andersson, A., Jansson, G. Jansson, A. (2006) The Swedish monitoring of pesticide residues in food of plant origin: 2006. Report nr 4, 2008. *National Food Administration,* Uppsala, ISSN 1104-7089.

Beenen R, Roques A (2010) Leaf and Seed Beetles (Coleoptera, Chrysomelidae). Chapter 8.3. In: Roques A et al. (Eds) Alien terrestrial arthropods of Europe. BioRisk 4(1): 267–292.

Bellinger, R. G., (1996) Pest Resistance to Pesticides. [online] Available from: http://entweb.clemson.edu/pesticid/issues/pestrest.pdf [2010-12-07]

Boyd, J., Banzhaf, S., (2007) What are ecosystem services? The need for standardized environmental accounting units [online]. Available from: http://www.epa.gov/nheerl/arm/streameco/docs/BoydBanzhaf07.pdf [2010-12-07]

Butt T.M., Jackson, C. W. & Magan, N. (Eds) (2001) *Fungi as biocontrol agents: Progress, Problems and Potential.* Wallingford, Oxon: CAB International.

Carolyn Randall (Ed) (1998) *General Pest Management, A Guide for Commercial Applicators.* Chap. 8, Michigan: Michigan State University Extension.

Caston, Makaka (2008) The efficacy of two isolates of *Metarhizium anisopliae* (Metschin) Sorokin *(Deuteromycotina: Hyphomycetes)* against the adults of the black maize beetle *Heteronychus licas Klug (Coleoptera: Scarabidae)* under laboratory conditions. *African Journal of Agricultural Research*, Vol. 3 (4), pp. 259-265.

CBM – Centrum för Biologsik Mångfald. Vad är biologisk mångfald? [online]. Available from: http://www.cbm.slu.se/biodiv.php [2010-10-06]

Chen, M.C. (2005) Pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae* on the Asiatic palm weevil, *Rhabdoscelus lineatocollis (Heller)(Coleoptera: Rhynchophoridae) Reserch bulletin of Kaohsiung district agricultural research and extension station,* Vol . 16, No.4, December 2005.

Dhaliwal, G.S and Koul, Opender (2007) *Biopesticides and pest management - conventional and biotechnical approaches.* Rajinder Nagar: Kalyani publishers.

Eilenberg, J., Hajek, A. and Lomer, C. (2001) Suggestions for unifying the terminology in biological control. *Biocontrol* 46: 387-400.

Eilenberg, J and Hokkanen, H.M.T (Eds) (2006) An Ecological and Societal Approach to Biological control. Dordrecht, Springer. Chapter 1, the vision.

Ekelund, Lena, (2009). Personal communication.

Ghidiu,G. M.,(2005) Been weevil- Insect Pests of the Home Garden Series, New Jersey Rutgers Cooperative Research & Extension,, October 2005.

Hails, C. (Ed) (2008) *Living Planet report 2008*. Gland, Switzerland, WWF – World Wide Fund For Nature.

Inra (2010). French National Institute for Agricultural research [online]. Available from: http://www.inra.fr/hyppz/RAVAGEUR/6acaobt.htm [2010-06-03]

Jones. A.L. Centro Internacional de Agricultura Tropical (CIAT) (1999) Compendium on Post-harvest Operations: CHAPTER IV PHASEOLUS BEAN [online] Available from: http://www.fao.org/inpho/content/compend/text/ch04-03.htm [2010-06-03]

Kannan, S. Kamala, Murugan, K., Kumar, A. Naresh, Ramasubramanian, N. And Mathiyazhagan, P. (2008) Adulticidal effect of fungal pathogen, *Metarhizium anisopliae* on malarial vector *Anopheles stephensi (Diptera: Culicidae). African Journal of Biotechnology*, Vol. 7 (6), pp. 838-841.

Kassa, A., (2003), Development and testing of mycoinsecticides based on submerged spores and aerial conidia of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae (Deuteromycotina: Hyphomycetes)* for control of locusts, grasshoppers and storage pests**,** *Georg-August-University Göttingen, Institute for Plant Pathology and Plant Protection,* Germany, May 2003.

Keals, N., Hardie, D., and Emery, R (1998) Bruchids: secret seed eaters. *Proceedings of the Australian Postharvest Technical Conference*, pp. 52–54.

Koona, P and Bouda, H (2006) Biological Activity of *Pachypodanthium staudtii (Annonaceae)* Against the Bean Beetle *Acanthoscelides obtectus* Say *(Coleoptera: Bruchidae). Journal of Applied Sciences Research*, 2(12), pp. 1129-1131.

Krutmuanga, Patcharin and Mekchayb, Supamit (2005) Pathogenicity of Enthomopathogenic Fungi *Metarhizium anisopliae* Against Termites. *Conference on International Agricultural Research for Development*. Stuttgart-Hohenheim, October 11-13, 2005, pp.

Lacey, L (Ed) (1997) *Manual of Techniques in Insect Pathology.* San Diego: Academic press.

Lacey, L. A., et al. (2001) Insect Pathogens as Biological Control Agents: Do They Have a Future? *Biological control*, Volume 21, number 3, pp. 230-248.

Lord, Jeffrey C. (2005) From Metchnikoff to Monsanto and beyond: The path of microbial control, *Journal of invertebrate pathology 89*, pp. 19-29.

Längle, T (2006) *Beauveria bassiana (Bals.-Criv.) Vuill.* – A biocontrol agent with more than 100 years of history of safe use. *Workshop on Current Risk Assessment and Regulation Practice.* Salzau, Germany, September 18-22,2006.

Messmer T., Dahl G., Wildlife and Pesticides: A practical guide to reducing the risk. 15 june 2009 [online] Available from: http://www.ag.ndsu.edu/pubs/ansci/wildlife/wl1017-1.htm [2010-12-07]

Metcalf, C. L. and Flint, W. P. (Eds) (1979) *Destructive and useful insects.* 4 e.d. New Delhi: TATA McGraw-Hill Publishing Company Ltd.

Mohammad A. Al-Deghairi (2008) Bioassay Evaluation of the Entomophatogenic Fungi *Beauveria bassiana Vuellemin* Against Eggs and Nymphs of *Bemisia tabaci Gennadius (homoptera:Aleyrodidae). Pakistan Journal of Biological Sciences* 11 (12).

Nguyen Thi Loc, Vo Thi Bich Chi, Nguyen Thi Nhan, Nguyen Duc Thanh, Tran Thi Be Hong and Pham Quang Hung (2004) Biocontrol potential of *Metarhizium anisopliae* against coconut beetle, *Brontispa longissima. Omonrice* 12, pp. 85-91.

Muir, W. E. White, N. D. G. (2000) *Grain preservation biosystems.* Chap. 5, University of Manitoba, Department of Biosystems Engineering, Canada.

Pettersson, M.L and Åkesson, I, (2003) *Växtskydd i trädgård.* 2. e.d. Stockholm: Natur och Kultur/LTs förlag.

Scholte, Ernst-Jan, Knols, Bart GJ and Takken, Willem (2004) Autodissemination of the entomopathogenic fungus *Metarhizium anisopliae* amongst adults of the malaria vector *Anopheles gambiae s.s. Malaria Journal* 2004, Vol. 3, pp. 1-7.

UCONN (2010) University of Connecticut- Integrated pest management (IPM) [online]. Available from: http://www.ipm.uconn.edu/IPM/general/htms/bassiana.htm [2010-07-20]

Wordiq (2010). Biodiversity – Definition. [online]. Available from: http://www.wordiq.com/definition/Biodiversity [2010-10-06]
Wortmann, C.S.; Kirkby, R.A.; Eledu, C.A.; Allen, D.J. (2004) Atlas of common bean *(Phaseolus vulgaris L.)* production in Africa, *National Agricultural Research Organization* (NARU), Uganda, October 2004.