Adane Kassa

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
Entomology Section

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To my sister who passed away at the start of this study

Contents

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AH	Adámek half concentrated liquid medium
AS	Adámek standard liquid medium
a. s. l.	Above see level
AC	Aerial conidia
AEZs	Agroecological zones
AGRYMET	Regional Centre for Agro-Meteorology and Hydrology
BBA	Biologische Bundesanstalt Für Land- und Forstwirtschaft
BCAs	Biological Control Agents
BH	Biomalt-yeast medium
CD	Codacide
CFU	Colony-forming unit
CL	Confidence limit
CM	Catroux medium
CRT	*Cryptocatantops haemorrhoidalis*
DAAD	German Academic Exchange Service
DAT	Days after treatment
Den	Denomenator
df	Degrees of freedom
DP	Dustable powder
EARO	Ethiopian Agricultural Research Organization
EO/E-oil	Experimental oil
FD	Freeze-dried
Fig.	Figure
GLM	Generalized linear model
GM	Green Muscle
GTZ	Deutsche Gesellschaft für Technische Zusammenarbeit GmBH
HDA	*Hieroglyphus daganensis*
L_3/L_4	Third or fourth larval stages
LD_{50}	Doses that kill 50% of the test insect
LD_{90}	Doses that kill 90% of the test insect
LMI	*Locusta migratoria*
LP	Lag phase (time to 5% germination of spores)
LUBILOSA	LUtte BIologique contre les LOcustes et les SAuteriaux (a French abbreviation for biological control of locust and grasshopper, which is collaborative research programme being executed by IIBC, IITA, CILSS/AGRHYMET and Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ).
MC	Moisture content
MEA	Malt extract agar
MST	Median survival time
N	Number of samples/insects/replicates
Num	Numerator
OE1	Oil-in-water emulsion 1
OE2	Oil-in-water emulsion 2
OF	Oil flowable
OFC	Oil flowable concentrate
PPP	Public Private Partnership
RGR	Roy's Greatest root
RH	Relative humidity

rpm/ rev min^{-1}	Revolution parts per minute
SD	Spray-dried
SE/SEM	Standard error of the mean
sp/spp	Species
SM	Sámsináková medium
SNK	Student-Newman-Keuls Test
TG_{95}	Time for 95% germination of spores
ULV	Ultra low volume
UV	Ultra violet light
Var.	Variety
w/v	Weight by volume
W1	Water based formulation 1
W2	Water based formulation 2

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CHAPTER 1

GENERAL INTRODUCTION

In many countries, especially in the tropics, locusts and grasshoppers are considered to be the major pests in agriculture causing significant damage in many field crops since the biblical times (Geddes, 1990; Steedman, 1990). Under favourable conditions, certain species exhibit gregarious and migratory behaviour, leading to the formation of spectacular swarms (Lomer *et al.*, 2001). The desert locust, *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae) is the most important locust species with an estimated invasion area of some 29 million km^2 affecting 57 countries, which is more than 20% the total land surface of the world (Steedman, 1990). During plagues the desert locust has the potential to damage the livelihood of a tenth of the world's population and in some countries they were reported as the determining factor between sufficient food for the people and starvation (Steedman, 1990). Similarly, the migratory locust, *Locusta migratoria* (R. & F.), the red locust, *Nomadcris septemfasciata* (Serville) and the brown locust, *Locustana pardalina* (Walker) (all belong to Orthoptera: Acrididae) found in most African countries south of the Sahara and during plagues, they cause damage comparable to that of the desert locust (COPR 1982; Steedman, 1990). On the other hand, grasshopper species such as *Hieroglyphus daganensis* (Krauss), *Zonocerus variegatus* (L.), *Cryptocatantops haemorrhoidalis* (Krauss), *Kraussaria angulifera* (Krauss) and *Cataloipus fuscocoerulipes* (Sjöstedt) are regular serious pests of subsistence and cash crops in most African countries (COPR, 1982). Control strategies for locusts and grasshoppers rely almost exclusively on the use of chemical insecticides (Prior and Streett, 1997). For example, the most recent outbreak of locusts and grasshoppers in Africa beginning in 1988 affected 23 nations and over US $ 250 million were spent for control operation and relief for damage caused by locusts (Showler and Potter, 1991; Showler, 1995).

In good production years farmers in Africa harvest and store cereals and pulse crops for several months mostly in a simple storage structures at farm or village levels. However, as in field crops, all stored products are attacked by a wide range of insect pests (Hill, 1983, Subramanyam and Hagstrum, 1995). The maize weevil, *Sitophilus zeamais* (Motsch.) (Coleoptera: Curculionidae), and the larger grain borer, *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) are the two most important and destructive pests of stored grain in the tropics (Dobie *et al.,* 1984; Dick, 1988). The maize weevil was regarded as a cosmopolitan pest in tropical countries while the larger grain borer, native to Central America

and Mexico, was accidentally introduced into East and West Africa between the 1970s and 1980s and became a serious pest of stored maize and cassava (Dick, 1988). The favourable climatic conditions and poor storage systems in Africa often favour growth and development of these pests, resulting in considerable crop losses. For example, losses as high as 40 % were reported on stored maize due to *P. truncatus* and *S. zeamais* (Meikle *et al.*, 1998). In Africa, where subsistence grain production supports the population, such grain losses may be substantial (Golob & Tyler, 1994). In addition to grain weight loss, pests of stored grain also cause secondary fungal infection, resulting in a reduction in seed vigour, quality and commercial value. Synthetic chemical insecticides have been widely used for the control of pests of stored grain.

The widespread use of insecticides for the control of locusts and grasshoppers as well as stored-product insect pests is of global concern with respect to environmental hazards, insecticide resistance development, chemical residues in foodstuffs, side-effects on non-target organisms and the associated high costs (Morallo-Rejesus, 1987; Beeman & Wright, 1990; Krall *et al.*, 1997). To this effect, the increased public awareness and concern for environmental safety has directed research to the development of alternative control strategies such as the use of microbial control agents for locusts and grasshoppers (Inglis *et al.*, 1997; Lomer *et al.*, 2001) as well as for stored-product insect pests (Brower *et al.*, 1995). Entomopathogenic fungi have been shown to be effective biological control agents against several insect pests (Müller-Kögler, 1965; Burges, 1981, Inglis *et al.*, 2001). There are more than 100 genera of fungi that contain numerous species that are pathogenic to insects (Hall & Papierok, 1982; Zimmermann, 1986). However, the most important groups of insect pathogenic fungi presently used in biological control belong to the order Deuteromycotina (Butt and Goettel, 2000; Inglis *et al.*, 2001). This is partly because of their easiness to culture them in *vitro* for mass production and their wide host range (Payne, 1988; Charnley, 1991). Fungi are unique among the insect pathogens in that they infect their hosts primarily through the external insect cuticle although infection through the digestive tract occurs with some species (Butt and Goettel, 2000; Inglis *et al.*, 2001). Their spores attach to the cuticle, germinate, and penetrate the integument by means of a combination of physical pressure and enzymatic degradation of the cuticle. Once the fungus reaches the haemocoel, it grows as hyphal bodies (= blastospores). Host death usually occurs due to a combination of nutrient depletion, invasion of organs and the action of fungal toxins (Butt and Goettel, 2000; Inglis *et al.*, 2001).

Fungal biological control agents (BCAs) are also proved to offer alternative more rapid prospects for implementation (Prior and Greathead, 1989; Prior, 1997). Among these, entomopathogenic fungi such as *Metarhizium anisopliae, Beauveria bassiana*, *B. brongniartii, Paecilomyces fumosoroseus* and *Verticillium lecanii* have shown considerable potential for the management of a variety of insect pests (Inglis *et al.*, 2001). Recently, considerable progress has been made in the development of fungal BCAs and to date there are more than 30 trade-named mycoinsecticide products registered or under-development world-wide (Wraight *et al.*, 2001). As regard to locusts and grasshoppers, two fungi have been developed as commercially available biopesticides. The first is Mycotrol®-GH OF, based on aerial conidia of *B. bassiana* strain GH produced by Mycotech (Wraight and Carruthers, 1999). The other fungal product is Green Muscle™ based on aerial conidia of *M. anisopliae* (*flavoviride*) var. *acridum* strain IMI 330189 and developed by the LUBILOSA research project (Bateman, 1997; Lomer *et al.*, 1999). Both mycoinsectide products are developed as an oil formulation suitable for application by ULV. On the other hand "Boverosil" a powder commercial formulation based on aerial conidia of *B. bassiana* was registered for use in the former Czechoslovakia for the control of residual infestation of stored-product pests in grain stores and silos (Brower *et al.*, 1995).

More recently, submerged spores/conidia of *M. anisopliae* var. *acridum* were successfully produced in liquid culture (Kleespies and Zimmermann, 1992; Jenkins and Prior, 1993; Jenkins and Thomas, 1996; Stephan and Zimmermann, 1998) and dried using various drying techniques (Stephan and Zimmermann, 1998, 2001). Similarly, much progress has been made in recent years on the production and stabilisation of submerged spores/conidia of *B. bassiana* (Rombach *et al.*, 1988). However, the commercial-scale production/stabilisation systems developed so far are not competitive with existing technologies for conidia production (Wraight and Carruthers, 1999). Furthermore, submerged spores/conidia are hydrophilic in nature and it is very difficult to develop a pure oil formulation for ULV application. For the development of microbial control system, new isolates are continuously sought that require characterization and testing against specific target pests. Moreover, studies on the potential use of submerged spores/conidia of *B. bassiana* and *M. anisopliae* for control of storage pests are limited. Therefore, the central aim of this study was to fill the existing information-gap by addressing several pertinent questions with regard to the selection, production, stabilisation and formulation of *B. bassiana* and *M. anisopliae* for control of locusts, grasshoppers and key stored-product insect pests.

Objectives of the study

1. Establish optimal condition for production and processing of *B. bassiana* and *M. anisopliae* submerged spores and submerged conidia and test their suitability for dry powder or oil flowable concentrate formulation development.

2. Develop water-based, emulsions, and oil flowable concentrate formulations of submerged spores/conidia of *M. anisopliae* strain IMI 330189 and evaluate their efficacy against locusts and grasshoppers under laboratory and field conditions. All formulations were compared with the standard mycoinsecticide, Green Muscle™, with respect to the direct and residual infection of *H. daganensis* (HDA), *C. haemorrhoidalis* (CRT), and *L. migratoria* (LMI) (Plate 1).

3. Evaluate the effect of temperature on spore germination and vegetative growth of entomopathogenic Hyphomycetes collected in different agroecological zones (AEZs) of Ethiopia.

4. Investigate the susceptibility of *S. zeamais* and *P. truncatus* (Plate 1) to entomopathogenic fungi from Ethiopia.

5. Develop different dustable powder (DP) formulations based on aerial conidia and submerged spores/conidia of virulent *B. bassiana* and *M. anisopliae* isolates; assess their efficacy against *S. zeamais* and *P. truncatus* in simulated storage condition in the laboratory; investigate the persistence of DP formulations after application on stored grains and analyse their storage properties under different temperature conditions.

In order to address all these specific objectives, a series of experiments were conducted at the Federal Biological Research Centre for Agriculture and Forestry (BBA), Institute for Biological Control, Darmstadt under the frame work of the Public Private Partnership (PPP) project supported by the Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ). The need for alternative stored-product insect pest management strategies for subsistence agriculture in Ethiopia has provided the decisive inspiration for part of this work.

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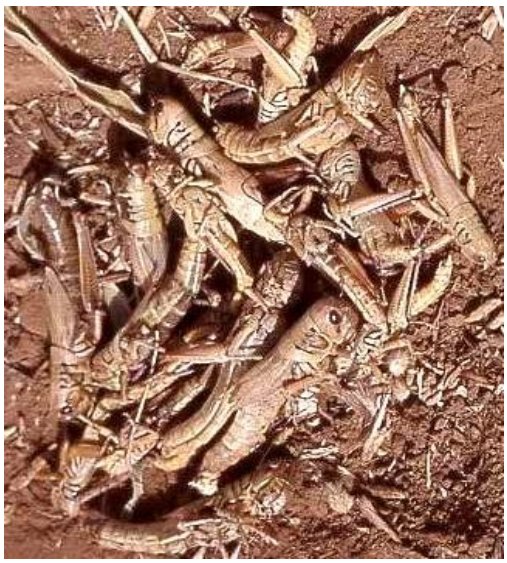
a) *Locusta migratoria,* 4th larval stage.

b) Adult *Cryptocatantops haemorrhoidalis* (Dead).



c) Adult *H. daganensis* (Dead).

d) Adult *Sitophilus zeamais*.

e) Adult *Prostephanus truncatus*.

Plate 1. Test insects used for the laboratory and field studies.

CHAPTER 2

Production and processing of *Metarhizium anisopliae* var. *acridum* submerged conidia for locusts and grasshoppers control

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Currently, mycopesticide development for locusts and grasshoppers control depends on aerial conidia or submerged spores of entomopathogenic fungi. In our study, the production of submerged conidia of *Metarhizium anisopliae* var. *acridum* (IMI 330189) was investigated in a BH liquid medium (3 % biomalt and 1 % yeast extract). The effects of freeze and spray drying techniques on the quality of submerged conidia were determined. Moreover, we assessed the influence of different additives on the viability of fresh submerged conidia and their suitability for oil flowable concentrate formulation development. In a BH medium maintained at 180 rev min^{-1} and at 30 °C for 72 h, IMI 330189 produced a green pigmented biomass of submerged conidia where as in Adámek medium it produced a yellowish biomass of submerged spores. The spore concentration was high in both mediums; however, the size of the spores produced in BH medium was significantly lower than of those produced in Adámek medium ($p < 0.001$). Submerged conidia of IMI 330189 can be effectively dried using either freeze or spray drying techniques. The viability and speed of germination were significantly affected by the drying and pulverizing process ($p < 0.001$). The initial viability was significantly higher for spray-dried submerged conidia than for freeze-dried spores. Pulverizing of freeze-dried submerged conidia reduced the speed of germination and the viability by 63 to 95 %. Dried submerged conidia can be stored over 45 weeks at low temperatures (< 10 °C) without suffering a significant loss in viability. Furthermore we have identified carriers that are suitable for oil flowable concentrate formulation development.

INTRODUCTION

Locusts and grasshoppers are major pests of agricultural crops in tropical and subtropical countries. Up to now, control strategies have been dependent on the use of synthetic chemical insecticides. However, recognition of such associated problems as non-target effects, environmental pollution as well as the high economic costs involved have prompted the development of alternative control strategies (Thomas, Wood & Lomer 1995, Inglis, Johnson & Goettel 1997). Entomopathogenic fungi such as *Metarhizium anisopliae* (Metchnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin have been intensively studied in order to develop commercial mycopesticides for the management of insect pests, including locusts and grasshoppers (Burges 1998, Butt, Jackson, & Magan, 2001). More recently various formulations have also been developed for the control of these specific pests in arid environments (Lomer, Prior & Kooyman 1995, Lomer *et al*. 2001).

Production and formulation technologyies have been developed specifically for aerial conidia of entomopathogenic fungi (Burges 1998, Butt, Jackson, & Magan, 2001). Industrial production of aerial conidia involves either surface cultivation or a two-stage production procedure in which the fungus is allowed to develop under submerged conditions prior to being transferred to a solid or semi-solid media to sporulate (Jenkins & Goettel 1997, Bartlett & Jaronski 1998). Arial conidia are of lipophilic nature, which allows for the development of an oil based formulation suitable for ULV application in arid environments (Bateman, 1993; Burges 1998, Lomer *et al*., 2001). Submerged spores however, which are produced in liquid culture, are hydrophilic, and are equally or even more virulent. (Hall 1981, Jenkins & Goettel 1997, Bartlett & Jaronski 1998, Burges 1998).

Submerged liquid fermentation is advantagous and can be used as an alternative for large scale production of entomopathogenic fungi as fermentation conditions can be controlled and manipulated thus inducing and increasing sporulation (Humphreys *et al*. 1989). The productivity of the strains may be further improved by using different additives during the production process (Adámek 1963, Inch & Trinci 1987, Kleespies & Zimmermann 1992, 1998; Stephan 1998). Stephan & Zimmermann (1998) were able to produce high amount of submerged spores of *M. anisopliae* var. *acridum* (IMI 330189) by using a medium described by Adámek (1963). In further studies the same strain was produced in a fermenter with a capacity of up to 300 l (Stephan *et al*. 2000). Moreover, they were able to improve drying techniques for long term storage of the product. However, submerged spore production in

Adámek medium may result in an increased mycelium biomass production, which affects the subsequent processing of submerged spores for drying and formulation for ULV application. Our research was aimed at evaluating the submerged conidia production of *M. anisopliae* var. *acridum* (IMI 330189) in a newly developed liquid medium. Furthermore, the viability and storability of submerged conidia exposed to different drying techniques using different carriers was evaluated. In addition the effects of different additives on the viability of fresh submerged conidia and their suitability for oil flowable concentrate formulation development was assessed.

MATERIAL AND METHODS

Production of submerged spores

Submerged conidia of *M. anisopliae* var. *acridum* (isolate IMI 330189) were produced in a BH liquid medium containing 3 % biomalt and 1 % yeast extract (FZB Biotechnik, Germany). Adámek medium (3 % cornsteep, 4 % yeast extract, 4 % glucose and 0.4 % Tween 80®) was used as a standard control (Adámek 1963). Aliquots of 50 ml of the medium were autoclaved in Erlenmeyer flasks (250 ml capacity) at 121 °C for 20 min. The sterilised medium was inoculated with 5 x 10^7 conidia and incubated for 72 h in a G24 environmental incubator shaker (New Brunswick Scientific, Germany) at 180 rev min^{-1} and at 30 °C. These submerged spores were used as initial inoculums for experiments and mass production in a laboratory scale reactor.

Flask shake experiments

For the flask shake experiments, the medium was prepared as described above. Each flask containing 50 ml of the medium was inoculated with 5 x 10^7 submerged spores to achieve an initial concentration of 1 x 10^6 ml^{-1}. The inoculated flask cultures were incubated for 72 h in a G24 incubator shaker at 180 rev min^{-1} and at 30 °C. For each medium, four flasks were inoculated and after 72 h, three replicate samples were taken from each flask to count submerged spores/conidia ml^{-1} in a Thoma haemocytometer. Pictures for all spore types were taken using Olympus digital camera mounted on a Zeiss Axioplan microscope (Germany) and spore size was measured using soft imaging analysis system (SIS Germany). Aerial conidia grown on malt extract agar for two weeks at 30 °C were also used for size comparisons. In order to determine the biomass weight, a 25 ml sample was taken from each flask and centrifuged at 9630 g (r_{av} 6 cm for 5 min at 4 °C (Couiter Bioresearch, Germany). Fresh biomass weight and dry weight of the samples were determined using a sensitive balance and

a Sartorius moisture tester (Sartorius AG, Germany), respectively. The experiment was repeated twice.

Following a similar procedure, 3 l of BH medium were prepared and poured in a 3 l capacity fermenter (Infors, Switzerland) and autoclaved as described previously. The fermenter was inoculated with 3 x 10^9 submerged spores and incubated for 72 h at 600 rev min^{-1} and at 30 °C. After 72 h the biomass was harvested and centrifuged for 5 min at 9630 g (r_{av} 6 cm) and at 4 °C. The pellets were re-suspended in 200 ml deionised water and homogenised using a magnetic stirrer. Volume, fresh weight, dry weight and spore concentration of the fresh biomass were determined before the drying process. The total amount of fresh biomass obtained in each fermentation cycle was divided into two equal parts and used for the differing drying processes. The experiment was repeated four times. The FZB carried out a separate production in a 300 l fermenter. This biomass was used for the freeze-drying experiment in order to select the most suitable carriers to dry submerged conidia for an oil flowable concentrate formulation.

Comparison of different drying techniques

Spray-drying

Submerged conidia produced in BH medium were formulated with skimed milk powder and molasses at a ratio of 1:1:0.08 by wt and subsequently homogenised using an ultra-turrax (Jank & Kunkel KG, Germany) at a low speed. The ratio of mixtures was based on the dry weight of the fresh biomass of submerged conidia (Stephan & Zimmermann 1998, 2001). A laboratory spray dryer (Büchi 190 Mini Spray Dryer, Swizerland) was used for drying. Inlet and outlet temperature were adjusted to 64 ± 2 °C and 48 ± °C, respectively. During the spray–drying process, the flow of compressed air for the spray nozzle was 600 NL h^{-1}. The experiment was repeated four times.

Freeze-drying

For freeze-drying, fresh submerged conidia were formulated with Skimmed milk powder and glycerol at a ratio of 1:0.4:0.16 by wt and homogenised as described for the spray-drying process. Freeze-drying was carried out using a GKF L 05-60 freeze dryer (WKF, Germany). A 100 g of formulated submerged conidia were poured onto a plate and frozen using liquid nitrogen. The frozen material was then transferred to a freeze-dryer. The heating temperature of the plate was adjusted to 40 °C for the first 3 h and then reduced to 30 °C for the next 21 h. The drying process was terminated after 24 h when the temperature of the dried material

reached 25 °C. A portion of freeze-dried submerged conidia were pulverized through a 120 µm mesh sieve, type ZMI 1000 (Retsch GmbH & Co. KG Haan, Germany). The experiment was repeated four times.

The moisture content, the spore yield and the viability of submerged conidia were measured for all products. The moisture content was determined using a Sartorius moisture tester (Sartorius AG, Germany) and 2 g samples were used each time. For counting submerged conidia, 1 g (DW basis) of the respective product was suspended in 100 ml water and sonicated for 3 min using Sonorex RK 52 (Bandelin electronic, Germany). The spore concentration was counted using a haemocytometer in samples diluted to 1:100 and three samples were counted for the respective treatments at each drying process. The viabilities of fresh and dried submerged conidia were assessed using water agar (1.5 % agar amended with 30 mg l^{-1} of streptomycin sulphate, 50 mg l^{-1} chloramphenicol and 0.005 % Benomyl). Submerged conidia from the different treatments were suspended in deionised water containing Tween 80® (0.01 % by vol.) and the concentration was adjusted to 1M ml^{-1}. Several drops of a 2µl spore suspension were dropped onto the agar plate and incubated at 25 °C .Viability was assessed after 8 h, 16 h and 24 h incubation period by counting 300 spores in each sample using a Zeiss Axioplan microscope (Germany). Staining with lactophenol cotton blue was carried out if necessary. The test was repeated four times and each time three samples were taken in order to assess the viability.

Testing different carriers for drying submerged conidia

The freeze-dried submerged spores product currently in use was developed based on Skimed milk and glycerol carriers. This product is hydrophilic in nature and extremely difficult to mix with oil for ULV application. In order to modify these properties, 16 different carriers, obtained from different sources were tested for influence on the viability of freshly produced submerged conidia (Table 2). Various concentrations (0.01 to 100 % by vol. carrier in deionised water) were prepared and mixed with submerged conidia. Viability was tested as described previously. Only those carriers which had no influence on the viability of submerged conidia were chosen for the freeze-drying experiment. Accordingly, nine different formulations were prepared and freeze-dried using a Christ LMC-3 ESP 2-90 drier (Martin Christ Gefriertrocknungsanlagen, Germany). After drying, moisture content, colony forming unit, milling property and suitability for oil flowable concentrate formulations were assessed. The experiment was repeated once. In a further experiment, submerged conidia were

formulated with lignin (Lignosulfonic acid: sodium salt, Sigma, USA) and freeze or spray dried. These formulations were compared with the standard Skimed milk powder based formulation for viability and spore yield. Viability, spore yield, and moisture content of the different formulations were determined using the methods described previously. The experiment was repeated four times.

Long term storage experiment

Samples of spray and freeze dried submerged conidia were placed separately in aluminium bags containing silica gel (AppliChem, Germany). The bags were sealed and stored at 10 °C, 20 °C or 30 °C for 45 weeks. Four (for spray-dried) and three (for freeze-dried) sub-samples were drawn at intervals and after which the bag was re-sealed and maintained at the respective storage temperature. The viability of the spores in each sample was assessed following the procedure described previously.

Statistical analysis

Spore concentration and spore germination data were transformed into a logarithmic and arcsine scale, respectively. All data were analysed with analysis of variance using the PROC GLM procedure and the treatment means were separated using Student-Newman-Keuls (SNK) test (SAS 1989).

RESULTS

Production of submerged spores/conidia

The two media tested differed significantly in total biomass production ($F = 90.62$, $D.F. = 1$, $p < 0.001$) (Figure 1a). Adámek medium resulted in a higher (> 68 %) biomass production as compared to the BH medium. As regards spore production, neither the Adámek nor the BH medium revealed a significant variability in the mean number of spores produced per gram of dry biomass weight ($F = 0{,}15$, $D.F. = 1$, $p < 0.707$) (Figure 1a). However, we observed that spores produced in BH medium differed to those produced in Adámek medium with respect to particular morphological parameters. In BH medium, *M. anisopliae* var. *acridum* was able to produce green pigmented biomass of submerged conidia if the culture was maintained at 180 rev min^{-1} and at 30 °C. In contrast, in Adámek medium it produced submerged spores of a yellowish colour and excessive mycelium biomass under the same conditions. The comparison of spores produced in liquid culture (BH or Adámek medium) and solid substrate

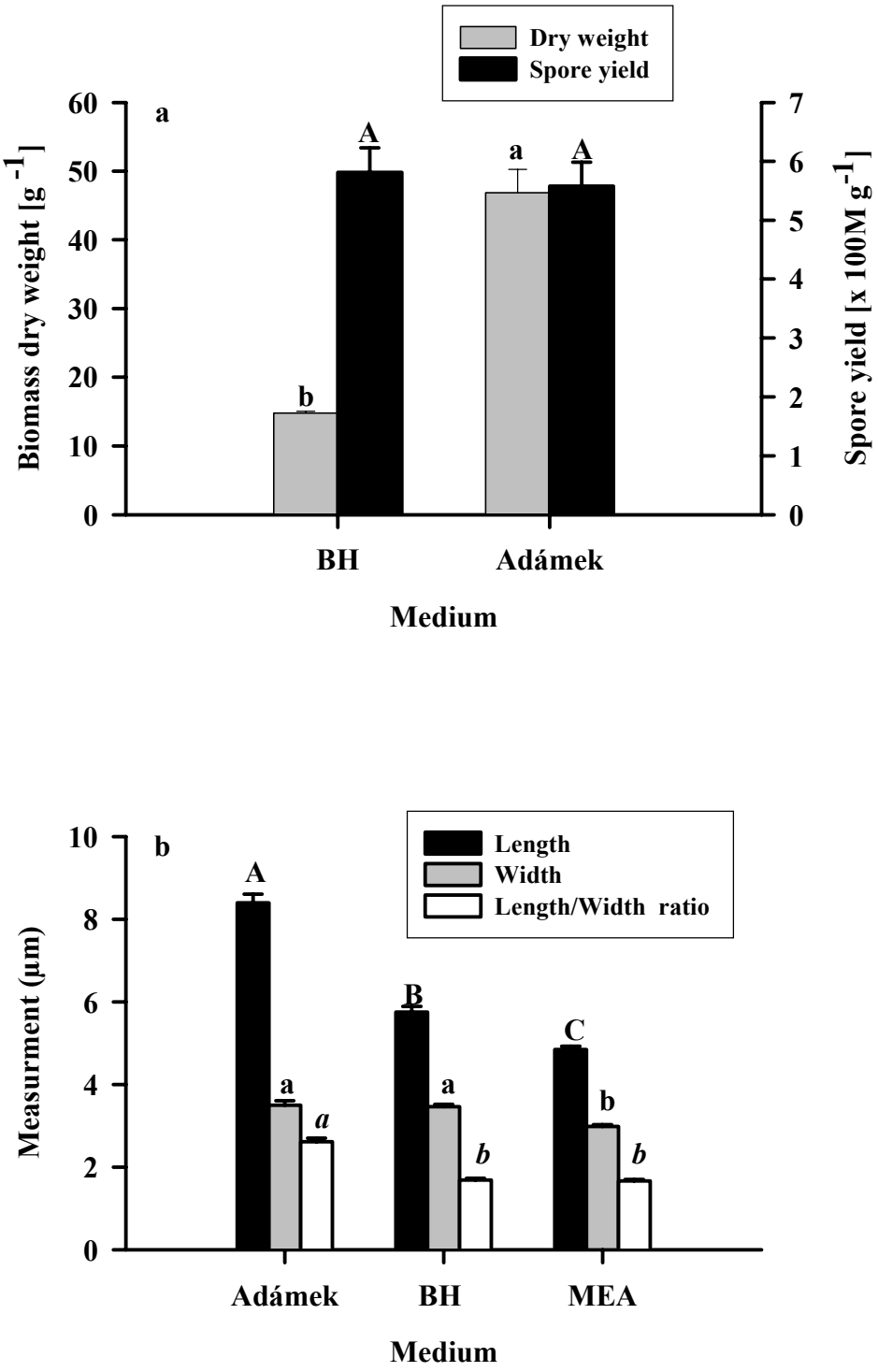

Figure 1. Mean biomass dry weight and spore concentration (a) and size (b) of *M. anisopliae* var.*acridum* (IMI 330189) produced in different liquid media after 72 h fermentation at 180 rev min^{-1} and at 30 °C (Bar ± SEM). Bars within a group followed by the same letters are not different ($p < 0.001$).

(malt extract agar) revealed clear differences in size and morphology (Figure 1b). Submerged spores produced in Adámek medium were much longer than those produced in BH medium as

well as from aerial conidia produced in malt extract agar (F = 136.65, $D.F.$ = 2, $p < 0.001$) (Figure 1b). Aerial conidia produced in malt extract agar showed reduced width compared to the other spore types (F = 11.59, $D.F.$ = 2, $p < 0.001$) (Figure 1b). Submerged spores produced in Adámek medium, exhibited a higher length to width ratio than those aerial and submerged conidia produced in malt extract agar and BH medium, respectively (F = 77.94, $D.F.$ = 2, $p < 0.001$) (Figure 1b). Spores produced in BH medium closely resembled aerial conidia in size and morphology where as submerged spores produced in Adámek medium were longer and relatively slender in shape. Following this basic production cycles, production was scaled-up to a 3 l fermenter using a BH medium. The results were comparable to the flask shake experiments in total number of spores produced, colour of the biomass after three days as well as in size and shape of submerged conidia (Data not shown). After 52 h the pigmentation of the culture started to change and was completely green after 72 h. The final biomass contained green submerged conidia and a small amount of mycelium.

Drying

The viability and speed of germination of submerged conidia of *M. anisopliae* var. *acridum* were affected significantly by the drying and pulverizing process ($p < 0.001$) (Table 1). The

Table 1. Effect of different drying techniques on moisture content, spore yield, viability and speed of germination of submerged spores of *M. anisopliae* var. *acridum* (IMI 330189) produced in BH medium (mean ± SEM, N = 4).

			Germination rate (%) at 25 °C[3]		
Drying method	Moisture content (%)[1]	Spore yield[2] x 10^{10} g^{-1}	8 h	16 h	24 h
Fresh submerged spore	91.04 (0.30)	7.02 (0.60) a	50.91 (2.43)a	97.47 (0.22)a	98.39 (0.18)a
Spray dried	5.02 (0.47)	3.85 (0.16) c	32.04 (3.41)b	87.12 (1.64)b	92.66 (0.69)b
Freeze dried	5.47 (0.64)	4.69 (0.17) b	17.97 (3.47)c	57.53 (0.80)c	66.64 (1.09)c
Freeze dried and pulverized	5.37 (0.68)	-	2.47 (0.20)d	20.91 (1.06)d	36.32 (2.19)d
P value		< 0.001	< 0.001	< 0.001	< 0.001
R^2		0.82	0.94	0.98	0.98
F-value		45.05	140.66	693.92	452.42

Means followed by the same letter within the same column are not significantly different (PROC GLM, SNK test).

[1] Statistical analysis was not performed.

[2] Analysis was done on logarithmic scale transformed values.

[3] Analysis was done on arcsine √ percentage transformed values

Values in parenthesis represent standard error of the mean.

most rapid and the highest rate of germination could be observed for un-dried fresh conidia followed by spray-dried submerged conidia (Table 1). Freeze-drying significantly reduced the speed of germination as well as the total viability by 32 to 65 % when compared to un-dried fresh conidia. When the freeze-dried submerged conidia were ground to fine powder, speed of germination and viability were reduced by 63 to 95 %. Moisture content of spray or freeze dried submerged conidia remained lower than 6 %. The spore concentration of the two dried products was reduced by nearly 50 % compared to the un-dried fresh submerged conidia.

Testing different carriers

Of the different carriers tested, 11 showed no influence on the viability of submerged conidia. Only 5 of them were however could be easily suspended or dissolved in diesel oil (Table 2). Those carriers with no negative effects were selected and different formulations were developed and freeze-dried. None of the new formulations however performed better than the standard formulation (Skimed milk powder + glycerol) as regards spore concentration, viability, pulverizing properties and suitability for an oil flowable concentrate formulation (data not shown).

In terms of spore yield, Skimed milk powder and lignin based formulations did not show variability, however, all are significantly different from fresh submerged conidia ($p = 0.05$) (Table 3). Fresh submerged conidia showed a significantly faster and higher germination rate than spray or freeze dried lignin based formulations ($p < 0.001$), followed by the standard Skimed milk powder formulation (Table 3). Spray-dried lignin based formulations of submerged conidia showed a relatively higher (58 %) germination rate compared to freeze-dried lignin based formulations, which gave a viability of lower than 41 % (Table 3).

Long term storage

Drying methods, storage temperature, storage duration and their interaction significantly affect the viability and speed of germination of submerged conidia (Table 4). Spray-dried submerged conidia displayed the highest initial viability as compared to freeze-dried submerged conidia (Figure 2a). When both spray and freeze dried submerged conidia were stored at 30 °C, the viability and speed of germination were higher for spray-dried submerged conidia (Figure 2a- b). When stored up to 45 weeks at 10 °C, there was only a small decline (9 %) in the viability of spray-dried submerged conidia (Figure 2a). Spray-dried submerged conidia stored at 20 °C or 30 °C, in contrast, showed reduction in viability of 19 and 23 %, respectively (Figure 2a).

There was no reduction in the viability of freeze-dried submerged conidia after 45 weeks of storage at 10 and 20 °C. A 16 % reduction in the viability was observed when freeze-dried submerged conidia were stored at 30 °C. Prolonged storage of dried submerged conidia at different temperatures also affected the speed of germination (Table 4).

Table 2. Effect of different carriers on the viability of fresh submerged spores of *M. anisopliae var. acridum*, (IMI 330189) and their suitability for oil flowable concentrate formulations.

No.	Carriers	Effect on viability of submerged spores	Compatibility with diesel fuel	Remarks
1	Plurafac LF223	No effect	+	Suitable for OFC formulation
2	P3- Ferrolin 8648	Lethal effect	-	Dissolve the Skimed milk powder (Form viscous fluid (good for emulsion)
3	Texapon (BASF)	Lethal effect	-	Eye irritant, difficult to mix
4	Plurafac LF400	Fast growth	+	Suitable for OFC formulation
5	Bioweb Dr 19	Retarded growth	-	Fast Phase separation
6	Lutensol ON 70	Lethal effect	+	Suitable for OFC formulation, Dissolve the Skimed milk powder
7	Emulgin RO 40	No effect	-	For emulsion
8	Texapon K12	No effect	+	Eye irritant, suspended in oil, dissolved in water
9	Propylene carbonate	No effect	+	Activator for Bentone 38
10	Bentone 38	No effect	+	Suspender in oil matrix
11	Telmion	Fast growth	-	Emulsified oil for temperate climate, good for emulsions
12	Lecithin	Fast growth	-	Emulsifier, Good for emulsions
13	Addit	No effect	-	Emulsified oil, spreader
14	Experimental oil	No effect	-	Emulsified oil, sticker
15	Codacide	No effect	-	Emulsified oil, sticker
16	Nu film	> 1% Lethal effect < 1 % v/v no effect	-	Wetting agent with water
17	Citowett	> 5% v/v lethal effect	-	Wetting agent with water

+ Compatible and – incompatible for oil flowable concentrate formulation.
Codacide and experimental oil are from Microcide Ltd. (UK), Addit from Koppert (The Netherlands), BENTONE ® 38 from Rheox Inc. (UK). Propylene carbonate and Lecithin from Sigma Co. Ltd (Germany). Citowett and Telmion from BASF (Germany). Nu film from Dr. Scaette, (Germany); all other materials from FZB Biotechnik, Germany.

Table 3. Effect of lignin based carriers and drying techniques on moisture content, spore yield, viability and speed of germination of submerged spores of *M. anisopliae* var. *acridum* (IMI 330189) (Mean, N = 4 ± SEM).

Carrier used	MC (%)[1]	Spore yield[2] x 10^{10} g^{-1}	Germination rate at 25 °C[3]		
			8 h	16 h	24 h
Fresh submerged spore	86.40	6.92 (0.24)a	76.80 (0.04)a	93.33 (0.88)a	99.00 (0.58)a
Skimed milk powder freeze dried (1:0.4:0.16)	12.6	5.85 (0.09)b	44.53 (3.84)b	66.63 (2.55)b	71.67 (3.53) b
Lignin spray dried (1:1:0.08)	5.25	5.76 (0.11)b	16.00 (0.00)c	45.00 (1.73)c	58.00 (0.50) c
Lignin freeze dried (1:0.4:0.16)	3.60	5.96 (0.28)b	15.43 (1.27)c	40.43 (4.95)c	40.57 (3.78) d
Lignin freeze dried (1:0.33:0.03)	3.76	5.76 (0.15)b	1.5 (0.10)d	11.00 (2.08)d	18.33 (2.19) e
P value		0.0362	< 0.001	< 0,001	< 0.001
R		0.70	0.99	0,99	0.98
F-value		4.38	252.8	144,73	129.10

Means followed by the same letter within the same column are not significantly different (PROC GLM, SNK test). Values in parenthesis represent standard error of the mean. Lignin stands for Lignosulfonic acid, sodium salt (Sigma, USA).

[1] Statistical analysis was not performed.

[2] Analysis was done on logarithmic scale transformed values.

[3] Analysis was done on arcsine √ percentage transformed values

Table 4. Results of a four-way ANOVA on the effects of drying, temperature and storage duration on the viability and speed of germination of *M. anisopliae* var. *acridum,* (IMI 330189).

Source	DF	Mean square	F value	Pr > F
Drying (D)	1	2.440	1911.57	< 0.0001
Temperature (T)	2	0.875	685.65	< 0.0001
Storage duration (SD)	4	0.026	20.20	< 0.0001
Speed of germination (SG)	2	5.861	4591.9	< 0.0001
D*SD*T*SG	52	0.0118	9.26	< 0.0001
D*T	2	0.006	4.65	0.0098
D*SD	4	0.010	7.86	< 0.0001
D*SG	2	0.049	38,28	< 0.0001
SD*T	8	0.001	0.43	0.9005
SD*SG	8	0.009	7.64	< 0.0001
T*SG	4	0.197	154.72	< 0.0001

DISCUSSION

Submerged conidia of *M. anisopliae* var. *acridum* (IMI330189) were produced in a newly developed BH liquid medium (3 % biomalt and 1 % yeast extract). Production was optimal and most consistent for cultures maintained at 180 rev min^{-1} and at 30 °C. Production of submerged conidia by *M. anisopliae* (= *M. flavoviride*) in a simple liquid medium was first described by Jenkins & Prior (1993). Generally, filamentous fungi seem to produce submerged conidia under conditions that limit vegetative growth (Hegedus *et al*. 1990, Bosch & Yantorno 1999). Manipulation of cultural and nutritional parameters can be exploited to favour the production of either submerged spores or submerged conidia (Hegedus *et al*. 1990). Sporulation for example can be induced by the exhaustion of the carbohydrate source in *Aspergillus niger* (Galbraith & Smith 1969) or by the depletion of the nitrogen source in *A. nidulans* (Carter & Bull 1969). In *B. bassiana,* submerged conidia production is associated with water activity (Inch & Trinci 1987, Humphreys *et al*. 1989) or with phosphate carbon and nitrogen levels (Thomas, Khachatourians & Ingledew 1987, Hegedus *et al*. 1990, Bosch & Yantorno 1999). The medium described by Adámek (1963) is a nutrient rich medium which induces the formation of submerged spores and large amounts of hyphal bodies. The morphological characteristics as well as the protein analysis provide further supportive evidence for the distinctive nature of submerged spores, aerial and submerged conidia of *M. anisopliae* var. *acridium* (IMI 330189) produced in different liquid cultures (Mager, unpublished data). Our research thus underlines the importance of medium selection and incubation temperature for the production of submerged conidia.

Submerged conidia of *M. anisopliae* var. *acridum* survived both the freeze-drying and spray-drying processes. However, a reduction in their viability and efficacy is apparent when compared with un-dried fresh submerged conidia. Similar results have been reported by Stephan & Zimmermann (2001) with regard to submerged spores of the same strain. Furthermore, our research demonstrates that the viability and the efficacy of freeze-dried submerged conidia is significantly reduced when ground to fine powder through a 120 µm mesh. In comparison to freeze-dried products, spray-dried submerged conidia maintained a significantly higher viability (> 90 %), although their germination rate was slightly retarded as compared to un-dried fresh submerged conidia. In this respect our results agree with those of Stephan & Zimmermann (1998). For appropriate product development, the viability of

submerged conidia should at least exceed 80 % and should maintain a high efficacy. As spray-drying is one of the most important industrial drying techniques it is crucial when

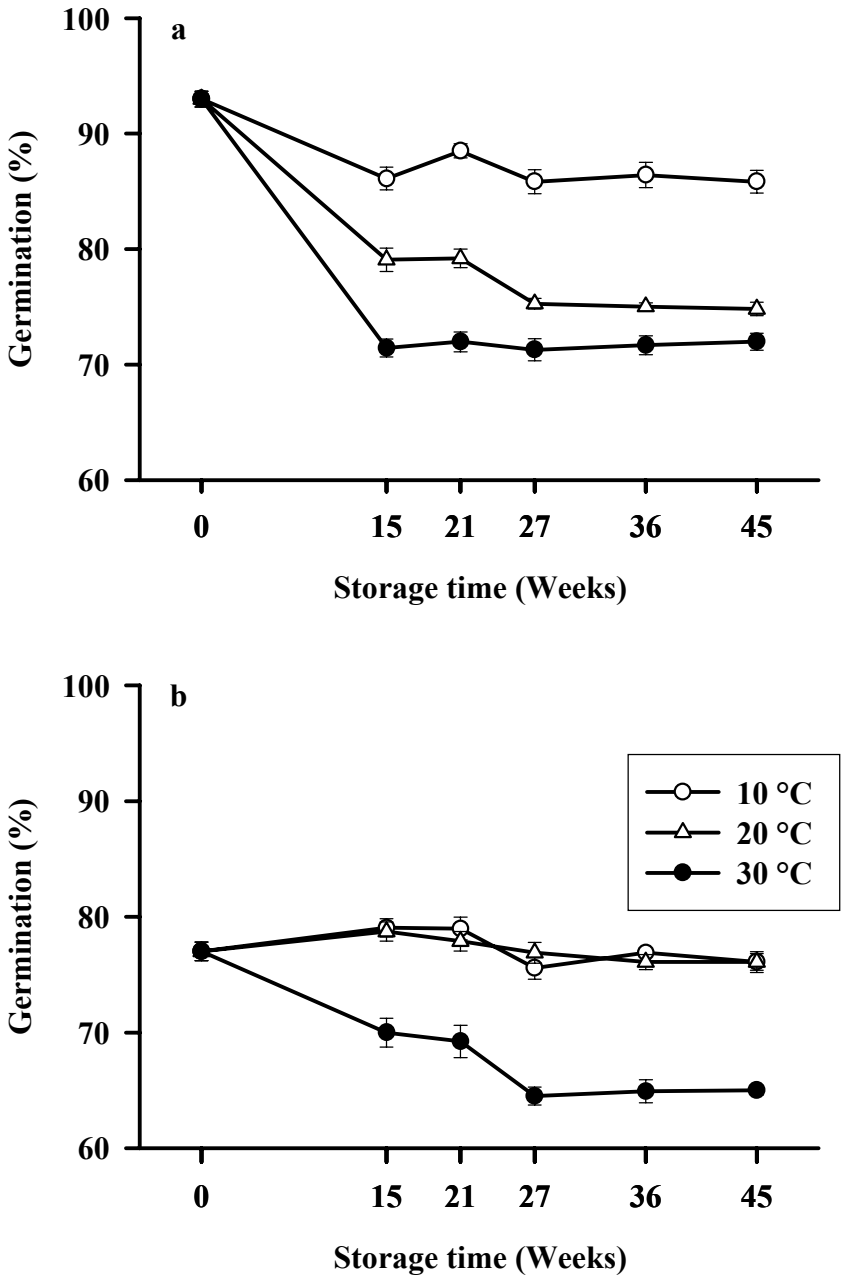

Figure 2. Viability of spray-dried (a) and freeze-dried (b) submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189) stored at different temperatures. (Bar ± SEM).

spray-drying microorganisms, to find appropriate protectants and temperature regimes in order to allow for drying without loss of viability (Stephan & Zimmermann 1998). In this respect the protectants and the spray-drying procedure used in our study are optimal for *M. anisopliae* var. *acridum* (IMI 330189). On the other hand, the reduced viability of freeze-

dried or freeze-dried and pulverized submerged conidia indicates the necessity to search for suitable protectants and of further improving the drying processes.

Leland *et al*. (2001) reported a viability of over 80 % for submerged spores of *M. anisopliae* var. *acridum* (IMI 330189) when freeze-dried with lignin protectants. In our research, however, none of the new carriers tested including the lignin product, performed better than the standard skimmed milk powder and glycerol based protectants. Spray-drying of *Beauveria bassiana* or *Metarhizium anisopliae* without the use of protectants results in complete loss of spore viability (Feng, Poprawski & Khachatourians 1994, Stephan & Zimmermann 1998). Our study showed that freeze-drying of *M. anisopliae* var. *acridum* (IMI 330189) submerged conidia separately caused a significant reduction (> 82 %) in the viability of the spores which would be critical for ULV application. Similar results have been reported by Leland *et al*. (2001).

The results of the long term storage experiment revealed that both freeze and spray-dried submerged conidia of *M. anisopliae* var. *acridum* can be stored at low temperatures (< 10 °C) for at least 11 months without suffering from a significant loss of viability. Long term storage at 20 °C results in a slight but significant reduction in the viability and speed of germination of spray-dried submerged conidia, while there was no reduction in freeze-dried ones. At 30°C, about 70 % and 60–70 % of spray-dried and freeze-dried submerged conidia are viable after nearly one year. Similar results have been reported previously (Stephan & Zimmermann 2001). Freeze-drying significantly reduced the viability and speed of germination. Those spores however, which were able to survive the drying process can be stored for even longer periods. It was possible to dry submerged conidia to a moisture level lower than 6 % using either spray-drying or freeze-drying techniques and this increased survival during storage. The results from other research work emphasized the importance of reducing conidial moisture content to approximately 5 % for long term survival and high temperature tolerance of some entomopathogenic fungi species (McClatchie *et al*. 1994, Hedgecock *et al*. 1995, Moore & Caudwell 1997). Couch & Ignoffo (1981) suggested a minimum shelf life of at least 18 months for microbial pesticides. With regard to locusts and grasshoppers, Moore & Caudwell (1997) suggested a shelf life of 3 to 6 months for mycoinsecticides and this agrees with our findings.

In conclusion, submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189) can be mass produced in a BH medium under specific culturing conditions. Moreover, submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189) can be effectively dried using either freeze-drying or spray-drying techniques. Storage at low temperatures (< 10 °C) is possible for long periods without significant loss in viability. The initial viability of spores remains higher for spray-dried submerged spores. In addition to retaining their viability during storage, microbial control agents must also maintain their virulence towards their target hosts (Burges 1998). Follow up research has indicated that dried submerged conidia formulated in oil flowable concentrate and emulsions show good control efficacy against locusts and grasshoppers in the laboratory and under field conditions (Kassa *et al*. unpublished).

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Plate 1. Submerged conidia production in liquid culture. A) Flask shaker culture, B) 3 L fermenter and C) 300 L fermenter.

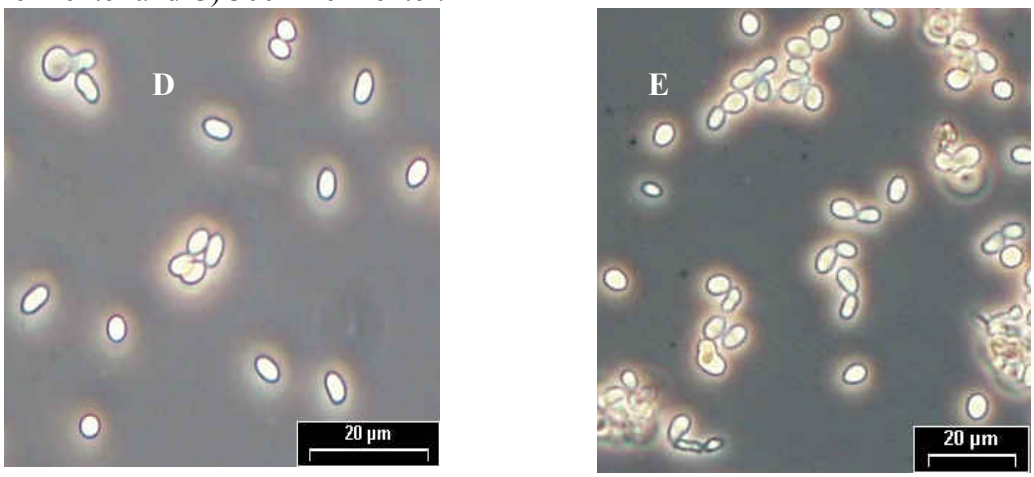

Plate 2. Aerial conidia (D) and submerged conidia (E) of *M. anisopliae* var *acridum* IMI 330189 produced in MEA and BH medium, respectively.

Plate 3. Spray-dryer (F) and freeze-dryer (G) machines used for drying of submerged conidia.

CHAPTER 3

Laboratory and field evaluation of different formulations of *Metarhizium anisopliae* var. *acridum* submerged spores and aerial conidia for the control of locusts and grasshoppers

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Two emulsions and two water-based-formulations of freeze dried submerged spores of *Metarhizium anisopliae* var. *acridum* (Metch.) Sorokin (isolate IMI 330189) were compared with aerial conidia (GREEN MUSCLE™) for their efficacy against *Hieroglyphus daganensis* (Krauss) and *Locusta migratoria* (R. & F.). The field experiments were conducted in East Niger on *H. daganensis* whereas the laboratory investigations were carried out in Germany using *L. migratoria*. In the fields, all formulations were applied on one hectare plots using ULV application techniques. Direct and residual spray effects were assessed. In all cases there were highly significant ($p < 0.001$) differences between formulations as regards to total mortalities and median survival times (MST). In both direct and spray residue effect assessments, aerial conidia formulated in diesel oil showed over 95 % mortality with significantly shorter MST (3 to 8 days) under field conditions. These were followed by emulsions of submerged spores, which resulted in a mortality ranging from 56 to 92 % (MST = 8 to 16 days) for the direct spray and 90 to 97 % (MST = 7 to 12 days) for spray residue effect assessments. Experiments in the laboratory positively confirmed these results. There were no apparent differences between water-based formulations and the control with respect to mortality and MST. These results emphasize the importance of employing oil carriers to protect spores from environmental stress and thus enhance efficacy. The study also demonstrated the importance of secondary spore pick up from the spray residues. Spores in all formulations persisted over five days and caused mortalities ranging from 62 to 100 % on healthy grasshoppers exposed to the spray residue from treated vegetation. The results of this research suggest that emulsions may be an effective option to improve efficacy of submerged spores for ultra low volume application under Sahelian conditions.

Key words: *conidia*, *emulsion*, *formulation*, Hieroglyphus daganensis; Locusta migratoria, Metarhizium anisopliae *var*. acridum, *mycoinsecticide*, *submerged spores, ULV*, *water-based formulation*

Introduction

Locusts and grasshoppers represent the most conspicuous of all insect pests and are abundant insects of dry grassland and desert. When populations of these insects increase, certain species exhibit gregarious and migratory behaviour, leading to the formation of spectacular swarms (Lomer et al., 2001). Locusts and grasshoppers cause significant damage throughout Africa, the Middle East and Australia, parts of Asia and North and South America (Office of Technology Assessment, 1990). During the last major outbreak of the desert locust (*Schistocerca gregaria* Forskål) (Orthoptera: Acrididae) in the Sahel between 1986 and 1989, over US $ 200 million were spent on the application of 15 million litres of chemical pesticides to control grasshoppers and locusts (Symmons, 1992).

Control strategies for locusts and grasshoppers rely to a large extent on the use of chemical insecticides. However, increased concern as to their impact on the environment and human health has directed research towards the development of microbial insecticides for the control of locusts and grasshoppers (Johnson and Goettel, 1993; Prior and Streett 1997; Lomer et al., 2001). With this objective, the LUBILOSA (LUtte BIologique contre les LOcustes et les SAuteriaux) project has developed a biological product, GREEN MUSCLE™, based on an oil formulation of aerial conidia of *Metarhizium anisopliae* var. *acridum* (Deuteromycotina: Hyphomycetes), isolate IMI 330189 (Bateman, 1997; Lomer et al., 1999; Lomer et al., 2001). The effectiveness of oil-based formulations of aerial conidia has been reported for a range of locust and grasshopper species(Bateman et al., 1994; Douro-Kpindou et al., 1995; Jenkins and Thomas, 1996; Kooyman et al., 1997; Kooyman and Godonou, 1997; Langewald et al., 1997; Lomer et al., 1997; Thomas et al., 1998). However, large scale production of aerial conidia requires much time and is expensive (Cherry et al., 1999). Biological control of locusts and grasshoppers using entomopathogenic fungi will only become feasible if economic methods of mass production are available (Kleespies and Zimmermann, 1992). Recent investigations indicated the potential use of submerged spores and/or conidia of the strain IMI 330189 for the control of locusts and grasshoppers (Jenkins and Prior, 1993; Jenkins and Thomas, 1996; Stephan et al., 1997). Submerged spores can be produced in a single step using liquid fermentation, and industrial scale-up could be enhanced, compared with solid-state production of aerial conidia (Jenkins and Goettel, 1997; Stephan, 1998; Burges, 1998). However, the development of both persistent and effective formulations for submerged spores still remains a key problem. Because of their hydrophilic properties, submerged spores are more difficult to

formulate in pure oil than the corresponding aerial conidia (Jenkins and Thomas, 1996; Burges, 1998).

To alleviate the formulation and storage problems of submerged spores, (Stephan and Zimmermann, 1998) developed various drying techniques. They demonstrated that spray dried submerged spores of *M. anisopliae* can be stored up to four years at 5 °C without a significant reduction in the viability of spores (Stephan and Zimmermann, 2001). Furthermore, the authors revealed the possibility of formulating spray-dried submerged spores of *M. anisopliae* var. *acridum* as water-based, oil-in-water emulsion and oil formulation for use in ultra low volume (ULV) spray applications for the control of the desert locust, *Schistocerca gregaria* in dry areas such as Mauritania. Earlier, (Jenkins and Thomas, 1996), targeting the variegated grasshopper, *Zonocerus* variegatus (L.) (Orthoptera: Acrididae) and the desert locust, *S. gregaria*, used submerged conidia formulated either in water or oil-in-water emulsion for use at higher volume application rates under humid conditions. The work of Lomer et al. (1997) on the rice grasshopper, *Hieroglyphus daganensis* Krauss (Orthoptera: Acrididae) suggested the possible advantage of water-based ULV formulations to improve the initial impact of a mycopesticide. Our research, which is a continuation of the work of Stephan et al. (1997) within a Public Private Partnership (PPP) Project, focussed on the development of emulsions and water-based ULV formulations of submerged spores of *M. anisopliae* var. *acridum* isolate IMI 330189 for use under Sahelian conditions. We evaluated four different formulations of freeze-dried submerged spores with regard to direct and residual infection of *Hieroglyphus daganensis* Krauss and the migratory locust, *Locusta migratoria* (R. & F.) (Orthoptera: Acrididae) under field and laboratory conditions, respectively, and compared these with the efficacy of aerial conidia formulated in diesel oil.

Material and methods

Field experiments

Experimental site and field layout

Research was carried out in the Thaua area, East Niger in October and November 1999 at the end of the rainy season. The site was positioned at 15º 27′ 55″ N and 05º 37′ 83″ E at an altitude of 500 m (a. s. l.). This experimental site was selected because of a high population density of *H. daganensis* (15-20 reproductive adults/m^2) and its accessibility. A mixture of sorghum and pearl millet predominantly covered the site. Six 100 m x 100 m (1 ha) plots,

each separated by at least 75-100 m, were selected and each plot was assigned to a different treatment. All plots shared a high plant density with plant height reaching up to four meters.

Products tested

Freeze-dried submerged spores and aerial conidia (GREEN MUSCLE™) of *Metarhizium anisopliae* var. *acridum* (isolate IMI 330189) were used for this study. The freeze dried product of submerged spores was obtained from FZB Biotechnik GmbH, Germany and gently crushed by hand and sieved to pass through a 125 µm mesh using a vibrotronic sieve (Retsch GmBH, Germany). The sieved powder contained 4 x 10^{10} viable submerged spores g^{-1} and samples were kept at 4 °C until required. GREEN MUSCLE™ (aerial conidia) is a standard bio-pesticide developed by the LUBILOSA research team (Bateman et al., 1993; Jenkins and Thomas, 1996; Kooyman et al., 1997; Langewald et al., 1999). The samples used in this study were obtained from LUBILOSA/AGRYMET in Niamey and had been stored for approximately 16 months at -8 °C. At the time of packing, the product contained over 4.5 x 10^{10} viable conidia g^{-1} (LUBILOSA, 1999). Shortly after application, germination rate ranging from 65–75% and 65–71% was observed for aerial conidia and submerged spores, respectively.

Formulation and application

For submerged spores, two oil-in-water emulsions and two water-based formulations were prepared (Table 1) and compared to standard ultra low volume (ULV) applications of aerial conidia (AC) formulated in diesel oil. All formulations were prepared the night before application and subsequently stored at 4 °C. An oil and water sensitive fluorescent tracer (Lumogen) was used at 2.5 % w/v for oil or water based formulations, respectively. Submerged spores were applied at 1 x 10^{13} viable spores/ha and aerial conidia were applied at

Table 1. Type of formulations tested and their compositions.

Formulation code	Compositions (w/v)	Formulation type
W1	4.3 % submerged spores in water	Water-based
W2	4.3 % submerged spores, 20 % molasses, water	Water-based
OE1	4.3 % submerged spores, 15 % Telmion*, water	Emulsion
OE2	4.3 % submerged spores, 1 % lecithin (Sigma, USA), 10 % soya bean oil, water	Emulsion
AC	3.3 % aerial conidia (GREEN MUSCLE™), diesel oil	Oil

* Telmion, is a commercial product, containing 85 % vegetable oil + 15 % emulsifier (Temmen, Germany).

5 x 10^{12} conidia/ha. The formulations were applied in 3 l/ha, with the exception of OE1, which was applied at 6 l/ha. All treatments were applied on successive days using ULVA-Plus hand held sprayer (Micron Sprayers Ltd., Bromyard, UK) at a flow rate of 70 ml/min. adjusted by appropriate nozzles. The formulations were sprayed before 11:00 a.m. at wind speeds varying between 7 and 10 km/h. During spraying, temperature ranged from 31 to 44 °C and the relative humidity varied between 54 % early in the morning to 14 % towards mid-day. The control plots were left unsprayed. Water and oil-sensitive paper-cards (CIBA-GEIGY LIMITED, Switzerland) were used to assess the spray coverage. Twelve sensitive paper-cards were placed at 0, 0.5 and 1 m above ground and at different positions within the plot by fixing the paper to the leaves using paper clips or by placing them in open Petri-dishes on the ground. Droplet densities for each card were determined with the help of a droplet-counting aid supplied by CIBA-GEIGY using a stereo-microscope (Zeiss, Germany).

Direct spray effect assessment

Immediately after spraying (0 day after treatment) 150 *H. daganensis* were collected from each plot and transferred to the field station. Fifty *H. daganensis* were used to assess the initial direct hit rate by searching for the fluorescent tracer droplets on the grasshoppers using ultra violet light (Jenkins and Thomas, 1996; Kooyman et al., 1997; Langewald et al., 1999). The remaining 100 insects were divided randomly into four groups (each containing 25 *H. daganensis*) and placed in wooden cages covered with wire mesh (25 x 30 x 25 cm). Subsequent samples in the treated plots were collected at 5, 10, 15 and 20 days after treatment (DAT). Because of the limited availability of wooden cages, sample insects from the control plot were collected only at the first two sampling dates (0 and 5 DAT). All caged insects were maintained under shaded conditions for 21 days and were fed daily with millet, sorghum or other grass leaves. In order to avoid cross-infection, the control groups were kept at separate sites under similar conditions. Temperature and relative humidity ranged from 22 – 35 °C (night-day) and 17 – 70 % (day-night), respectively. Differences between cage and ambient temperature and relative humidity at the field station were minimal throughout the study period.

Spray residue effect assessment

The persistence and infectivity of the spray residues over time was assessed using a field bioassay technique (Jenkins and Thomas, 1996; Kooyman et al., 1997; Thomas et al., 1998;

Langewald et al., 1999). Shortly after spraying (0 DAT), four open-bottomed foldable field cages made of mosquito screen (50 x 50 x 50 cm, with a zip on the top) were placed randomly over the vegetation (height lower than 50 cm) in the approximate centre of each treated plot. Any naturally occurring insects were removed and cracks in the ground were filled with soil prior to positioning the cage. One hundred *H. daganensis* were collected in the centre of the control plot, and 25 insects were introduced into each field cage. After 72 hours of exposure, the insects were removed from each field cage, transferred into perforated plastic bottles with the bottom replaced by a mosquito screen and kept for 21 days under similar conditions to those described in the direct spray effect assessment. This procedure was repeated 4, 7, 10, 13, 16, 19 and 21 DAT. At each sampling date the position of the cage was changed and healthy insects were introduced. Because of the limitations in the numbers of cages, in the untreated control plot, foldable cages were placed only at 10, 13 and 16 DAT.

Laboratory test

Following the field experiments, a spray trial was conducted in the laboratory using three different formulations of *M. anisopliae* var. *acridum* (isolate IMI 330189): AC, OE1 and OE2. Formulations were developed using similar batches of spore products as those that had been used for the field experiments. An oil and water sensitive fluorescent tracer (Lumogen) was added at 2.5 % w/v for oil or water based formulations, respectively. The test was conducted using laboratory reared *L. migratoria* (L_3 – L_4) and potted cabbage plants. All formulations were applied at 5 x 10^{12} spores/ha at an application rate of 2 l/ha. Treatments were applied using a ULVAFAN-MK2 hand held sprayer (Micron Sprayers Ltd., Bromyard, UK) at a flow rate of 65 ml/min. Water and oil-sensitive paper-cards were used to assess the spray coverage. During spraying, the temperature of the room ranged from 33 to 35 °C.

Direct spray effect

To assess the direct spray effect, 20 nymphs of *L. migratoria* were introduced into each of the four metal cages (20 x 15 x 15 cm with 6 mm mesh size) which were kept at different positions on the spray table. After spraying, treated nymphs from each treatment were transferred into wooden cages and kept for 21days at 25/35 °C night/day temperatures. Five nymphs from each cage were used to assess the fluorescent tracer droplets as previously described. Untreated insects were used for control treatments. Nymphs were provided daily with untreated fresh wheat and cabbage leaves. The assay was repeated four times.

Spray residues effect

The infectivity of the spray residues were monitored using potted cabbage. At 1, 4, 7, 10 and 17 days after treatment (DAT), a single treated cabbage plant was kept inside a plexi-glass cylinder cage (12 cm diameter and 20 cm height, covered by nylon mesh at the top) and 10-25 nymphs were introduced into each cage. Leaf samples were observed under UV light for fluorescent tracer droplets. Untreated cabbage plants were used for control insects. After 72 hours of exposure, the insects were transferred into a new plexi-glass cage and kept for 21 days following the procedure described for the direct spray effect assessment. Treated cabbage plants were also kept under the same conditions and watered every day. Four replications were arranged for the first spray residue effect assessment (1 DAT) whereas no replications were carried out on samples collected at 4, 7, 10 and 17 days after treatment. For both field and laboratory experiments, mortalities of the treated insects were recorded daily. In order to monitor fungal infection, cadavers were maintained in moist chambers.

Table 2. Mean cumulative mortality (%) and median survival time (MST in days) of *H. daganensis* sampled at 0 and 5 DAT with different formulations of *M. anisopliae* var. *acridum* in East Niger (Mean ± SE, N = 4).

	0 DAT		5 DAT	
Formulation	Mortality [1] (%)	MST[2] (Days)	Mortality[1] (%)	MST[2] (Days)
AC	98.0 (2.38) a	8.4 (0.33) a	100.0 (0.00) a	4.5 (0,27) a
OE1	67.0 (4.68) b	16.3 (0.62) b	80.0 (2.92) c	13.0 (0.63) c
OE2	56.2 (6,36) b	16.3 (0.59) b	92.3 (4.49) b	8.1 (0.55) b
W1	58.4 (3.65) b	17.0 (0.62) b	71.0 (3.15) c	13.0 (0.66) c
W2	68.0 (6,25) b	16.3 (0.63) b	77.0 (4.31) c	14.0 (0.63) c
Control	58.0 (7.20) b	18.2 (0.46) b	69.0 (3.42) c	15.0 (0.69) d

Means within the same column followed by the same letter are not significantly different
[1] (0 DAT, F = 14.04, df = 5, $p < 0.0001$; 5 DAT, F = 16.47, df = 5, $p < 0.0001$, SNK), analysis was made on arcsine transformed percentage value.
[2] (0 DAT, log-rank test, Chi-square = 239.6, df = 5, $p < 0.001$; 5 DAT, log-rank test, Chi-square = 270, df = 5, $p < 0.001$) for survival curves.

Statistical analysis

Analyses were performed on arcsine transformed mean cumulative percentage mortality data. Treatment effects were tested using the generalized linear model procedure (PROC GLM) followed by Student-Newman-Keuls (SNK)-Test for mean separations (SAS Institute Inc., 1989). The median survival time (MST) for each treatment was estimated using the Kaplan-Meier estimate, and the homogeneity of the survival curves (= mortality curves) amongst the

treatments was tested using the Log-rank test in the LIFETEST procedure (SAS Institute Inc., 1989). For laboratory spray residue effect assessments sampled at 4, 7, 10 and 17 days after treatment, the Log–rank test was used in order to compare the treatments. The infection data obtained under field conditions was used to evaluate the trend on the persistence of the formulations with time; statistical analysis was not performed.

Results

Field experiment

Spray deposit

Examination of the fluorescent tracer droplets on the insect bodies indicated that in all treatments, 88 to 100 % of the test insects received spray droplets. The number of droplets per insect exceeded three in all treatments. Similarly, depending on the treatments and the height of the plants, droplet numbers ranging from 15-75/cm^2 were counted on water or oil-sensitive paper-cards.

Direct spray effect

Mean cumulative mortality and MST for *H. daganensis* samples collected at 0 and 5 DAT are shown in Table 2. At both collecting dates, mortality was significantly higher ($p < 0.001$) and increased faster for insects collected from plots treated with aerial conidia (AC) formulation. For 0 DAT and 5 DAT samples, mortality in AC treated insects exceeded 85 % after 11 and 6 days, respectively, resulting in a significantly shorter MST ($p < 0.001$) than the corresponding submerged spores formulations (Table 2 and Figure 1a-b). For insects sampled 0 DAT, mortality in all other treatments, was lower, occurred more slowly, and did not differ significantly from the control (Table 2 and Figure 1a). Mortality of *H. daganensis* sampled 5 DAT was higher and increased faster with significant differences ($p < 0.001$) between the treatments (Table 2 and Figure 1b). The highest mortality and lowest MST was observed for insects treated with aerial conidia (AC) followed by insects treated with the OE2 formulation of submerged spores (Table 2). The mean cumulative mortality of the remaining treatments did not differ significantly from the control. A significant difference was however observed for MST (Table 2 and Figure 1b). For samples that were taken 10, 15 and 20 DAT, mean cumulative mortality in all treatments ranged between 91-100 % and there were no apparent differences between various formulations ($P = 0.05$).

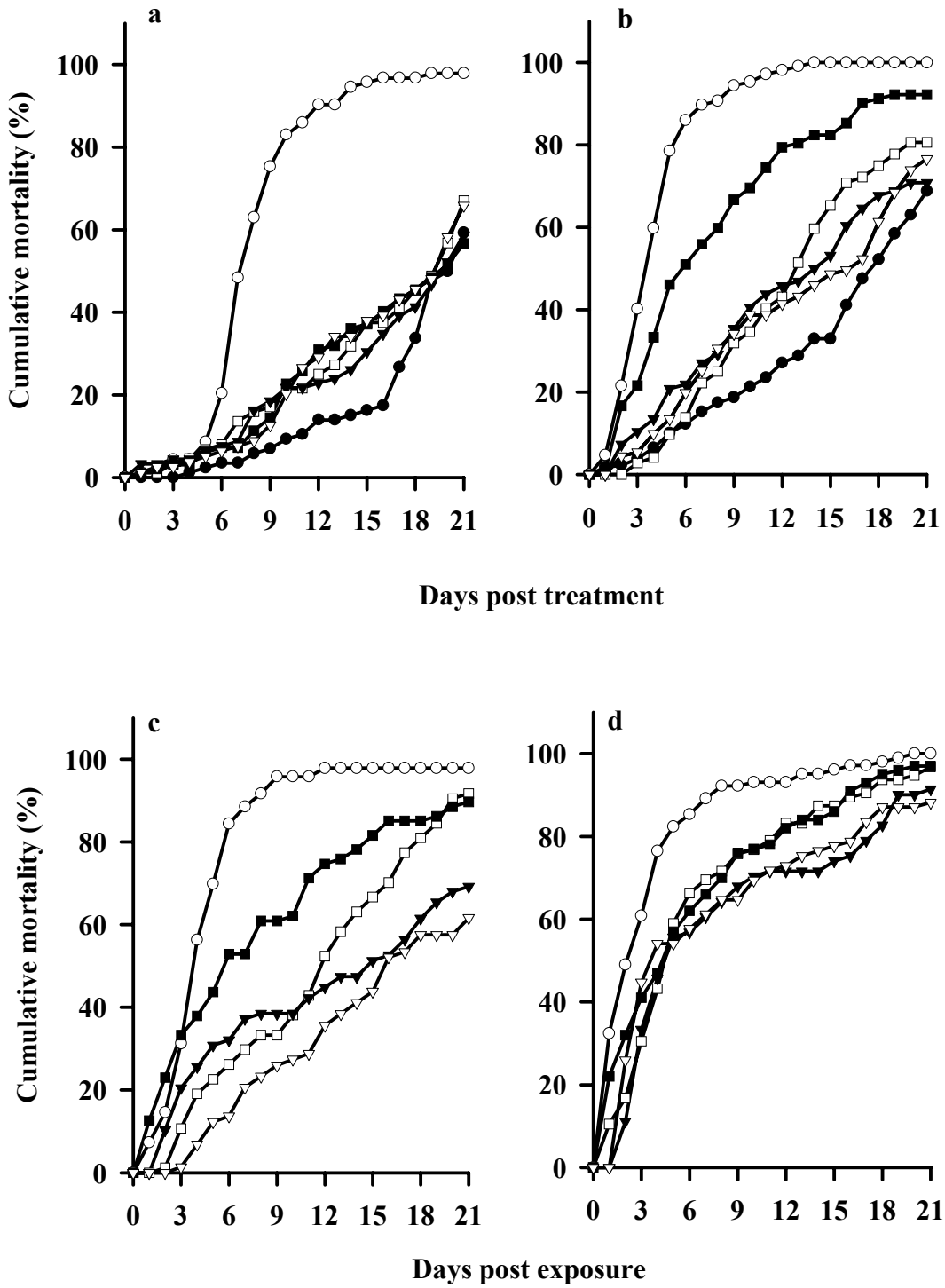

Figure 1. **Cumulative mortality (%) of *H. daganensis* treated with different formulations of *M. anisopliae* var. *acridum*. (a) direct spray effect 0 DAT; (b) direct spray effect 5 DAT, (c) spray residue 0 DAT, (d) spray residue 4 DAT. (○) Aerial conidia, (□) Oil emulsion 1 (OE1), (■) Oil emulsion 2 (OE2), (▼) Water-based 1 (W1) and (∇) Water-based 2 (W2), (●) Control.**

Total percent infections of *H. daganensis* at different time intervals subsequent to ULV spray application of the different formulations are presented in Figure 2. At 0 and 5 DAT, relatively higher infection occurred on insects that had been treated with aerial conidia. For the same treatment, infection dropped to lower than 20 % for the remaining sampling days. For water-based formulations 24-50 % and for oil emulsions 33–42 % infection was observed for insects that were sampled immediately after application. Although a relatively high infection existed on day 10 samplings, the general trend indicated a gradual decline in the infectivity of the different submerged spore formulations over time (Figure 2).

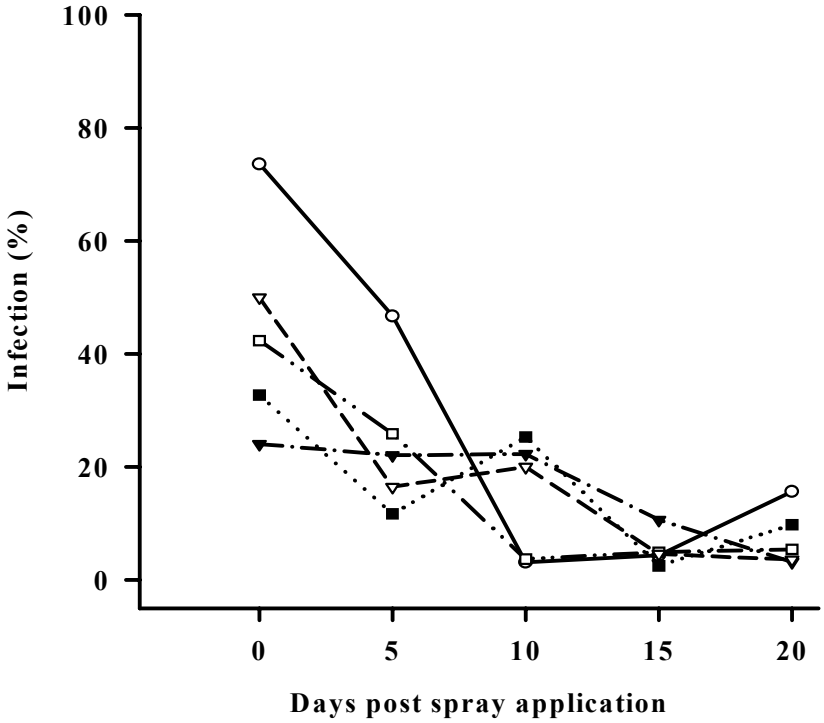

Figure 2. Total infection (%) in *H. daganensis* samples collected at different time intervals following ULV spray application of different formulations of *M. anisopliae* var. *acridum* in Niger. (○) Aerial conidia, (□) Oil emulsion 1(OE1), (■) Oil emulsion 2 (OE2), (▼) Water-based 1 (W1) and (∇) Water-based 2 (W2),

Spray residue effect

The infectivity of the spray residues followed patterns comparable to the direct spray effects (Table 3 and Figure 1c-d). Average mortality of *H. daganensis* was significantly higher ($p < 0.001$) when exposed to residues of all oil formulations immediately after spraying. The two water-based formulations (W1 and W2) caused the lowest mortality (Table 3). The survival

curve analysis for day 0 exposure also revealed a significant ($p < 0.001$) difference between treatment residues in speed of kill (Table 3 and Figure 1c). Significantly shorter MST was observed on insects that were treated with aerial conidia followed by OE2 and OE1 formulations of submerged spores in that order (Table 3 and Figure 1c). For the two water-based formulations of submerged spores, mortality increased slowly and this contributed to longer MST (Table 3 and Figure 1c).

Cumulative mortality of *H. daganensis* exposed to treated vegetation 4 DAT was generally higher than 0 DAT exposures, although there were no significant differences between the formulations in average final mortality (Table 3). Differences between the treatments were observed only in survival curves (Table 3 and Figure 1d). The MST was significantly shorter for aerial conidia treatment ($p < 0.001$). MST for the remaining submerged spore formulations exceeded 7 days and there were no apparent differences between these formulations (Table 3 and Figure 1c). Cumulative mortality of *H. daganensis* exposed to treated vegetation 7-19

Table 3. Mean cumulative mortality (%) and median survival time (MST in days) of *H. daganensis* exposed to vegetation at 0 and 4 DAT with different formulations of *M. anisopliae* var. *acridum* in East Niger (Mean ± SE, N = 4).

Formulation	0 DAT		4 DAT	
	Mortality[1] (%)	MST [2] (days)	Mortality[1] (%)	MST[2] (days)
AC	98.1 (1.92) a	4.5 (0.25) a	100.0 (0.00) a	3.8 (0.39) a
OE1	92.1 (5.36) a	12.0 (0.66) c	97.1 (1.79) a	6.9 (0.58) b
OE2	90.4 (3.66) a	8.5 (0.72) b	97.5 (2.50) a	6.6 (0.58) b
W1	69.5 (7.05) b	13.0 (0.87) d	93.0 (5.74) a	8.6 (0.77) b
W2	62.2 (4.78) b	15.0 (0.74) d	87.0 (5.37) a	8.1 (0.76) b

Means within the same column followed by the same letter are not significantly different (SNK)
[1] (0 DAT, F = 8.44, df = 4, $p = 0.0018$; 5 DAT, F = 1.74, df = 4, $p = 0.2056$, SNK), analysis was made on arcsine transformed percentage value.
[2] (0 DAT, log-rank test, Chi-square = 137.4, df = 4, $p < 0.001$; 4 DAT, log-rank test, Chi-square = 50.65, df = 4, $p < 0.001$) for survival curves.
Control mortality = 63.0 ± 3.85 % (10–16 DAT).

DAT was generally higher than that of immediate sampling (0 DAT), however, there were no significant differences ($p = 0.05$) between formulations (data not shown). Mortality of *H. daganensis* that had been exposed to the untreated vegetation 10, 13 and 16 DAT ranged from 62 to 72 % (mean = 63 ± 3.85 %). This mortality level was recorded after 72 hours of exposure and no fungal infections were observed after incubation, indicating the high level of

natural mortality that occurred in the untreated *H. daganensis* populations. The total infection ranged from 25 to 46 % for *H. daganensis* exposed to residues of W2 and W1 formulations immediately after spraying followed by OE1, AC and OE2 formulations. On subsequent exposure dates the infection rate declined for all formulations (Figure 3).

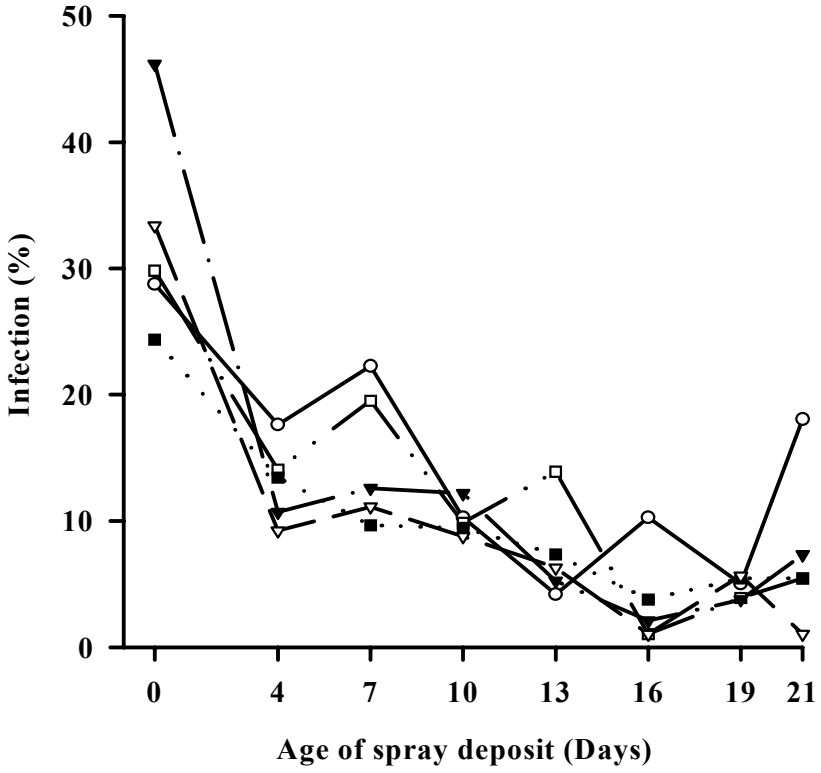

Figure 3. Total infection (%) in *H. daganensis* following exposure to treated vegetation subsequent to ULV spray application of different formulations of *M. anisopliae* var. *acridum* in Niger. (○) Aerial conidia, (□) Oil emulsion 1 (OE1), (■) Oil emulsion 2 (OE2), (▼) Water-based 1 (W1) and (∇) Water-based 2 (W2).

Laboratory test

All insects and leaves observed under UV light showed high droplet numbers, indicating adequate spray coverage for all formulations tested. For all treatments, the droplet numbers ranged between 120-150/cm^2 on the indicator paper.

Mean percentage mortalities and median survival time of *L. migratoria* for the direct spray and spray residue a day after treatment are presented in Table 4. In all cases, the efficacy of the tested formulations followed patterns comparable to the field results obtained for *H. daganensis*. Mortality of *L. migratoria* was significantly higher ($p < 0.001$) for aerial conidia

(AC) treatment both in direct spray and spray residue (Table 4). This was followed by the two oil-in-water emulsion formulations of submerged spores (OE1 and OE2). Survival curves for direct spray and spray residues are presented in Figure 4. The survival curve analysis revealed significant differences ($p < 0.001$) in median survival time of *L. migratoria*. For the direct spray, the median survival time of *L. migratoria* was significantly lower for aerial conidia treatment followed by emulsion formulations of submerged spores (Table 4, Figure 4a). For all formulations, the median survival times of *L. migratoria* exposed to the spray residues 1, 4 and 7 days after treatment ranged from 8 to 13 days and there were no differences between formulations ($p = 0.05$). All treatments differed from the control, ($p < 0.001$) (Figure 4b-d). Exposure of *L. migratoria* to the spray residue 10 and 17 days after treatment did not reveal any variability among the various treatments in median survival time and exceeded 12 days for all formulations (Figure 4e-f). For most exposure dates, mortality of *L. migratoria* remained over 60 % and decreased to lower than 40 % with increasing age of the spray deposits to 17 days (Figure 4b-f).

Table 4. Mean cumulative mortality (%) and median survival time (MST in days) of *L. migratoria* treated with different formulations of *M. anisopliae* var. *acridum* in the laboratory (Mean ± SE, N = 4).

	Direct spray		Spray residue	
Formulation	Mortality[1] (%)	MST[2] (Days)	Mortality[1] (%)	MST[2] (Days)
Control	34.0 (4.77) [c]	16.0 (0.61)[a]	28.0 (6.81) [c]	15.2 (0.58)[a]
OE1	72.0 (2.09) [b]	15.0 (0.47)[b]	71.0 (4.36) [b]	9.0 (0.82)[b]
OE2	76,3 (1.43) [b]	13.0 (0.56)[b]	82.3 (3.13) [b]	10.0 (0.88)[b]
AC	92.0 (3.23) [a]	12.0 (0.54)[c]	93.3 (1.07) [a]	8.3 (0.68)[b]

Means within the same column followed by the same letter are not significantly different

[1] (Direct spray, $F = 22.83$, $df = 3$, $p < 0.001$; Spray residue, $F = 22.83$, $df = 3$, $p < 0.001$; SNK), analysis was made on arcsine transformed percentage value.

[2] (Driect spray, log-rank test, Chi-square = 48.5, $df = 3$, $p < 0.001$; Spray residue, log-rank test, Chi-square = 79.7, $df = 3$, $p < 0.001$) for survival curves.

Discussion

This research compares the efficacy of four different formulations of *M. anisopliae* var. *acridum* (IMI 330189) to control locust and grasshopper species under arid conditions. Efficacy, as assessed by mortality and median survival time, varied depending on the nature of infectious spores and the type of formulation used. The enhanced infectivity of conidia in

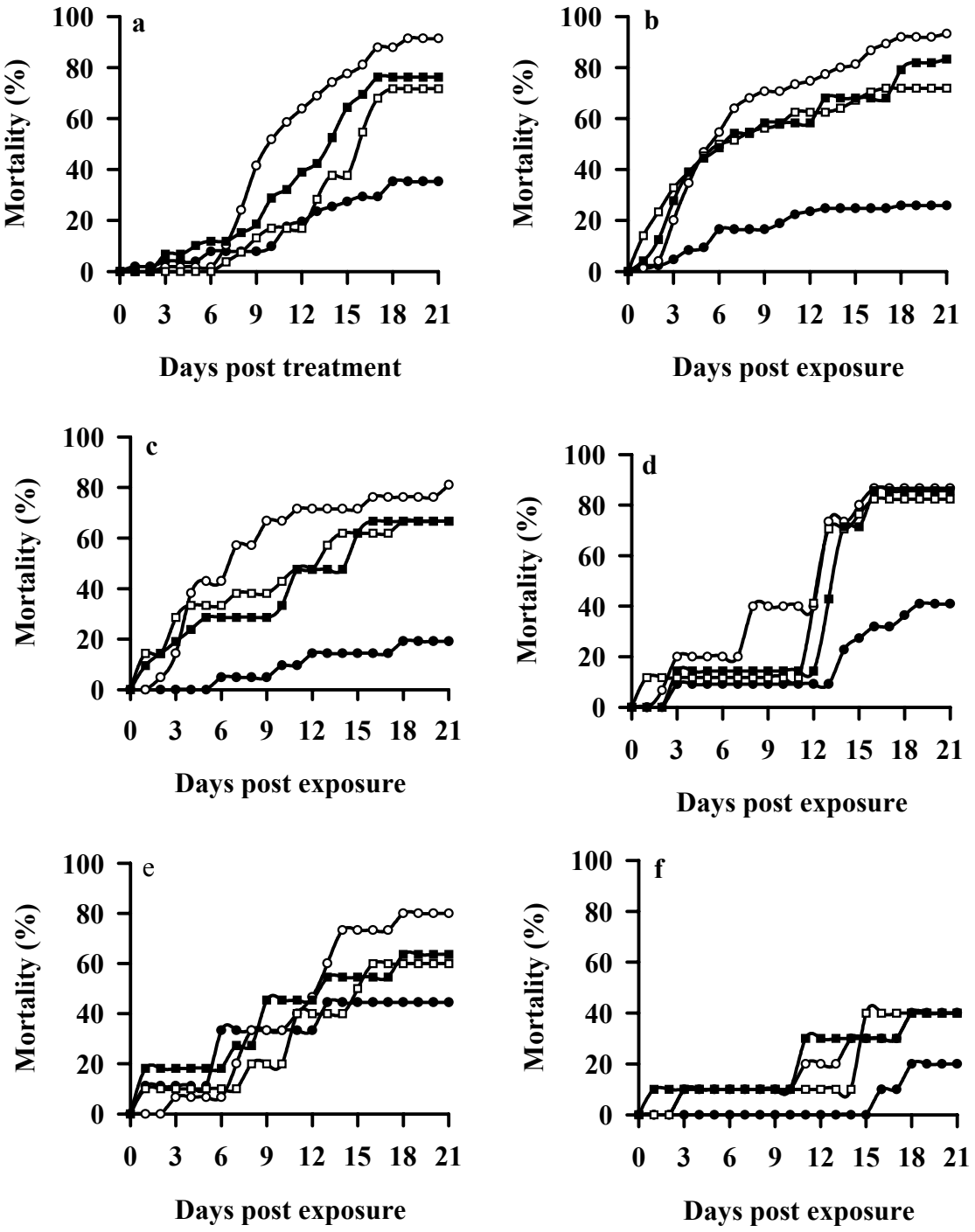

Figure 4. **Cumulative mortality (%) of *L. migratoria* treated with different formulations of *M. anisopliae* var. *acridum* in the laboratory. (a) direct spray effect; (b) spray residue 0 DAT, (c) spray residue 4 DAT, (d) spray residue 7 DAT , (e) spray residue 10 DAT and (f) spray residue 17 DAT. (○) Aerial conidia, (□) Oil emulsion 1 (OE1), (■) Oil emulsion 2 (OE2), (▼) Water-based 1 (W1) and (∇) Water-based 2 (W2), (●) Control.**

oil formulations has been reported previously (Prior et al., 1988; Bateman et al., 1993). Various authors have demonstrated the field efficacy of aerial conidia of *M. anisopliae* var. *acridum* to various Sahelian grasshoppers (Jenkins and Thomas, 1996; Langewald et al., 1997; Thomas et al., 1998; Langewald et al., 1999). In the present study, aerial conidia formulated in diesel oil were highly effective with an acceptable residual effect on *H. daganensis* under Sahelian conditions. Despite the use of higher concentrations, freeze dried submerged spores showed a lower efficacy as compared to aerial conidia. However, among the various submerged spore formulations, the two oil-in-water emulsions exhibited better control and persistence than the two water-based formulations. These findings correspond with the results obtained in the laboratory. Similar results have been reported by Jenkins and Thomas (1996) targeting the variegated grasshopper, *Zonocerus variegatus* and the desert locust, *Schistocerca gregaria*. These results demonstrate again the advantage of using oil carriers for protecting spores from environmental stress. Furthermore the oil carrier enhances the infectivity and improves the adherence and the spread of spores on the target pest (Inglis et al., 1996). The work of Lomer et al. (1997) on *H. daganensis* suggested a possible advantage of water-based ULV formulations (among others) in order to improve the initial impact of myco-pesticides by increasing the application volume, which optimises the coverage and the initial contact to the target insects. On the other hand, Stephan et al. (1997) tested different formulations of spray dried submerged spores against *Schistocerca gregaria* in Mauritania. They found that submerged spores, applied directly onto the insect as water-based ULV formulations, displayed better control than either oil-in-water emulsion or oil formulations. Our results, however, deviate from these findings. Under the conditions of the research at hand and under the harsh environmental conditions that occur in the Sahel, water-based formulations of submerged spores are not suitable for grasshopper control using ULV applications. *H. daganensis* usually aggregates near the bottom of the plant (Kassa's observation) and direct spray contact may be limited in dense vegetation. Direct spray impact could be improved by using a high volume application, a strategy suitable for less arid conditions (Jenkins and Thomas, 1996). On the other hand, the increased efficacy and persistence of oil-in-water emulsion formulations of submerged spores under Sahelian conditions indicated the potential for further development of oil-in-water emulsion formulations for ULV as well as for high volume spray application techniques. Further research into grasshopper control in the Sahel should be directed towards developing an effective emulsion formulation for ULV application.

Mortalities in *H. daganensis* collected immediately after spraying with different submerged spore formulations were lower than expected from the level of direct spray hits observed by the fluorescent tracer. This indicated that the amount of infectious spores carried by the droplets might not be sufficient to cause high mortality. In contrast, mortality rates of *H. daganensis* sampled 5 DAT were higher, comparable with the mortality rates observed from spray residues, indicating the increased exposure of the treated insects to the spray residues in the field. Thus, the spore load acquired by the target insects is lower from direct spray impact than that acquired from the spray residues alone or from the combination of both. Similar results have been reported for *H. daganensis* (Lomer et al., 1997; Thomas et al., 1998) and for *S. gregaria* (Langewald et al., 1997). The high rate of natural mortality observed for *H. daganensis* was associated with the age of the insects used. However, this naturally occurring high mortality did not impair the comparison between different formulations with respect to efficacy.

Incubation of the cadavers resulted in varying infection rates depending on the type of formulations tested and the time of exposure. The infection rate for direct spray and spray residues of all formulations declined with time. This decline in infection rates was faster in insects treated with the two water-based and OE1 formulations of submerged spores. During the field experimentation, the average ambient temperature exceeded 40 °C between 12:30-15:00 h and relative humidity dropped to less than 15 %. No rain fell during the duration of the experiment. These climatic conditions probably contributed to the low infection rate recorded particularly for submerged spore formulations. Evidence as to the effect of high temperature on the viability of *M. anisopliae* spores was shown by Zimmermann (1982). Furthermore, the effect of light on the infection rate of *B. bassiana* in grasshopper has been recently demonstrated by Inglis et al. (1997). They indicated that, direct exposure of treated insect to full spectrum sunlight conditions significantly reduced the prevalence and total infection rate of *B. bassiana* on grasshoppers. In our study we kept the caged insect under shade conditions. Furthermore, the cages were covered by a cloth mesh, which further contribute the reduction in the amount of light getting into the cage (Inglis et al., 1997). Although sunlight is important and would affect infection rates especially time to kill, this should not impair the comparison between different formulations with respect to efficacy and persistence.

Research has been carried out worldwide into developing mycoinsecticides for locusts and grasshopper control based on aerial conidia of *M. anisopliae* and *Beauveria bassiana* (Balsamo) Vuillemin (Lomer et al., 2001). Most of this research relied on the use of solid state fermentation for the large scale production of conidia. However, in industrial terms, the liquid fermentation of microbial control agents is not only more advanced but could potentially result in significantly lower production costs (Jenkins and Goettel, 1997; Stephan, 1998). In this respect, the results presented in this paper are interesting, indicating the potential for future development of mycoinsecticides based on submerged spores of *M. anisopliae* var. *acridum.* Future research should focus on improving the quality of submerged spores products and formulations with respect to their persistence and efficacy in the field. Furthermore attempts should be made to develop stable and effective formulations for use under Sahelian conditions.

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Plate 1. Vegetation cover sprayed with mycoinsecticides and field cages used for spray residue assessments.

Plate 2. Wooden cages and plastic bottles used for incubation of treated insects.

CHAPTER 4

Laboratory and field evaluation of emulsions and oil formulations of *Metarhizium anisopliae* var. *acridum* submerged and aerial conidia for the control of locusts and grasshoppers

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A series of studies based on submerged and aerial conidia of Metarhizium anisopliae *var.* acridum *(IMI 330189) were conducted. Initially, the suitability of freeze and spray dried submerged conidia for oil flowable concentrate (OFC) formulations were investigated in the laboratory. The results revealed that freeze and spray dried submerged conidia of IMI 330189, formulated in OFC formulations, are equally infectious for* Locusta migratoria *(R. & F.). Subsequently, two OFC and four emulsions were developed based on freeze dried submerged conidia of IMI 330189. These were compared with Green Muscle™ for efficacy against* L. migratoria *(in the laboratory and field) and* Cryptocatantops haemorrhoidalis *(Krauss) under field conditions. For the laboratory experiment I and for the field studies, submerged and aerial conidia were applied at 1 x 10^{13} and 5 x 10^{12} spores ha^{-1}, respectively. In laboratory experiment II the treatments were applied at 5 x 10^{12} spores ha^{-1}. The direct and residual spray effects were assessed. The results revealed that the formulations differed in efficacy ($p < 0.001$). Over all, the aerial conidia formulation performed better than the submerged conidia formulations. As regards submerged conidia, OFC2 formulation showed good biocontrol potential under both laboratory and field conditions followed by the emulsions. Conidia in all formulations persisted for at least 10 days and depending on the test insect, caused mortalities ranging from 19 to 99 %. It could be observed that* C. haemorrhoidalis *was more susceptible to the mycopathogen than* L. migratoria*. Further research should focus on improving the quality of submerged conidia and the formulations.*

Key words: Cryptocatantops haemorrhoidalis, *conidia*, *emulsion*, *formulation*, Locusta migratoria, Metarhizium anisopliae var. acridum, *mycoinsecticide*, *oil flowable concentrate, submerged conidia,*

INTRODUCTION

In many countries, especially in the tropics, locusts and grasshoppers are considered to be major pests in agriculture causing significant damage in many crops (Steedman, 1990). Control strategies for locusts and grasshoppers rely almost exclusively on the use of chemical insecticides (Prior and Streett, 1997). During the 1985-89 outbreaks of desert locusts in northern and central Africa for example over US $ 275 million were spent on a massive insecticide spraying programme (Bateman, 1992). However, a growing awareness of environmental issues associated with the use of excessive pesticides has prompted the development of environmentally safe alternative control strategies such as the use of microbial control agents (Inglis *et al*., 1997; Lomer *et al*., 2001) as well as use of insect growth regulators and botanical plant extracts (Krall *et al*., 1997; Musuna and Mugisha, 1997). Biological control based on mycopesticides is an alternative which offers more rapid prospects for implementation (Prior, 1997; Prior and Greathead, 1989) and entomopathogenic fungi such as *Metarhizium anisopliae* (Metsch.) Sorkin and *Beauveria bassiana* (Balsamo) Vuillemin have shown considerable potential for the management of locusts and grasshoppers (Feng *et al*., 1994; Lomer *et al*., 2001).

Recently, the formulation and application of biopesticides have received much attention and different formulations have been developed and tested against a variety of insect pests (Burges, 1998). Due to the diverse nature of climatic situations, targets and user preferences, a single organism has often to be formulated in different forms (Jones and Burges, 1998). With regard to locusts and grasshoppers, for example, the LUBILOSA project has developed two formulations (oil and oil flowable concentrate) based on aerial conidia of *M. anisopliae* var. *acridum* (IMI 330189) suitable for ultra-low volume (ULV) application (Bateman, 1997; Lomer *et al*., 2001). Aqueous, oil and flowable oil formulations have also been developed based on aerial conidia of *Beauveria bassiana* and tested against grasshoppers (Johnson *et al*., 1992; Jaronski and Goettel, 1997; Delagado *et al*., 1997).

More recently, submerged spores/conidia of *M. anisopliae* var. *acridum* were successfully produced in liquid culture (Kleespies, 1992; Jenkins and Prior, 1993; Jenkins and Thomas, 1996; Stephan, 1998) and dried using various drying techniques (Stephan and Zimmermann, 1998, 2001). Submerged liquid fermentation can be used as an alternative for large scale production of entomopathogenic fungi as the fermentation conditions can be controlled and manipulated to induce and increase sporulation (Humphreys *et al.*, 1989). The enhanced

infectivity of conidia of *B. bassiana* and *M. anisopliae* var *acridum (= M. flavoviride* Gams and Rozsypal) in oil formulations has been described previously (Prior *et al.*, 1988; Bateman *et al.*, 1993). The oil in the formulation also provides enhanced protection to the conidia after spraying (Jenkins and Thomas 1996). Furthermore, the none-evaporative oil carriers are advantageous in order to use ultra-low volume (ULV) spraying techniques (Bateman *et al.*, 1998). However, submerged spores/conidia are hydrophilic in nature and are more difficult to formulate in oil than aerial conidia (Jenkins and Prior, 1993; Jenkins and Thomas, 1996). An aqueous formulation was recently developed for submerged spores/conidia of *M. anisopliae* var. *acridum* (IMI 330189) and tested against locusts and grasshoppers (Jenkins and Thomas, 1996; Stephan and Zimmermann, 2001; Kassa *et al*., unpublished). However, research results on oil based formulations of submerged spores/conidia for ULV application remain rare. Therefore, our study focused on developing emulsions and oil based formulations for submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189). Our specific objectives were: 1) to compare the suitability of freeze and spray dried submerged conidia for oil flowable concentrate formulations and 2) to develop different formulations and compare their efficacy with aerial conidia under laboratory and field conditions. For all laboratory studies, *Locusta migratoria* (R. & F.) (Orthoptera: Acrididae) was used as a test insect whereas for the field studies, both *L. migratoria* and *Cryptocatantops haemorrhoidalis* (Krauss) (Orthoptera: Acrididae) were used.

MATERIALS AND METHODS

Fungal Isolate

Green Muscle™ is a mycoinsecticide product developed by LUBILOSA (LUtte BIologique contre les LOcustes et les SAuteriaux) based on aerial conidia of *Metarhizium anisopliae* var. *acridum* isolate (IMI 330189) (formerly, *M. flavoviride*) isolated from *Ornithacris cavroisi* (Finot) (Orthoptera: Acrididae) in Niger (Bateman et al., 1997; Neethling and Dent 1998). Samples of Green Muscle™ were obtained from AGRHYMET (Regional Centre for Agro-Meteorology and Hydrology). For this study, the same isolate was used for submerged conidia production and formulation development. The isolate was grown on malt extract agar at 25°C in darkness and stalk cultures were maintained at 4°C.

Laboratory experiments

Formulation

Three different oil flowable concentrate formulations (OFC) were developed based on freeze or spray dried submerged conidia and compared with Green Muscle™ (aerial conidia) for efficacy against *L. migratoria* (Table 1). Submerged conidia were produced in a three litre fermenter using a BH liquid medium (3 % biomalt and 1 % yeast extract) in the laboratory (Kassa *et al*. submitted). Production was carried out in four different fermentation cycles. Similarly, spray and freeze drying of the submerged conidia were carried out in four different occasions following the methods described previously (Stephan *et al*. 1998, 2001). Dried samples were reconstituted to get one working sample for further efficacy test. In order to mix the components of the OFC formulation, the procedure described by Lisansky *et al*. (1993) was adopted. Components of the formulations are indicated in Table 1. They did not affect the viability of the submerged conidia (Kassa *et al*., submitted). The suspender (Bentone 38) and 10% of the oil (carrier) were mixed at maximum shear in an ultra-turrax (Jank and Kunkel KG, Germany) for 45 min. The Propylene carbonate (activator of Bentone 38) was added rapidly while mixing at high shear for 5 min to get the thick pre gel. The remaining oil was added into the pre gel and was mixed for 10 min. Using a low speed, the required amount of the technical powder (freeze or spray dried submerged conidia) was mixed in batches into the pre gel mix until all clumps dispersed. The resulting formulations were not filtered and were used directly after formulation.

Laboratory spray experiment I

Laboratory experiment I consisted of two components, (a) direct spray effect and (b) spray residues effect assessments that are described in detail further below. The test was conducted using laboratory reared *L. migratoria* (L_3/L_4) (Kleespies, 1993) and four weeks old potted cabbage plants grown on LD 80 (Industrie-Erdenwerk Archut, Germany). All submerged conidia formulations (OFC) were applied at 1 x 10^{13} spores ha $^{-1}$ (Stephan *et al.*, 1997) while Green Muscle™ was applied at 5 x 10^{12} conidia ha^{-1} (Langewald *et al.*, 1999). Spraying was done at a volume application rate of 3 L ha^{-1}. Treatments were applied using an ULVAFAN-MK2 hand held sprayer (Micron Sprayers Ltd., Bromyard, UK) at a flow rate of 18.5 ml min^{-1}. In order to assess the spray coverage, an oil sensitive fluorescent tracer (Lumogen) was added at 2.5 % w/v for all formulations. Furthermore, oil-sensitive paper-cards (Ciba-Geigy Ltd, Switzerland) that were fixed with small paper clips onto the leaves or placed inside the cages.

Table 1. List of various formulations and their components tested in the laboratory and under field conditions.

Formulation code.	Components % w/v	Type of formulation
	Laboratory spray experiment I	
SD-OFC2	9.33 % spray dried (SD)submerged spores, 0.99 % Benton 38 and 0.32 % propylene carbonate, diesel oil	Oil flowable concentrate
SD-OFC3	6.22 % spray dried submerged spores, 23.28 % PF- 400, 0.69 % Benton 38 and 0.23 % propylene carbonate, diesel oil	Oil flowable concentrate
FD-OFC2	14. 43 % freeze dried (FD) submerged spores, 0.99 %Benton 38 and 0.23 % propylene carbonate, diesel oil	Oil flowable concentrate
Green Muscle™ (GM)	5.69 % Green Muscle™ (aerial conidia), diesel oil	Oil
	Field and Laboratory spray experiment II	
OFC1[a]	4. 02 % freeze dried submerged spores, 0.98 % Benton 38 and 0.32 % propylene carbonate, shellsol	Oil flowable concentrate
OFC2	4.02 % freeze dried submerged spores, 0.98 % Benton 38 and 0.32 % propylene carbonate, diesel oil	Oil flowable concentrate
Codacide (CD)	3.47 % freeze dried submerged spores, 3.47 % Codacide ,water	Emulsion
EO4	3.47 % freeze dried submerged spores, 3.47 % experimental oil 4-12728 ,water	Emulsion
Addit™	4.27 % freeze dried submerged spores, 20 % Addit™, 5 % v/v Tween 80, water	Emulsion
E-oil	20 % Experimental oil 4.27 % freeze dried submerged spores 5 % v/v Tween 80, water	Emulsion
OE2[b]	4.27 % submerged spores, 1 % lecithin (Sigma, USA), 10 % soya bean oil, water	Emulsion
Green Muscle™ (GM)	5.69 % Green Muscle ™ (aerial conidia), diesel oil	Oil
Control	No spray	-

OFC: Oil flowable concentrate,
SD: Spray-dried submerged spores
FD: Freeze-dried submerged spores
[a] Not tested in the laboratory,
[b] Promoted from pervious studies (Kassa *et al* unpublished) and not tested in the laboratory.
Codacide and Experimental oil number 4-12728 are commercial and test emulsifiers oil, respectively from Microcide Ltd. (UK), Addit and E-oil are commercial and experimental emulsifiers, respectively, from Koppert (The Netherlands), BENTONE ® 38 is a product of Rheox Inc. UK). Propylene carbonate is a product of Merck, Germany. The carriers were added in the formulation up to 100 %.

were used to assess the spray coverage. Density of the droplet in each card were assessed using droplet counting aid supplied by (Ciba-Geigy) under Zeiss stereo-microscope, whereas droplets on the leaves and on the insect body were assessed using UV light. During spraying, the temperature of the room ranged from 33 to 35 °C and the RH between 35 to 45 %. The assay was replicated three times.

Direct spray effect. To assess the direct spray effect, 20 nymphs of *L. migratoria* were introduced into each of three metal cages (20 x 15 x 15 cm with 6 mm mesh size) and were placed at different positions on a spray table. After spraying, the treated nymphs in each treatment were transferred into a wooden cage (30 x 30 x 20 cm, covered with nylon (0.1mm mesh size) and were kept for 21 days under controlled environmental conditions at 35/25 °C (day/night) temperature; 35/45 % RH (day/night) and 12:12 h light: dark photoperiod. Prior the development of the formulations, a preliminary test was carried out to assess the effect of the carriers on the survival of *L. migratoria* using topical inoculation of 1 µl of the preparation through the pronotum. However, none of them (alone or in combination) caused mortality on tested insects. Because of this, untreated insects were used for control treatments. Furthermore, including blank formulations could create a prohibitively large number of treatments that needs several test insects. Insects in all treatments were provided daily with untreated fresh wheat and cabbage leaves. Mortalities of the insects in all treatments were recorded daily. Cadavers were maintained in Petri-dishes with moistened tissue paper to monitor fungal infection.

Spray residues effect. The infectivity of the spray residues was monitored using potted cabbage plants. Immediately after being sprayed, a single treated cabbage plant was placed inside a plexi glass cylinder cage (12 cm diameter and 20 cm height, at the top covered by nylon mesh) and 15 nymphs were introduced into each cage. Additional treated cabbage plants were provided in case the test insects consumed the whole plant within 72 h. Untreated cabbage plants were used for control insects. After 72 hours of exposure, the insects were transferred into a new plexi glass cage and kept for 21 days following a similar procedure as described for the direct spray effect assessment. Treated cabbage plants were also kept under the same conditions and watered every day. Mortalities of the treated insects were recorded daily. Cadavers were maintained in Petri-dishes with moistened tissue paper to monitor fungal infection.

Laboratory spray experiment II

Pervious studies (Kassa *et al.*, submitted) and the present laboratory experiment I has lead to the development of new emulsions and OFC formulations. Furthermore, the industrial partner FZB Biotechnik GmbH (Germany) produced submerged conidia of *M. anisopliae* var. *acridum* (isolate IMI 330189) in a 300 l fermenter and the efficacy of this product needs to be evaluated along the formulation before the field trial. The product was gently crushed by hand and sieved to pass a 125 µm mesh size using a vibrotronic sieve (Retsch GmbH, Germany). The sieved powder contained 3.84 x 10^{10} viable spores g^{-1}. One OFC and four emulsions were developed and tested for their efficacy against *L. migratoria* (L_3/L_4) (Table 1). All formulations were applied at 5 x 10^{12} submerged spores ha^{-1} and at a volume application rate of 3 L ha^{-1}. For the direct spray effect assessment, 25 nymphs per replicate were used and for each treatment, there were four replicates. For spray residue effect assessment, 25 nymphs per treatment were used and because of limited number of cages available, the assay was not replicated. Spraying, handling and assessment of the treated insects followed similar procedures as described in the laboratory spray experiment I. Treatments that showed higher efficacy were chosen for further testing under field conditions.

Field spray experiment

The field experiment also had two components (a) direct spray and (b) spray residue effect assessments. The study was conducted in Niamey (Niger) at the experimental site of AGRHYMET in large field cages (9 m x 2m x 3m length) covered with fine wire mesh. The four sides of the cage were covered with bamboo matting to minimize the displacement of the droplets by the natural wind during application (Bateman *et al.*, 1998). Prior to the spray application, millet seedlings that were grown in the field were transplanted into plastic pots (10 cm diameter) and kept in one of the large field cages. The test insects were *L. migratoria* and *C. haemorrhoidalis*. Adult *C. haemorrhoidalis* required for the entire experiments were collected in the field while L_3-L_5 instar larval stages of *L. migratoria* were obtained from the laboratory rearing of AGRHYMET, Niamey. Three emulsions (CD, EO4 and OE2) and two oil flowable concentrate formulations (OFC1 and OFC2) of submerged spores were prepared and compared with Green Muscle™ (GM) formulated in diesel oil (Table 1). All formulations were applied using a hand held sprayer (ULVAFAN-MK2). Spraying was carried out in one path in order to cover the 27 m^2 floor area of the cage within 11 to 17 seconds depending on

the type of formulation (flow rate) maintaining an application rate equivalent to 3 L ha^{-1}. Submerged spores were applied at 1 x 10^{13} spores ha^{-1} (Stephan *et al.*, 1997) while Green Muscle™ was applied at 5 x 10^{12} conidia ha^{-1} (Langewald *et al.*, 1999). The control insects remained untreated and were kept separately to avoid cross infection. Spraying took place under calm conditions in the morning. Temperature and relative humidity (measured during the day) varied between 31–46 °C and 10–35 %, respectively. Sensitive indicator paper cards were used to monitor droplet deposits on the plants and in the cages as well as on the ground following similar methodology described previously. The direct spray effect and the secondary pick-up from the spray residues on millet were assessed as described below.

Direct spray effect. For each treatment, 100 *C. haemorrhoidalis* or *L. migratoria* were distributed equally into five small cages (20 cm x 10 cm x 10 cm) with 5 mm mesh size. The cages were maintained within the large field cages described previously and considered as replicates. Cages containing insects were placed at 1.5 m distance from each other and 1 m from the sprayer tip in a row and sprayed with the respective formulations. Because of limitation in the number of cages available, shortly after spraying, the treated insects were collected and placed in one cage for *C. haemorrhoidalis* and two cages (each with 50 insects) for *L. migratoria* and kept in the shade for 21 days. The treated insects were provided daily with untreated millet plants. Mortality was monitored daily and cadavers were maintained in Petri-dishes with high relative humidity to verify the infection.

Spray residue effect. The effect of spray residues was assessed at 0, 5 and 10 days after treatment (DAT) using potted and sprayed millet plants. The potted millet plants were placed at 0.5 m distance from the sprayer tip and sprayed in one row along with the caged insects. At each assessment time, 60 *C. haemorrhoidalis* (in one cage) or 60 *L. migratoria* (in two cages, each with 30 insects) were exposed to treated plants for 72 h under field conditions. Additional treated plants were provided in case of 100 % consumption of the previous plants. Because of limitation in the number of cages available, the experiment was not replicated. After 72 h exposure, the treated plants were removed from the cage, and insects were fed with untreated millet plants and kept for 21 days for mortality assessment. In all cases, cadavers were maintained in Petri-dishes with high relative humidity to verify the infection.

Data analysis

Data were analysed by the general linear model procedure (PROC GLM, SAS, 1989) after logarithmic (log_{10}) and arcsine square-root transformation of count and percentage data, respectively. Student-Newman-Keuls (SNK) test was used to separate means (SAS, 1989). Non-replicated experiments were analysed using the LIFETEST procedure and the homogeneity of the survival curves was tested using pair-wise Log-Rank statistics (SAS, 1989). Median survival time of the treated insects was estimated using the LIFETEST procedure (SAS, 1989).

RESULTS

Laboratory experiments

Laboratory spray experiment I

For all formulations, the number of droplets ranged from 100 to 150 per cm^2 of indicator paper. Similarly, all insects and leaves observed under UV light showed good coverage of spray droplets for all formulations. Mortality of *L. migratoria* differed significantly (F = 10.91 and 11.27; df = 4, 4; $p < 0.01$) among the different formulations in the direct spray and spray residues, respectively (Figure 1). For the direct spray, mortality was higher for Green Muscle™ (aerial conidia) than for submerged spore formulations (Figure 1a). There was no apparent difference in mortality between all submerged spore formulations, however, all differed significantly from the control (Figure 1a). When *L. migratoria* was exposed to the spray residues of the different formulations, mortality was over 93 %, and there were no apparent differences between formulations, however, all differed significantly from the control (Figure 1a). The survival curve analysis revealed a significant difference in median survival time (χ^2 = 34.58, 66.92; df = 4, 4; $p < 0.001$) among the treatments in the direct spray and spray residues, respectively. For the direct spray, insects treated with SD-OFC2 showed longer survival time than the other treatments (Figure 1b). Insects exposed to the spray residues of Green Muscle™ and SD-OFC2 showed significantly lower median survival time than the remaining formulations.

Laboratory spray experiment II

For the direct spray effect assessment, highly significant differences were observed between formulations in mortality (F = 27.98; df = 5; $p < 0.001$) and in median survival time (χ^2 = 111.87; df = 5; $p < 0.001$). Mortality of *L. migratoria* was higher and faster in OFC2

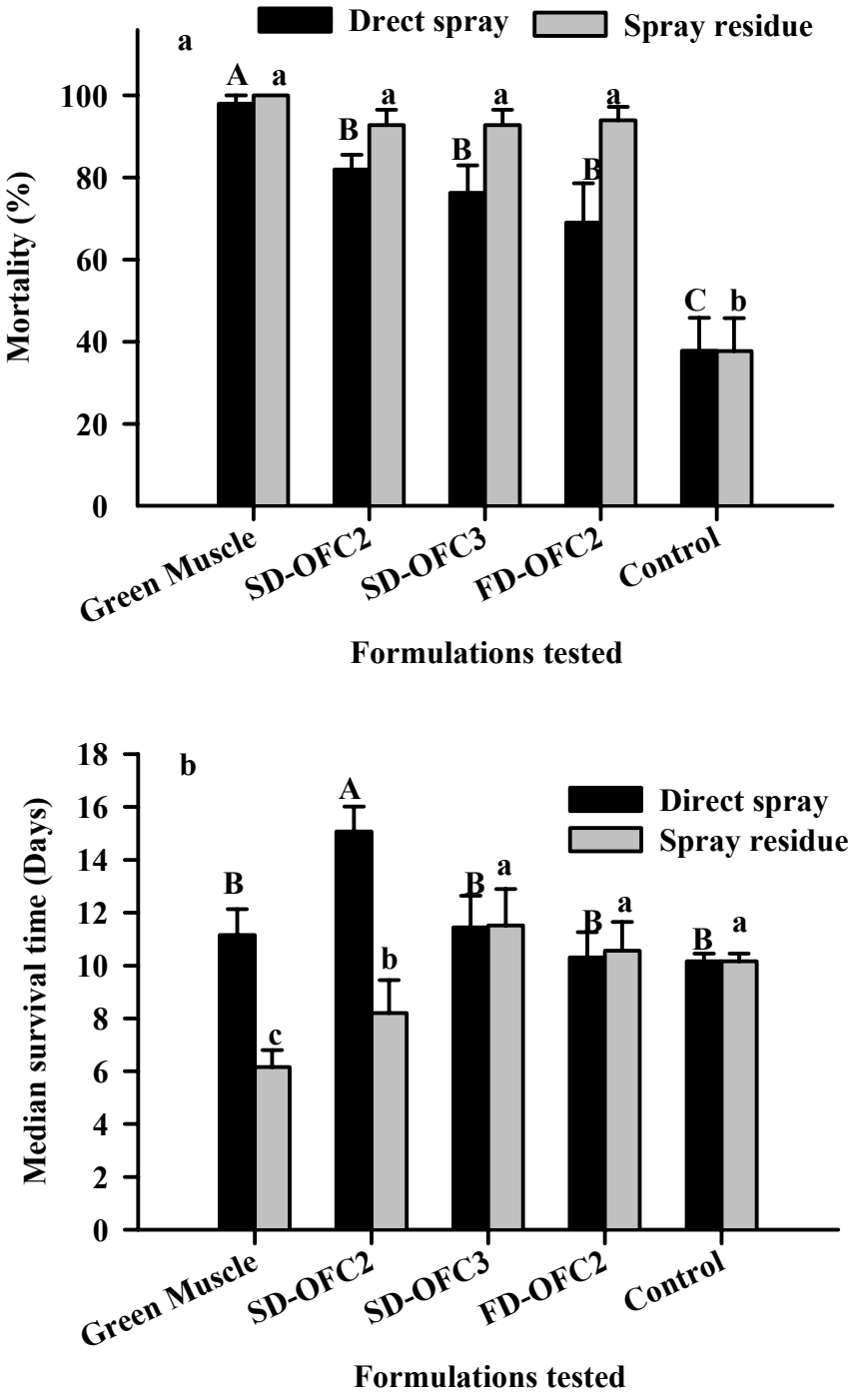

Figure 1. Mean percent mortality (a) and median survival time (b) of *L. migratoria* treated with different formulations of *M. anisopliae* var. *acridum*, (IMI 330189) in the laboratory. The same shaded bars followed by the same letter are not significantly different (SNK test, $p < 0.001$) for mortality and (log-rank test, $p < 0.001$) for median survival time.

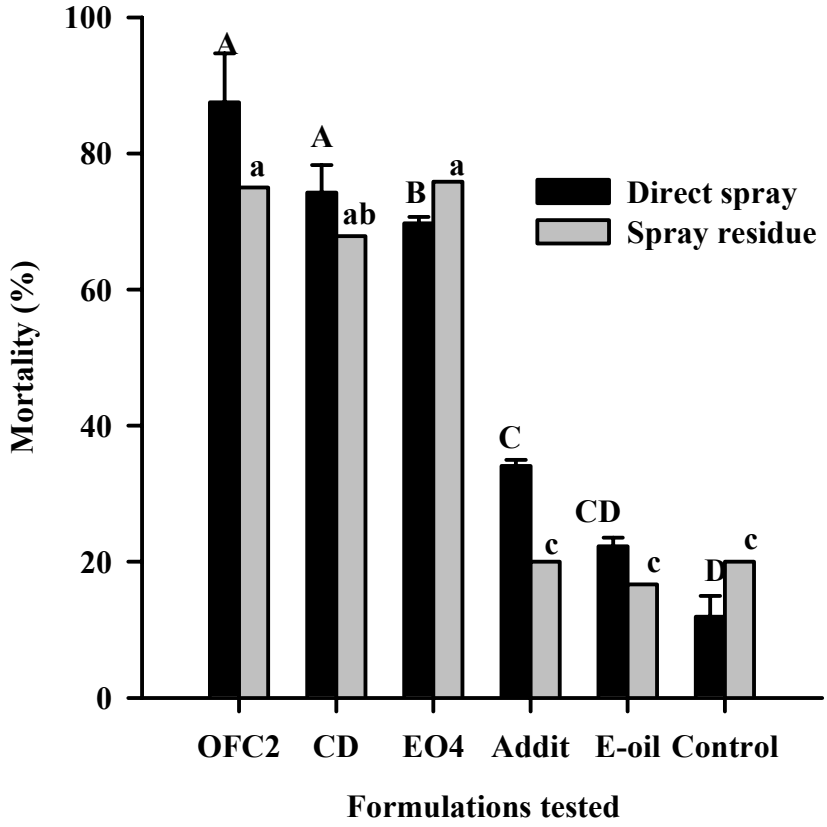

Figure 2. Mean percent mortality of *L. migratoria* treated with different formulations of *M. anisopliae* var. *acridum*, (IMI 330189) in the laboratory. Bars with the same shading followed by the same letters are not significantly different (SNK test, $p < 0.001$) for direct spray, (log-rank test, $p < 0.001$) for spray residues.

formulation of submerged spores followed by the Codacide and EO4 emulsions (Figure 2). Median survival time for these three formulations ranged from 12 to 14 days. Submerged spores formulated with Addit and E-oil emulsions showed less than 35 % mortality (Figure 2). The survival curve analysis revealed a highly significant difference in survival curves of *L. migratoria* exposed to the spray residues of the different submerged spore formulations ($\chi^2 = 46.23$; $df = 5$; $p < 0.001$). Exposure to the spray residues of OFC2 and EO4 formulations resulted in higher mortality of *L. migratoria* followed by the Codacide emulsion (Figure 2) and the median survival time ranged from 13 to 16 days. The Addit™ and E-oil emulsions did not differ with the control in respect to mortality and median survival time. Based on the results obtained in both laboratory spray experiments I and II, OFC2, codacide and EO4 formulations of submerged spores were chosen for the field experiment.

Field experiment

Direct spray effect. None of the formulations affected the viability and the speed of spore germination immediately after formulation (Figure 3). The germination rates obtained after formulation of the spores are comparable to the viability of spores before formulation. The droplet density observed ranged from 85 to 125 per cm^2 and 95 to 185 per cm^2 on the indicator paper cards inside the cage and on plants, respectively, indicating adequate coverage during application.

Survival curve analysis revealed a highly significant difference in median survival time (χ^2 = 295.83, 421.38; df = 6; 6 $p < 0.001$) among the formulations for *C. haemorrhoidalis* and *L. migratoria*, respectively. For both test insects, mortality was faster and higher for Green Muscle™, resulting in a short median survival time (Table 2). For *C. haemorrhoidalis*, among the submerged spore formulations tested, OFC2 caused the highest mortality, showed short median survival time and differed significantly from the remaining submerged spore formulations ($p < 0.001$) (Table 2). The remaining formulations caused 76 to 84 % mortality on *C. haemorrhoidalis*, and there were no differences in median survival time. With regard to *L. migratoria*, all submerged spore formulations showed lower mortality and a higher median survival time when compared to Green Muscle™. The differences among the submerged

Table 2. Cumulative mortality, mycosis and median survival time (MST) of *C. haemorrhoidalis* and *L. migratoria* treated with different formulations of *M. anisopliae* var. *acridum* in Niger (n = 70 to 100 for *C. haemorrhoidalis* and n = 55 to 100 for *L. migratoria*).

	C. haemorrhoidalis			*L. migratoria*		
Treatments[a]	Mortality (%)	Mycosi s (%)	MST (Days)	Mortality (%)	Mycosis (%)	MST (Days)
GM	98.2	82.6	6 ± 0.2 a	99.0	54.1	10 ± 0.4 a
OFC2	90.0	83.8	8 ± 0.4 b	31.0	39.0	18 ± 0.8 b
EO4	84.3	76.3	10 ± 0.8 c	22.1	36.0	17 ± 0.8 b
OFC1	76.3	94.6	11 ± 0.4 c	28.3	19.0	18 ± 0.8 b
CD	77.5	87.0	10 ± 0.5 c	12.0	8.0	20 ± 0.4 b
OE2	77.0	77.5	11 ± 0.7 c	27.0	44.0	19 ± 0.5 b
Control	3.0	0.0	10 ± 0.0 d	1.0	0.0	4 ± 0.0 d

[a] GM was applied at 5 x 10^{12} conidia ha^{-1} whereas all the other formulations were applied at 1 x 10^{13} conidia ha^{-1}. Mean within a column followed by the same letter are not significantly different in their survival curves (Log rank test, $p < 0.001$).

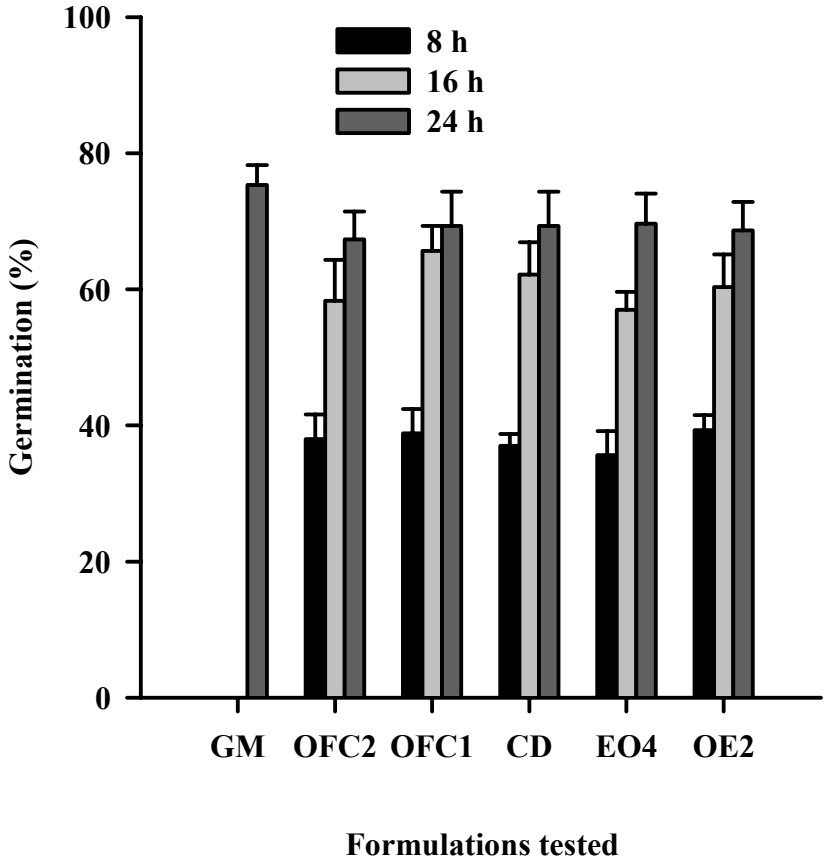

Figure 3. Effect of formulation carriers on viability and speed of germination of aerial conidia and freeze dried submerged spores of *M. anisopliae* var. *acridum* (IMI 330189). ($F = 1.90$, $df = 4$, $p = 0.2045$) for 8 hr, ($F = 2.94$, $df = 4$, $p = 0.0906$) for *16* h and ($F = 2.02$, $df = 5$, $p = 0.1612$) for 24 h after incubation at 30°C. For GM, germination rate at 8 h and 16 h was not assessed.

conidia formulations were not significant, however, OFC2, OFC1 and OE2 formulations resulted in a relatively higher mortality than the remaining treatments (Table 2). For all formulations, the infection rate on *C. haemorrhoidalis* exceeded 75 % whereas it ranged from 8 to 54 % on *L. migratoria* (Table 2).

Spray residue effect. For both test insects, the infectivity of the spray residues followed patterns comparable to the direct spray effects (Tables 3 & 4). For all exposure dates, mortality of *C. haemorrhoidalis* was higher and faster for spray residues of Green Muscle™ ($p < 0.001$) (Tables 3 & 4). For day 0 exposures (immediately after application), Codacide, OFC2 and EO4 formulations showed higher mortality in *C. haemorrhoidalis* with a prolonged median survival time. The OE2 formulation produced a mortality of only 25 % (Tables 3). At

5 days after treatment exposures, all emulsions caused over 83 % mortality on *C. haemorrhoidalis*. For these formulations, the median survival time exceeded 17 days and was significantly different from OFC2 (Table 4). The OFC2 formulation caused over 74 % mortality on *C. haemorrhoidalis* with shorter median survival time where as OFC1 showed lower mortality and longer median survival time (Table 3 & 4). Exposure of *C. haemorrhoidalis* to spray residues of the different formulations 10 days after treatment resulted in 71 to 83 % total mortality and the median survival time differed (Tables 3 & 4). A prolonged median survival time was observed for OE2 and OE4 emulsions followed by OFC1, OFC2 and CD formulations. Green Muscle™ showed lower median survival time when compared to the other formulations. Regardless of the age and type of formulations tested, the infection rate on *C. haemorrhoidalis* for the spray residue ranged from 19 to 98 % (Table 3).

The activity of spray residues of submerged conidia formulations on *L. migratoria* follows a different pattern (Tables 3 & 4). For all exposure dates, mortality of *L. migratoria* was higher and faster for Green Muscle™ followed by OFC2 and OFC1 with the exception of OFC2 on 5 days after treatment exposure (Tables 3 & 4). Following the Green Muscle™, mortality on 5 days after treatment exposures was relatively faster and higher for OFC1, CD and OE2

Table 3. Cumulative percent mortality and mycosis of *C. haemorrhoidalis* and *L. migratoria* exposed to spray residues of different formulations of *M. anisopliae* var. *acridum* (isolate IMI 330189) submerged spores and aerial conidia at different time intervals after application in Niger (DAT: Days after treatment).

	Percent mortality and mycosis					
	C. haemorrhoidalis			*L. migratoria*		
Treatments[a]	0 DAT	5 DAT	10 DAT	0 DAT	5 DAT	10 DAT
GM	99.1 (98.2)	93.2 (92.7)	82.0 (84.1)	88.1 (38.5)	80.0 (31.3)	78.2 (29.6)
OFC2	72.0 (27.8)	74.3 (65.4)	86.1 (91.9)	54.2 (3.9)	30.0 (27.8)	62.1 (50.0)
EO4	70.5 (18.6)	86.0 (81.4)	76.7 (82.6)	24.6 (0.0)	25.0 (26.7)	25.0 (13.3)
OFC1	56.5 (42.3)	65.0 (89.3)	83.0 (64.1)	43.1 (0.0)	47.5 (35.7)	72.9 (37.2)
CD	74.4 (25.8)	83.0 (82.5)	79.5 (58.1)	21.7 (23.1)	61.0 (30.7)	23.3 (7.1)
OE2	25.0 (30.0)	88.0 (84.1)	71.4 (66.7)	19.0 (18.2)	50.0 (51.7)	43.3 (16.0)
Control	6.7 (0.0)	0.0	0.0	8.3 (0.0)	25.0 (0.0)	8.3 (0.0)

[a] GM was applied at 5 x 10^{12} conidia ha^{-1} whereas all the other formulations were applied at 1 x 10^{13} conidia ha^{-1}. Significant log rank test ($p < 0.001$) for survival curves. Values in parenthesis represent percent mycosis. (n = 69 for *C. haemorrhoidalis* , and n = 65 for *L. migratoria*).

formulations. The OFC2 and EO4 formulations caused lower mortality with longer median survival time. Ten days after treatment, the median survival time was significantly lower for all oil-based formulations compared to emulsions (Tables 3 & 4). For this exposure date, there were no differences among the emulsions in median survival time but all differed from the control, which showed a median survival time of over 20 days. Regardless of the age and type of formulations tested, the infection rate on *L. migratoria* for the spray residue ranged from 0 to 52 % (Table 3).

DISCUSSION

In the present study, different formulations of submerged conidia of *M. anisopliae* var. *acridum* were developed and their efficacy was tested in the laboratory and in the field. This is the first time that oil flowable concentrate formulations of freeze or spray dried submerged conidia of this fungus have been examined for locust and grasshopper control. As demonstrated, the efficacy, measured by mortality and median survival time, varied among the different formulations tested. Green Muscle™ (aerial conidia) formulated in diesel oil was

Table 4. Median survival time of *C. haemorrhoidalis* and *L. migratoria* exposed to spray residues of different formulations of *M. anisopliae* var. *acridum* (isolate IMI330189) at different time intervals after application in Niger (DAT: Days after treatment).

	Median survival time (Days ± SE)					
	C. haemorrhoidalis			*L. migratoria*		
Treatments[a]	0 DAT	5 DAT	10 DAT	0 DAT	5 DAT	10 DAT
GM	7 (0.2) a	13 (0.5) a	16 (0.5) a	13 (0.6) a	18 (0.4) a	16 (0.5) a
OFC2	16 (1.2) b	16 (1.2) b	17 (0.7) b	18 (0.5) b	20 (0.5) c	16 (0.6) a
EO4	16 (0.7) b	17 (0.7) c	20 (0.4) c	18 (0.5) b	20 (0.4) c	18 (0.2) b
OFC1	17 (0.8) b	20 (0.6) d	18 (0.6) b	18 (0.6) b	19 (0.4) b	16 (0.6) a
CD	15 (1.0) b	18 (0.6) d	16 (0.9) b	19 (0.5) c	19 (0.5) b	18 (0.3) b
OE2	9 (0.5) b	18 (0.7) d	18 (0.7) c	20 (0.5) c	19 (0.4) b	18 (0.7) b
Control	16 (4.5) c	-	-	19 (0.3) d	20 (0.4) c	18 (0.3) d
χ^2	277.98	186.79	111.38	201,73	75,31	75,31
p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a] GM was applied at 5 x 10^{12} conidia ha^{-1} whereas all the other formulations were applied at 1 x 10^{13} conidia ha^{-1}. Means within column followed by the same letter are not significantly different in their survival curves (Log rank test, $p < 0.001$). (n = 69 for *C. haemorrhoidalis* , and n = 65 for *L. migratoria*).

highly effective for both pests tested, with adequate residual effect under laboratory and field conditions. These results agree with previous studies carried out with *Hieroglyphus daganensis* and other grasshopper species (Prior *et al*., 1988; Bateman *et al*., 1993; Kassa *et al*., submitted). Direct application of submerged conidia at the rate of 1 x 10^{13} conidia ha^{-1} in an oil flowable concentrate or in an emulsion achieved adequate control of *C. haemorrhoidalis* under field conditions. As regards to *L. migratoria*, all submerged conidia formulations performed better under laboratory conditions than in the field. The spray residue effect assessment indicated differential persistence of all submerged conidia formulations up to 10 days under unfavourable environmental conditions. Regardless of the formulations tested in the laboratory, mortality of *L. migratoria* was higher for the spray residues than for the direct spray alone. This is probably linked to the high spore up-take which occurs during insect foraging on treated leaves, underlining the importance of secondary spore pick-up from treated plants (Thomas *et al*., 1998). Furthermore, the oil has the added advantage of protecting the spores from desiccation, irradiation and enhancing the efficacy (Moore and Caudwell, 1997; Prior *et al*., 1988).

Of the two oil flowable concentrate formulations tested, the OFC2 appeared to be more effective. A flowable oil formulation and emulsifiable suspensions have been developed for aerial conidia of *B. bassiana* (Delgado *et al*., 1997) and an oil flowable concentrate for *M. anisopliae* (Bateman, 1997) and for bacterial biopesticides (Lisanky *et al*., 1993). Our results also indicate the potential of emulsions of submerged conidia for locust and grasshopper control under Sahelian conditions. The potential use of emulsions for the control of locusts and grasshoppers was also demonstrated by others (Jenkins and Thomas, 1996; Delgado *et al*., 1997; Stephan and Zimmermann, 2001; Kassa *et al*., submitted).

Adequate host targeting is imperative for successful inundative control of insects with entomopathogenic fungi (Inglis *et al*., 1997). For all formulations, we observed large numbers of droplets on the cover-slips, Petri-dishes and indicator paper-cards. Furthermore, all droplets found on Petri-dishes containing nutrient agar were able to germinate and form colonies after incubation at 30 °C in the laboratory (Data not shown). Despite the depositions of a large number of droplets, the efficacy observed in all submerged conidia formulations was lower than that observed on Green Muscle™. There could be several explanations for this; the most important reasons however, may be associated with the fitness of submerged spores in hot and dry conditions, the relatively large particle size of the products, the nature of the

formulations and the associated environmental stress. Therefore, further studies are required in order to investigate the effects of these factors and thus improve the quality and efficacy of submerged spore formulations.

Studies have shown that environmental temperature and host thermal biology are central in determining the performance of mycoinsecticides (Lomer *et al*., 2001). In our research we observed differences among the grasshopper species in susceptibility to various mycoinsecticide formulations. *C. haemorrhoidalis* was found to be more susceptible to all formulations than *L. migratoria*. This might be partially explained by the behaviour and relative body size of the two species and the amount of viable inoculum load acquired during spray application. Under conditions that enable locusts and grasshoppers to thermoregulate optimally, pathogens are unable to develop inside infected insects and incubation time will increase (Lomer *et al*., 2001). Thus *C. haemorrhoidalis* is much smaller than *L. migratoria* and probably needs only a small number of droplets for infection to occur and that the thermal ecology of the host-pathogen interaction might have favoured the pathogen to cause infection more rapidly. The existence of some behavioural responses (fever) in reaction to the infection caused by entomopathogens has been reported for various grasshopper species (Inglis *et al*., 1996; Blanford *et al*., 1998, 2000; Ouedraogo *et al*., 2002).

In general, our study established a number of relevant results: (1) when aerial and submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189) were tested in different formulations in the laboratory and under field conditions, aerial conidia formulated in diesel oil displayed a higher efficacy at a lower application rates of 5 x 10^{12} conidia ha^{-1} than all submerged conidia formulations, which were applied at 1 x 10^{13} conidia ha^{-1}; (2) freeze or spray dried submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189) formulated in an oil flowable concentrate formulation are equally infectious to *L. migratoria* under laboratory conditions at a higher rate 1 x 10^{13} spores ha^{-1}; (3) submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189) can be formulated as emulsions or as an oil flowable concentrate and one oil flowable concentrate (OFC2) formulation revealed good biocontrol potential under both laboratory and field conditions followed by CD, EO4 and OE2 emulsions; 4) insects varied in their susceptibility to mycoinsecticide formulations, *C. haemorrhoidalis* being more susceptible to the mycopathogen than *L. migratoria*. Overall, the results indicated that Green Muscle™ was superior to the submerged conidia formulations tested.

Overall, our results provide evidence on the potential use of submerged conidia of *M. anisopliae* var. *acridum* under Sahelian environmental conditions. However, these facts and the variability in the results amongst the different treatments underline the critical role of the production and formulation process and the importance of formulation research. Therefore, if further large scale field experiments are anticipated using submerged spores/conidia of *M. anisopliae* var. *acridum*, improvements on the production process along with the quality of the formulation is indispensable. We believe that mycopesticides based on submerged conidia of *M. anisopliae* var. *acridum* represent a promising future for locust and grasshopper control.

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Plate 1. Freeze-dried submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189).

Plate 2. ULVAFAN-MK2 sprayer mounted on a run-way bridge for laboratory spray experiments. a) A liquid pump pumping OFC2 formulation and a rubber tube for delivery of the suspension into the sprayer tip, b) power supply for the sprayer, c) Sprayer holder, d) Run-way line support (front view), e) Sprayer tip, f) fan, g) sample plants and caged insects used during laboratory spraying.

Plate 3. Potted cabage plant used for spray residue assessments in the laboratory. a) Sprayed cabbage plant and b) *L. migratoria* exposed to the spray residues on cabbage plant after 72 h.

Plate 4. Large field-cage experiment. c) Spraying of potted millet and caged insects and d) bulking of the treated insects into one cage for incubation.

a) Infected *C. haemorrhoidalis.*

b) Internal sporulation of *M. anisopliae* var *acridum* on infected *C. haemorrhoidalis* a day after incubation in moist chamber.

c) External sporulation of *M. anisopliae* var *acridum* on infected *C. haemorrhoidalis.*

Plate 5. *Metarhizium anisopliae* var. *acridum* (IMI 330189) infection development on *C. haemorrhoidalis.*

CHAPTER 5

Effect of temperature on spore germination and growth of entomopathogenic fungi isolates (Deuteromycotina: Hyphomycetes) from Ethiopia

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Investigating the interaction of temperature and entomopathogenic fungi are essential for the development of mycoinsecticides. To this end, we determined the effect of temperature on germination and vegetative growth of *Beauveria bassiana*, *Metarhizium anisopliae*, *B. brongniartii* and *Paecilomyces* sp. obtained from various agro-ecological zones in Ethiopia. The fungi were grown on malt extract agar at 20–35°C and at 8–37°C for germination and vegetative growth assessments, respectively. The results revealed that the lag phase and time to 95% germination levels were temperature and isolate dependent. Consequently, the temperature range producing the shortest lag phase was determined to be 25–30°C for most *M. anisopliae* isolates, 20–30°C for *Paecilomyces* sp. and 20–35°C for most *Beauveria* species. The observed optimal temperature range for germination of *M. anisopliae* isolates P2 and P-EE was 20–30°C and 25–35°C, respectively. Most *M. anisopliae* isolates reach 95% germination within 11–20 h at 25–30°C, compared with 11–26 h for *Paecilomyces* and *Beauveria* isolates at 20–30°C. *Metarhizium* anisopliae isolate P-EE requires 10–14 h for the initiation of germination and 57–70 h to reach 95% germination. The optimum temperature for vegetative growth was 30°C for seven and 20–30°C for three *M. anisopliae* isolates, compared with 20–25°C for the *Paecilomyces*; and 25–30°C for nine *B. bassiana* isolates. Two *Beauveria* isolates (P1 and P-BB) exhibited temperature optima at 25°C. The maximum thermal threshold occurred at 35–37°C for most isolates; and > 37°C for two isolates. None of the tested isolates grew at 8°C. At 25/35°C alternating temperature (8/16 h) all but three isolates tested exhibited growth. The thermal requirements established in this study would substantially facilitate the future selection of these entomopathogenic fungi for use in biocontrol.

Key words: conidia, entomopathogenic fungi, germination, vegetative growth, temperature.

INTRODUCTION

Temperature is an important factor that affects the efficacy of entomopathogenic fungi (Carruthers, Robson and Roberts, 1985; Benz, 1987; McCoy, Samson and Boucias, 1988; Glare and Milner, 1991). It also affects the survival of these pathogens and their ability to infect, the host's susceptibility and resistance, and the progress of the infection within the host (Ferron, Fargues and Riba, 1991). Thus, for successful development as microbial control agents, entomopathogenic fungi must be adapted to the environmental conditions in the area in which they intended to be applied (Ferron, Fargues and Riba, 1991).

Several authors have addressed inter-specific as well as intra-specific variability of temperature tolerance among the various groups of entomopathogenic fungi (Ferron, Fargues and Riba, 1991; Glare and Milner, 1991; Ouedraogo et al., 1997; Fargues et al., 1997; Vidal, Fargues and Lacey, 1997). Recent explorations for entomopathogens conducted in Ethiopia, have resulted in the collection of hundreds of isolates of entomopathogenic fungi with potential for control of a number of major insect pests (Kassa et al., unpublished). The isolates were collected from different hosts in diverse agro-ecological zones, but their thermal requirements are still not known. Therefore, the objective of this study was to determine the effect of temperature on *in vitro* spore germination and vegetative growth of some of these entomopathogenic fungi. Some isolates obtained from the Sudan were also included in the study whereas *Metarhizium anisopliae* var. *acridum*, isolate IMI330189, was included as a standard check.

MATERIALS AND METHODS

Fungal isolates and growth conditions

Ten *B. bassiana*, one *B. brongniartii*, ten *M. anisopliae* isolates and one *Paecileomyces* sp. were examined in this study. The list of the isolates, their hosts and their origin are described in Table 1. The isolates were grown and maintained on malt extract agar (MEA) (3% malt extract, 0.5% soya-peptone, 1.5% agar, all from VWR International, Darmstadt, Germany) under dark conditions at 25 °C.

Germination rate

The conidia from a two weeks old culture, harvested by scraping, were suspended in demineralised and sterile water containing Tween 80 (0.1% v/v). The stalk suspension was

Table 1. List of entomopathogenic fungal isolates tested, their host insect, place of origin and altitude.

Isolate No.[a]	Fungal species	Host insect	Place of origin and altitude (m)[b]
	Beauveria bassiana		
PPRC-9501 (P1)	*B. bassiana*	*Melolontha* sp. (Coleoptera: Melolonthidae)	West Showa (2500) (Tukurinchinii)
PPRC-9604 (P4)	*B. bassiana*	*Aceraea acerata* (Lepidoptera: Nymphalidae)	Arbaminch (1925)
PPRC-9609 (P9)	*B. bassiana*	*Blosyrus rugulosus* (Coleoptera: Curculionidae)	Dila (1875)
PPRC-9614 (p14)	*B. bassiana*	Ground beetles (Coleoptera)	Awassa (1750)
PPRC-9615 (P15)	*B. bassiana*	Spider (Arachnida)	Awassa (1750)
PPRC-CC (P-CC)	*B. bassiana*	Adult beetle (Coleoptera)	Woldiya (1950)
PPRC-DD (P-DD)	*B. bassiana*	Adult beetle (Coleoptera)	Debremarkos (2030)
PPRC-FF (P-FF)	*B. bassiana*	Adult beetle (Coleoptera)	Ashengae (2400)
PPRC-GG (PGG)	*B. bassiana*	Adult beetle (Coleoptera)	Ashengae (2400)
PPRC-HH (P-HH)	*B. bassiana*	Adult beetle (Coleoptera)	Ashengae (2450)
PPRC-BB (P-BB)	*B. brongniartii*	Spider (Arachnida)	Tigray (1950)
PPRC-AA (P-AA)	*Paecilomyces* sp.	Adult beetle (Coleoptera)	Korem (2450)
	Metarhizium anisopliae		
PPRC-EE (P-EE)	*M. anisopliae* var. a*nisopliae*	Crustacean (sow pill)	Alemata (1500)
PPRC-2 (P2)	*Metarhizium* var. *anisopliae*	*Pachnoda interrupta* (Coleoptera: Scarabaeidae)	North Showa (Ashan, 1900)
PPRC-4 (P4)	*Metarhizium* var. *anisopliae*	*P. interrupta* (Coleoptera: Scarabaeidae)	North Showa (Shewa Robit,1950)
PPRC-5 (P5)	*Metarhizium* var. *anisopliae*	*P. interrupta* (Coleoptera: Scarabaeidae)	North Showa (Shewa Robit, 1950)
PPRC-6 (P6)	*Metarhizium* var. *anisopliae*	*P. interrupta* (Coleoptera: Scarabaeidae)	North Showa (Kewot, 1950)
PPRC-55 (P55)	*Metarhizium* var. *anisopliae*	*P. interrupta* (Coleoptera: Scarabaeidae)	North Showa (Kewot, 1950)
IMI 330189 (Mfl6)	*Metarhizium anisopliae* var. *acridum*	*Ornithacris cavroisi*	Niger
S1a	*Metarhizium anisopliae*	Acridid	Sudan
S1b	*Metarhizium anisopliae*	Acridid	Sudan
S4	*Metarhizium anisopliae*	Acridid	Sudan

[a] Description in parentheses represents short names used in the text.

[b] All other places in the last column are found in Ethiopia.

sonicated for 3 min, filtered through a double layer of cloth mesh, and the spore density was ssessed using a haemocytometer. From all isolates, a conidial suspension containing 1 x 10^7 conidia ml^{-1} was prepared. The inoculation of the plates was carried out following the method used by Thomas and Jenkins (1997) with modification. Three drops, each 1 µl of the spore suspension, were aseptically applied onto the surface of MEA in a Petri-dish (60 mm

diameter) and allowed to dry under the clean bench before incubation. For each isolate, there were three replicates and a total of 32 plates were inoculated with the spore suspension. Inoculated plates were then incubated at 20, 25, 30 and 35°C for 8–48 h. Germination was assessed at various time intervals by counting 300 spores in each replicate using a Zeiss Axioplan microscope (Germany) at 400 x magnification. Spores were considered germinated if the germ tube is at least twice longer than the width of the conidia.

Radial growth rate

The conidial suspension was prepared as described above. A single drop (2 µl) of the spore suspension (1 x 10^7 conidia ml^{-1}) was aseptically applied onto the centre of a Petri-dish (90 mm diameter) containing malt extract agar. In all cases, the area of the applied and dried droplets was 4 mm diameter. Four replicate plates were prepared for each isolate-temperature combination and incubated for 12 days at a constant dark condition at 8, 15, 20, 25, 30, 35, and 37°C and 60–70% RH. One set of culture was also incubated for 12 days at 25/35°C (8/16 h) fluctuating temperature regimes and 40–60% RH. Radial growth was measured daily using two cardinal diameters previously drawn on the bottom of each plate.

Data Analysis

Percent germination data observed after 24 h incubation were square-root arcsine transformed and subjected to two-way ANOVA using the PROG GLM procedure (SAS, 1989). Percent germination data, observed for each isolate-temperature combination at various time intervals, were used in order to estimate the lag phase (LP) (time to 5% germination) and time to 95% germination (TG_{95}) (Hywel-Jones and Gillespie, 1990; Vanniasingham and Gilligan, 1988) using the PROC PROBIT procedure (SAS, 1989). Growth rates (velocity mm day^{-1}) were used as the main parameter to evaluate the influence of temperature on fungal growth (Fargues et al., 1992). Analysis of variance was done using a two-way ANOVA following the PROC GLM procedure (SAS, 1989). Data from 8 and 37°C treatments were excluded from the analyses because, for the most part, there was either no growth or growth was not significant at these temperatures. Where significant differences ($P < 0.05$) were detected with ANOVA, means were separated using Student- Newman-Keuls test (SNK) (SAS, 1989).

RESULTS

Germination rate

The germination rate of *M. anisopliae*, *B. bassiana* and *Paecilomyces* sp. was found to be temperature and isolate dependent, as can be seen from Figure 1a–b. In *M. anisopliae*, there was a highly significant difference between temperature (F = 481, df = 3, $P < 0.0001$) and isolate (F = 960.3, df = 9, $P < 0.0001$) as well as for isolate by temperature interaction (F = 56.2, df = 26, $P < 0.0001$). Four *M. anisopliae* isolates (Mfl6, S4, S1b and S1a) showed a consistently high germination rate at all temperatures tested whereas four other isolates (P5, P4, P55 and P2) exhibited a high germination rate at 20°, 25° and 30°C (Figure 1a). For these isolates, the germination rate declines rapidly at 35°C. At a lower temperature, P-EE showed no germination and at a higher (> 30°C) exhibited a reduced germination rate (Figure 1a). This isolate germinated best at 25°C. However, compared to the other isolates, the observed germination rate was much lower after 24 h. Similarly, isolate P6 showed lower than 5 % germination rate at all temperature ranges tested (Figure 1a).

As regards *B. bassiana*, there were also a highly significant difference between temperature (F = 382, df = 3, $P < 0.0001$) and isolate (F = 41.6, df = 11, $P < 0.0001$) as well as for isolate by temperature interaction (F = 20.98, df = 32, $P < 0.0001$). All tested isolates, exhibited 87-100% germination at 20–30°C after 24 h, and even at 35°C the germination rate of all isolates except P-AA was between 65 and 98 % (Figure 1b). Compared to the others, isolate P1 showed a reduced germination rate at 30 and 35°C (Figure 1b). The *Paecilomyces* sp. (P-AA) exhibited 98-100% germination at 20-30°C and only 5% at 35°C (Figure 1b).

The lag phase (LP) and time to 95% germination (GT_{95}) of *M. anisopliae* isolates tested were temperature dependent (Figure 2a-b). The LP of isolate S4 and Mfl6 had a similar response to the temperature range studied. In both cases, the LP ranged between 5-8 h. Similarly, for isolate S1b, the LP ranged from 9–12 h (Figure 2a). In isolate P-EE, the lag phase increased with increasing temperature up to 35°C. The shortest LP was at 25 °C for isolate P2 and P5 compared to 30°C for isolate P4 and S1a (Figure 2a). In contrast to the other isolates, P55 showed the shortest LP both at 25 and 30°C. Most isolates showed an extended LP at 20°C and 35°C (Figure 2a). On the other hand, no germination was observed at these both temperatures in P-EE and P2, respectively.

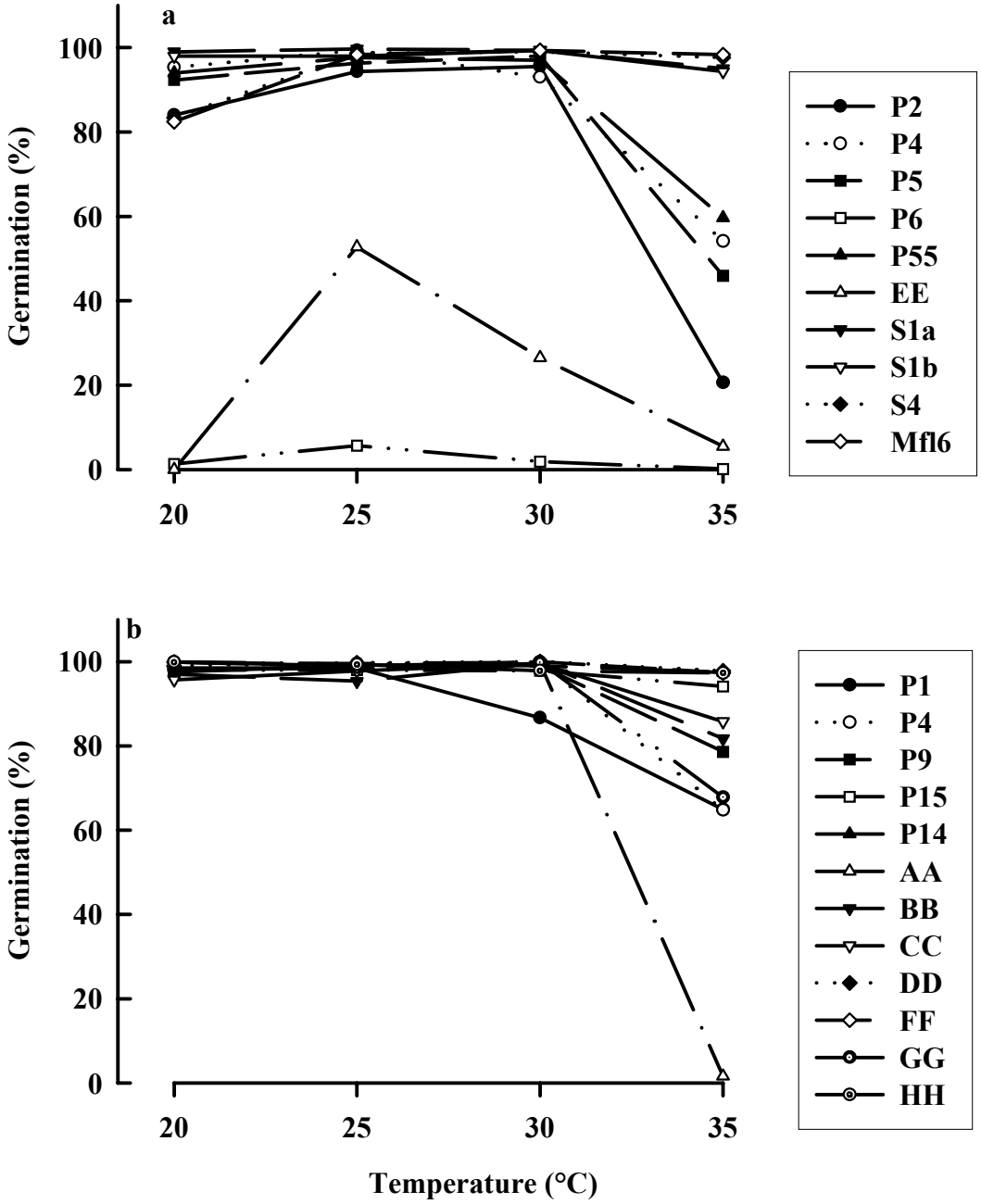

Figure 1. The effect of temperature on the mean germination (%) after 24 h of (a) *M. anisopliae* and (b) *B. bassiana, B. brongniaritii* and *Paecilomyces* sp. *in vitro.*

Compared to other isolates, the GT_{95} was extremely higher in P-EE at 25-35°C (Figure 2b). Two other isolates (S4 and Mfl6) required 37 h and 43 h to approach 100% germination at 20°C, respectively. For S4, Mfl6, S1a and S1b, the GT_{95} was reduced to lower than 24 h at 35°C. The remaining other isolates approached 100% germination between 20-30 h at 20°C, 15-20 h at 25°C and 13-25 h at 30°C (Figure 2b).

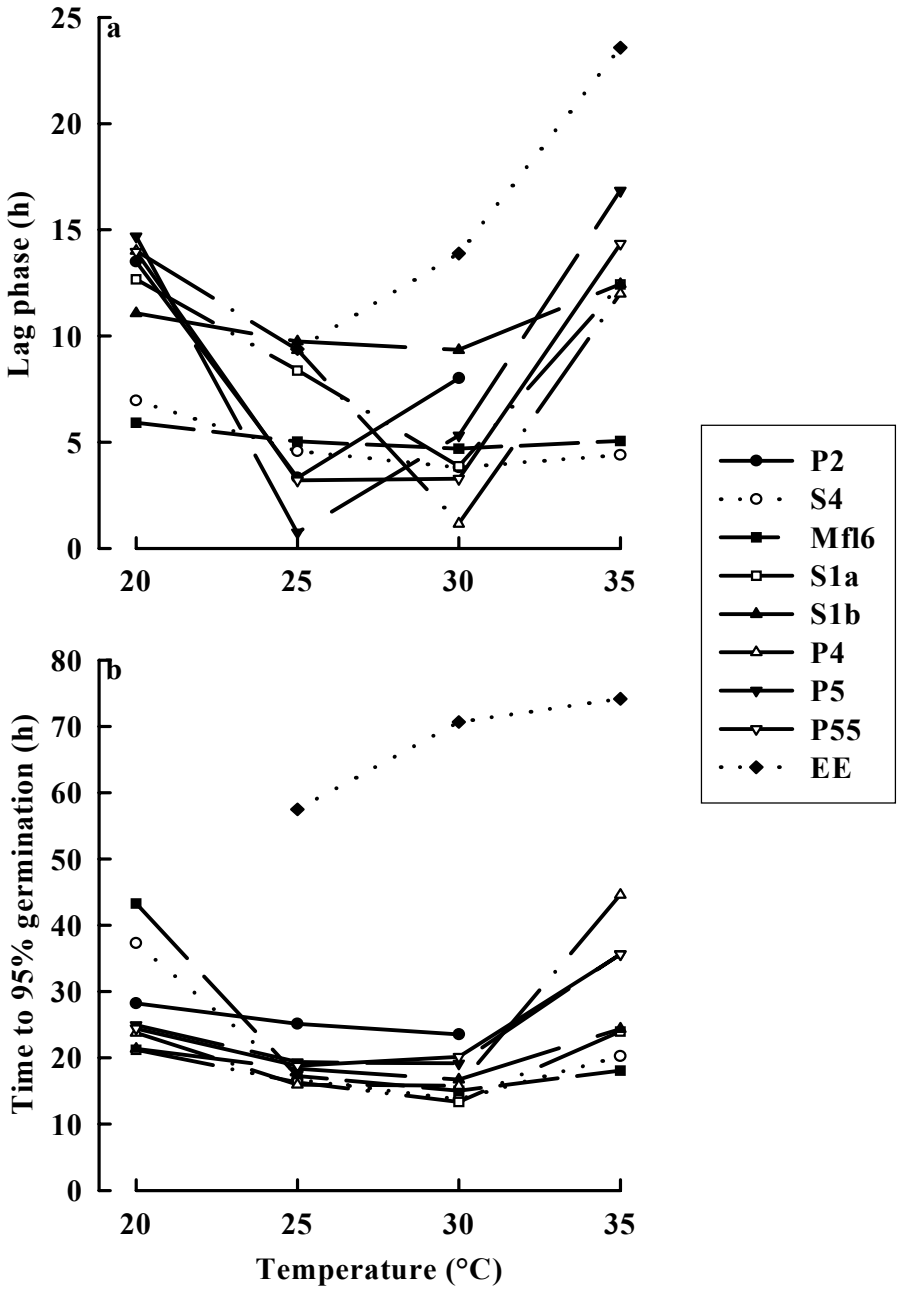

Figure 2. Effect of temperature on lag phase duration (a) and GT_{95} (b) of *M. anisopliae* isolates.

In the case of *B. bassiana*, the LP duration was reduced with increasing temperature (Figure 3a) and six isolates (P-HH, P-CC, P1, P4, P-GG, P-FF) showed the shortest (< 6 h) LP at all temperature ranges tested. The shortest LP was at 30°C and 35°C for isolate P1 and P9, compared with 35°C for P4 and P14. *Paecilomyces* sp. (isolate P-AA) showed the shortest LP at 30°C.

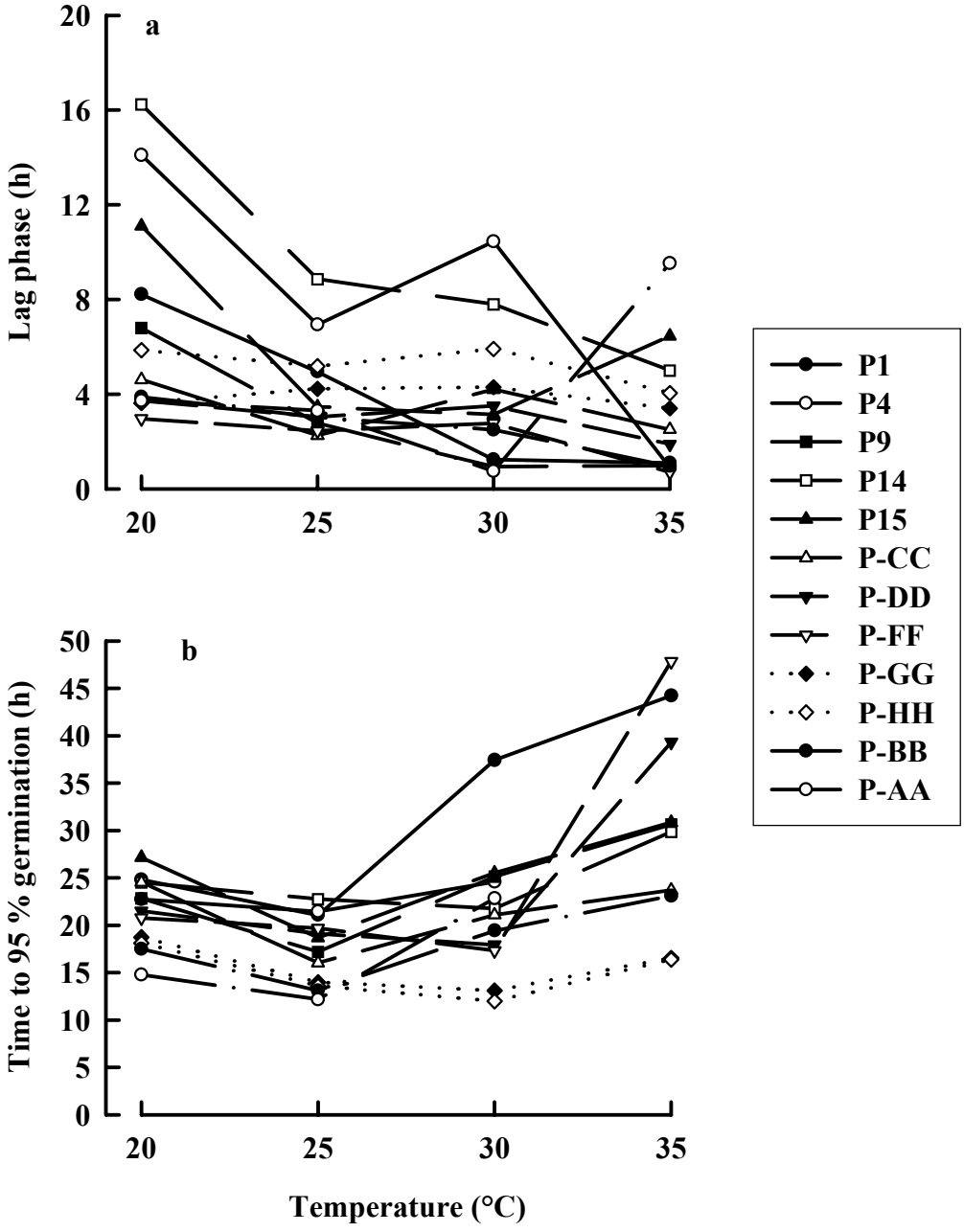

Figure 3. Effect of temperature on lag phase duration (a) and GT_{95} (b) of *B. bassiana*, *B. brongniartii* and *Paecilomyces* sp. isolates.

For most *B. bassiana* isolates, the GT_{95} remains lower than 30 h at all temperatures tested (Figure 3b). However, three isolates (P1, P-FF and P-DD) require over 39 h to approach 100% germination at 35°C. Isolate P1 requires 37 h to approach 100% germination at 30°C, compared with other isolates, which need 15–25 h to approach 100% germination at 30°C (Figure 3b). At 20°C and 25°C all isolates required 15–27 h and 15–23 h to approach 100%

germination, respectively (Figure 3b). Isolate P-HH and P-GG show a similar response to all temperatures tested.

Growth rate

Both *M. anisopliae* and *B. bassiana* grew at a wider temperature range (Figure 4a-b). For most *M. anisopliae* isolates a well defined maximum thermal threshold occurred at 37°C , compared with 35°C for one *M. anisopliae* isolate (P6) and > 37°C for two *M. anisopliae* isolates (Mfl6 and S4) (Figure 4a). In all *M. anisopliae* isolates tested, no growth was observed at 8°C, i.e. the minimum temperature threshold is between 8° and 15°C.

The radial growth of *M. anisopliae* was affected significantly by temperature ($F = 1671$, df = 5, $P < 0.0001$) and the isolate ($F = 211.5$, df = 8, $P < 0.0001$) with significant isolate by temperature interaction ($F = 31.5$, df = 40, $P < 0.0001$). A one-way analysis of variance on the radial growth rate of *M. anisopliae* isolates at each temperatures tested also showed a significant difference between isolates ($P < 0.0001$). For most *M. anisopliae* isolates the optimum growth occur at 30 °C, whereas for three *M. anisopliae* isolates (S1b, S1a and P-EE) the optimum growth rate was at 20–30 °C (Figure 4a). At 35°C, isolate P6 showed no growth at all whereas at the same temperature, the remaining isolates exhibited a reduced growth rate (Figure 4a).

In the case of *B. bassiana* the minimum and maximum temperature threshold were between 8°C and 15°C and 35° to 37°C, respectively, whereas for *Paecilomyces* sp. they were 8°C and 35°C, respectively. As regards to *Beauveria* and *Paecileomyces* isolates, radial growth was also affected significantly by temperature ($F = 929.6$, df = 4, $P < 0.0001$), the isolate ($F = 30.70$, df = 11, $P < 0.0001$) and by the isolate-temperature interaction ($F = 13.89$, df = 44, $P < 0.0001$) (Figure 4b). *Paecileomyces* sp. (P-AA) showed an optimum growth at 20–25°C, compared to 25-30°C for 9 *B. bassiana* isolates; and 25°C for isolates P1 and P-BB (Figure 4b).

The growth rates of *M. anisopliae*, *B. bassiana*, *B. brongniaritii* and *Paecilomyces* sp. were also tested at an alternating temperature of 25/35°C, 8:16 h (Figure 5). The results revealed significant differences among the *M. anisopliae* isolates ($F = 174.6$, df = 8, $P < 0.0001$) as well as among the *Beauveria* isolates and *Paecilomyces* sp. ($F = 46.25$, df = 11, $P < 0.0001$).

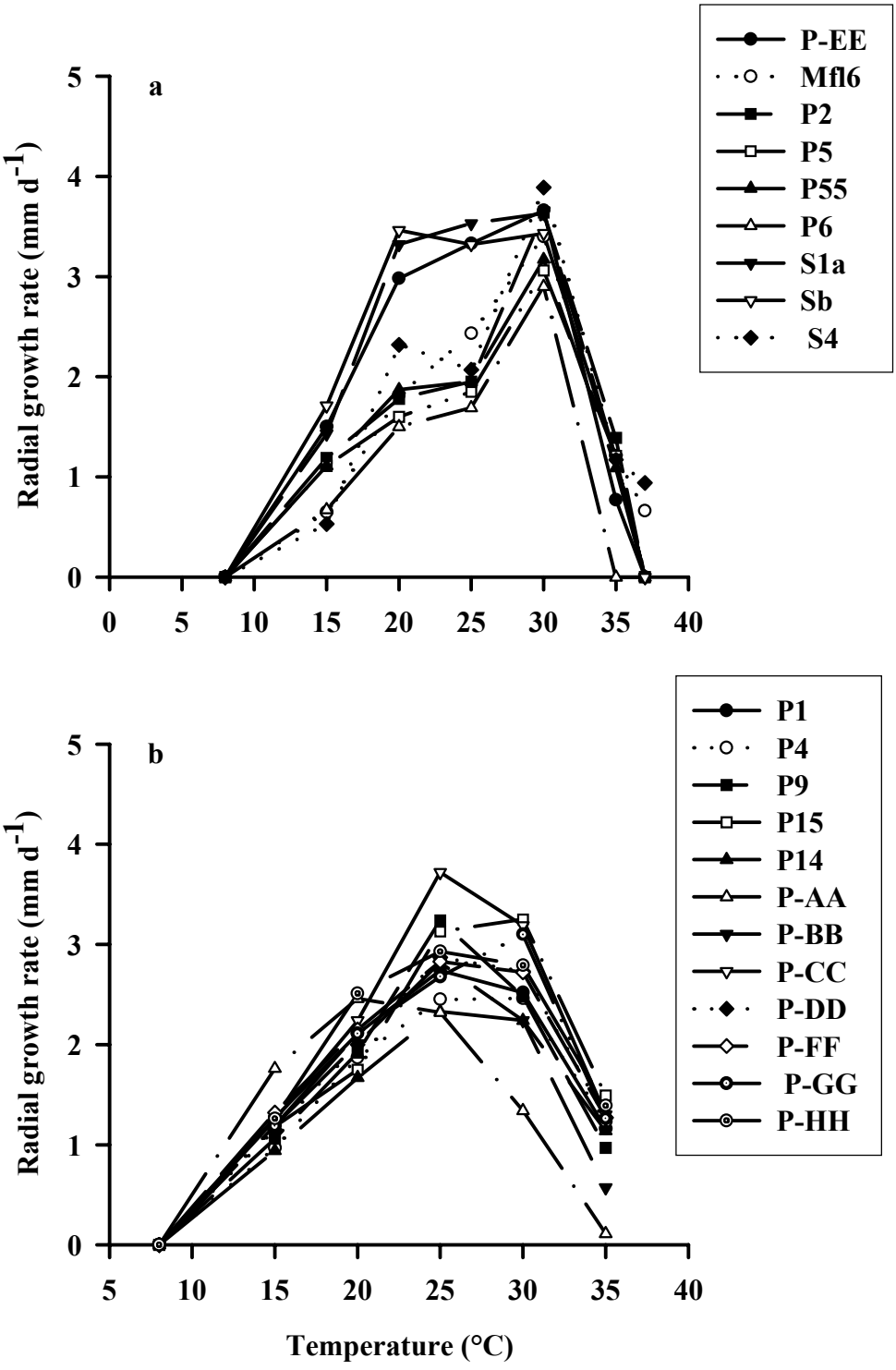

Figure 4. Effect of temperature on radial growth rate of (a) *M. anisopliae* and (b) *B. bassiana, B. brongniartii* and *Paecilomyces* sp. *in vitro*.

For *M. anisopliae,* a high radial growth rate was observed for S1a and S1b (Figure 5). Isolate P6 showed slower growth rate (1 mm d^{-1}) when compared with the remaining *M. anisopliae* isolates, which attained a growth rate of 1.4–1.7 mm d^{-1}. As regards the *B. bassiana* isolates,

all showed a slower (< 1.5 mm d^{-1}) growth rate at 25/35°C fluctuating temperature. However, a relatively high growth rate was observed for P14 and P-FF followed by P9, P-DD. Four other isolates (P15, P4, P1, P-CC) showed a growth rate of 0.7 mm d^{-1}, whereas isolate P-BB maintained a growth rate lower than 0.5 mm d^{-1} (Figure 5b). In contrast, isolate P-GG, P-HH and P-AA showed no growth at 25/35°C fluctuating temperature condition.

DISCUSSION

The search for commercially viable entomopathogenic fungi for use in integrated pest management programmes entails several steps including collection, evaluation, production and formulation (Butt and Goettel, 2000). Tolerance among fungal isolates to environmental factors such as sunlight and temperature (Fargues et al, 1996; Fargues et al, 1997) and speed of germination, growth and ability to sporulate on host cadaver (Hall, 1984) are some of the most important factors that needs to be considered during isolate selection process for mycopesticide development. The length of time when conditions are favourable for growth is critical and it was relevant to determine how long it takes for each strain to approach 100% germination (Hywel-Jones and Gillespie, 1990). The present study has demonstrated the effect of temperature on spore germination and growth rate of isolates of *M. anisopliae*, *B. bassiana* and *Paecilomyces* sp. that were collected from different agroecological zones of Ethiopia and Sudan. The results revealed that temperature-germination and temperature-growth responses varied considerably between isolates. The optimal temperature for germination of most *M. anisopliae* and *B. bassiana* isolates tested ranged between 20–30°C and for some isolates, the range extended to 35°C. Previously, it has been reported that grasshopper adopted isolates could be more heat tolerant than other isolates (Ouedraogo et al., 1997). Likewise, our results corroborate with their findings. We observed an increased heat tolerance for those *M. anisopliae* isolates, which were originally isolated from acridid hosts in Sudan and Niger. For few isolates a restricted optimal temperature of 25°C was observed for germination. These isolates do germinate neither at 20°C nor at 35°C. The optimal temperature for germination of the *Paecilomyces* sp. tested ranged between 20–35°C, however, we have observed that continuous exposure of *Paecilomyces* sp. as well as some isolates of *M. anisopliae* and *B. bassiana* over 24 h at 35°C causes a deleterious effect on spores and further vegetative growth of germinated spores. Determining the effects of temperature on germination, growth and sporulation is a fundamental step to optimize mass production and efficacy (Thomas and Jenkins, 1997). The ability of some of the tested isolates

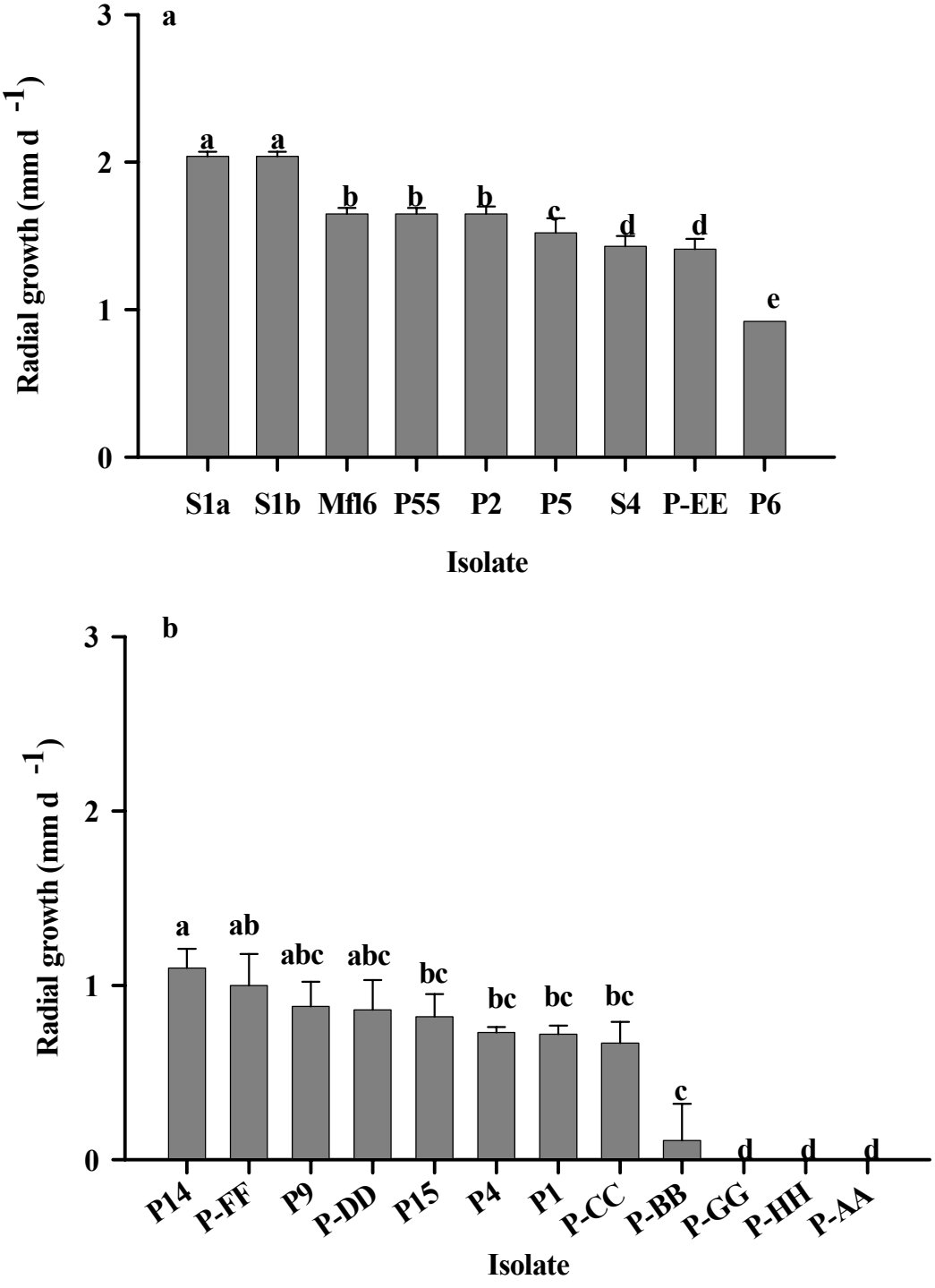

Figure 5. Effect of alternating temperatures of 25/35°C (8:16 h) on radial growth rate of (a) *M. anisopliae* and (b) *B. bassiana, B. brongniartii* and *Paecilomyces* sp. *in vitro*.

to germinate at 35°C could be an indication that heat shock could be deployed to speed up germination of these isolates during the production process. Previous studies demonstrated that temperature affected the lag phase and germination rate of pycnidiospores of *Leptosphaeria maculans* (Desm.) Ces & de Not (Vanniasingham and Gilligan, 1988) and conidia of *M. anisopliae* and *B. bassiana* isolates (Hywel-Jones and Gillespie, 1990). In our study, temperature also affected the lag phase and times to approach 100% germination. More interestingly, there were both inter and intra-specific variations in germination response. The lag phase of most *M. anisopliae* isolates tested was longer both at 20°C and at 35°C and germinates faster at 25°C and 30°C. For those isolates, which were originally isolated from Acridids, the lag phase was not affected much by the range of temperatures tested. Generally, at 25 and 30°C, 95 % of conidia of most *M. anisopliae* isolates germinated in 11-20 h. In their studies, Hywel-Jones and Gillespie (1990) reported similar results on some *M. anisopliae* isolates. In contrast to these, at 25–30°C the *M. anisopliae* isolate P-EE needs a longer time (10–14 h and 57–70 h) to initiate germination and to reach 95% germination, respectively. Contrary to *M. anisopliae*, some of the *B. bassiana* isolates tested showed a reduced lag phase (< 4 h) at 20–35°C and time to approach 95% germination ranged between 11–26 h. In their studies Hywel-Jones and Gillespie (1990) reported that some *B. bassiana* isolates approach 95% germination at 14–25 h after incubation. Thermal conditions strongly influence germination or sporulation, as well as growth of entomopathogenic fungi (Hall, 1981; Carruthers and Haynes, 1986; Hajek, Carruthers and Soper, 1990). Temperature ranges tolerated for germination or sporulations are generally narrower than those for growth (Griffin, 1981). In this study, inter as well as intra-specific variation was also observed between isolates with respect to the optimum temperature for vegetative growth. The optimal temperature for vegetative growth for the majority of *M. anisopliae* isolates studied was 30°C (20–30°C for three isolates) compared with 25–30°C for nine *B. bassiana* isolates; 25°C for two *Beauveria* isolates; and 20–25°C for *Paecilomyces* sp. tested. Except the two *M. anisopliae* isolates Mfl6 and S4, from Acridids, which showed a retarded growth at 37°, none of the remaining *M. anisopliae*, *B. bassiana*, *B. brongniaritii* and *Paecilomyces* sp. isolates tested grew at 8°C and at 37°C. Despite the high germination of conidia observed at 35°C, the *Paecilomyces* sp. showed a retarded growth rate at 35°C and no growth at 37°C. The observed optimal temperatures for growth of our tropical isolates tested in this study are similar to those previously published results (Hywel-Jones and Gillespie, 1990; Ferron, Fargues and Riba, 1991; Welling, Nachtigall and Zimmermann 1994; Ouedraogo et al., 1997; Fargues et al., 1997; Vidal, Fargues and Lacey 1997; Hallsworth and Magan, 1999). It has been indicated

that *B. bassiana* is mesophilic, capable of growing between 6 and 35°C (Ferron et al., 1997). Similarly, Vidal, Fargues and Lacey (1997) indicated, that the growth of *Paecilomyces fumosoroseus* isolates is between 8 and 32°C. However, in contrast to these, none of the *B. bassiana*, *M. anisopliae* and *Paecilomyces* sp. isolates we tested grew at 8°C. This probably suggests the adaptation of these isolates to tropical environments. A relationship between thermal tolerance and climate of origin has been shown for isolates of *B. bassiana*, *B. brongniartii*, *M. flavoviride*, *Nomuraea rileyi* (Fargues et al., 1992). Similarly, the variability in response to lower or higher temperature tolerance was evident between temperate, tropical and subtropical origin of *P. fumosoroseus* isolates (Vidal, Fargues and Lacey, 1997).

Strain selection according to thermal tolerance may be warranted when choosing an isolate for development as a microbial control agent (Fargues et al., 1997). Factors that govern *in vitro* growth of the fungus and infection can be related, even if the optimal conditions for growth and infectivity are not always identical (Mietkiewski et al., 1994). This suggests that a relationship between thermal tolerance as measured by *in vitro* vegetative growth and disease development needs to be first established (Ouedraogo et al, 1997). Similarly, Inglis, Johnson and Goettel (1996) found a strong correlation between vegetative growth and mycosis in *Melanoplus sanguinipes* at different temperatures. However, the effect of temperature on conidial germination was poorly correlated with disease incidence. In the present study, one of the *M. anisopliae* isolates (P-EE) showed a germination rate of 55 and 30% after 24 h incubation at 25 and 30°C, respectively and no germination within 24 h at 20°C. Data on the vegetative growth indicated that the optimum temperature for this specific isolate ranged between 20–30°C. On the other side, in virulence studies conducted by Kassa et al (2002) the same isolate caused 63 and 100% mortality (MST = 4–5 days) at 1 x 10^7 and 1 x 10^8 conidia ml^{-1} respectively, which was much higher when compared to *B. bassiana* isolates tested with a germination of 90–100% . This difference between temperature optimum for *in vitro* germination and growth and *in vivo* killing shows the inability to select strains for climatic tolerance only according to their *in vitro* temperature preferences (Ouedraogo et al, 1997). Furthermore, it has been reported that low germination does not affect the virulence of *B. bassiana* against the silver leaf whitefly, *Bemisia argentifolii* (James and Jaronski, 2000). These results could be due to live conidia retaining full infection capacity, or could be a result of the occurrence of high exotoxin levels in lots with low viability (James and Jaronski, 2000). The other possibility may be the impact of the host-pathogen interaction at the site of infection (Ferron, Fargues and Riba, 1991).

In general, the results presented here confirm that there is a considerable variability in germination and growth at different temperatures among isolates of *M. anisopliae*, *B. bassiana* and *Paecilomyces* sp. collected from various ecological zones in Ethiopia and Sudan. These comparative tests permit the establishment of a hierarchy among isolates according to their thermal requirements. Further selection, taking into account the tolerance to different climatic constraints, host-pathogen interaction (Benz, 1987; Ferron, Fargues and Riba, 1991; Hall, 1982) and productivity, storage properties and aspects of formulations (Butt and Goettel, 2000) will help to single out the most effective isolates for mycopesticide development for use in integrated pest management (IPM) programmes. Thus, the results presented in this study could be considered as an important contribution to the selection of strains against a range of insect pest species in distinct environmental condition.

ACKNOWLEDGMENTS

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M. anisopliae **isolates**

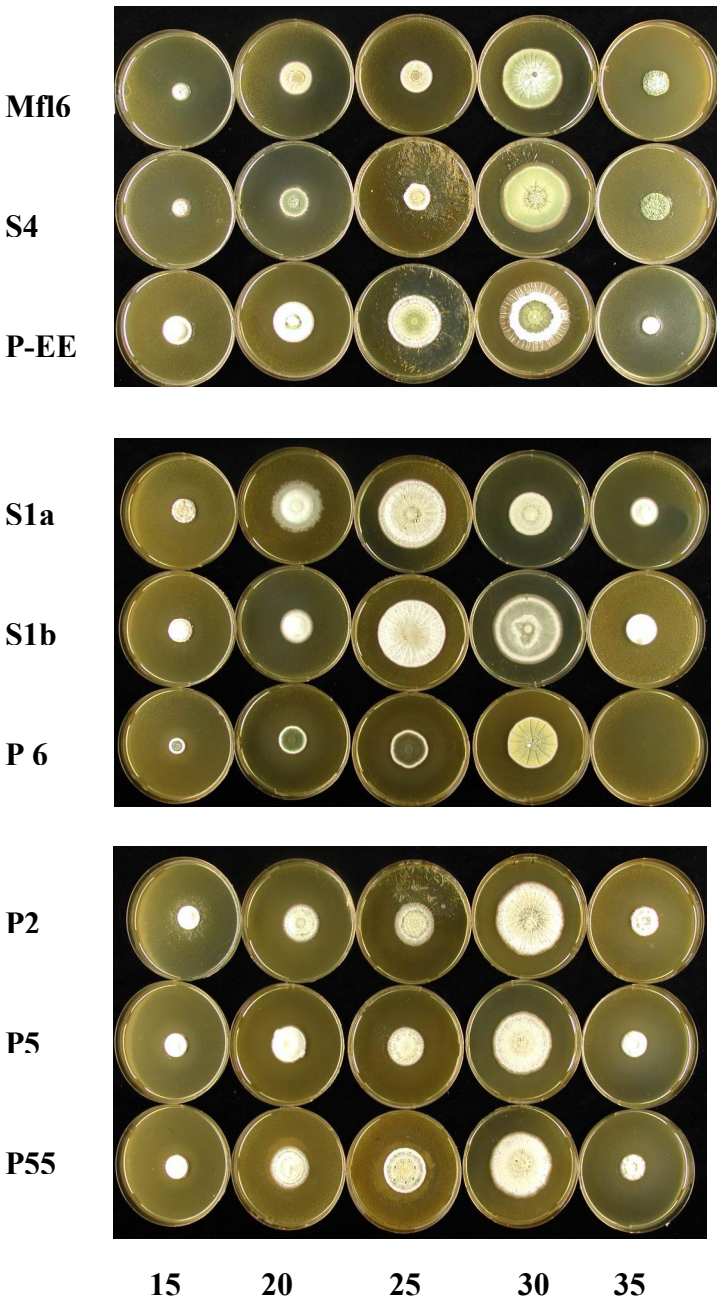

Plate 1. Growth pattern of *M. anisopliae* strains at different constant temperatures on MEA.

Beauveria and *Paecilomyces* isolates

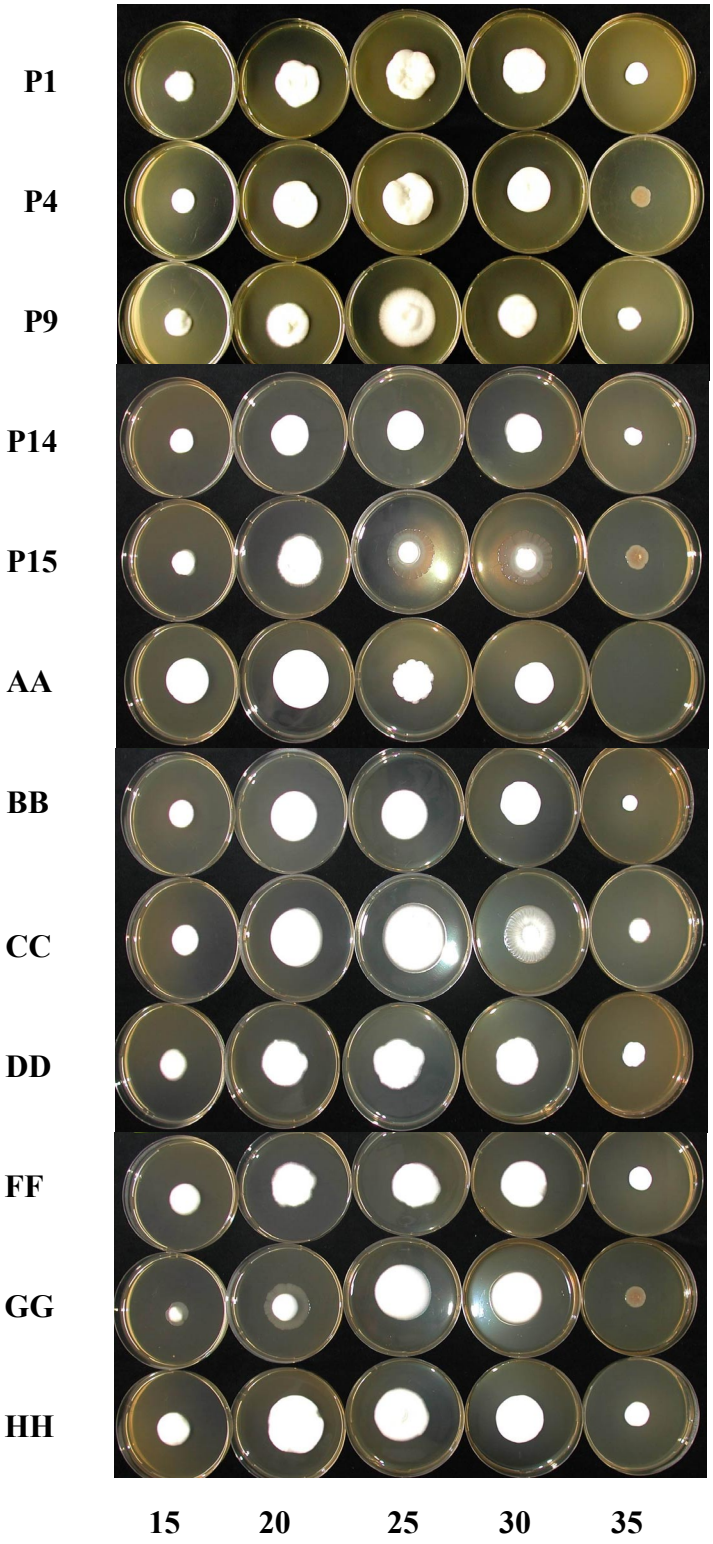

Plate 1. Growth pattern of *M. anisopliae* strains at different constant temperatures on MEA.

CHAPTER 6

Susceptibility of *Sitophilus zeamais* (Motsch.) (Coleoptera: Curculionidae) and *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) to entomopathogenic fungi from Ethiopia

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The efficacy of 13 isolates of entomopathogenic fungi belonging to *Beauveria*, *Metarhizium* or *Paecilomyces* spp. was assessed against *Sitophilus zeamais* (Coleoptera: Curculionidae) and *Prostephanus truncatus* (Coleoptera: Bostrichidae) using a total immersion bioassay technique in the laboratory. Fungi were applied at concentrations of 1 x 10^7 and 1 x 10^8 conidia ml^{-1} for *P. truncatus* and *S. zeamais*, respectively. All isolates tested were virulent to *P. truncatus* (98 – 100 % mortality, and median survival time (MST) ranged from 2.85 – 4.05 days). *Metarhizium anisopliae* and *B. bassiana* were also virulent to *S. zeamais* (92-100 % mortality, MST ranged from 3.58 – 6.28 days). The isolate of *Paecilomyces* sp. was found to be the least virulent against *S. zeamais*, causing only 26.32 ± 4.29 % mortality with MST of 10.38 ± 0.29 days. *Prostephanus truncatus* proved more susceptible to the entomopathogenic fungi tested than *S. zeamais*. One *M. anisopliae* (PPRC-EE) and three *B. bassiana* isolates (PPRC-HH, PPRC-9609 and PPRC-9614) were selected for further study and dose-mortality relationships were assessed on *S. zeamais*. The tested concentrations ranged from 1 x 10^4-1 x 10^7 conidia ml^{-1}. *Metarhizium anisopliae* (PPRC-EE) showed the lowest LC_{50} (3.39 x 10^5 conidia ml^{-1}) followed by *B. bassiana* PPRC-HH (2.04 x 10^6 conidia ml^{-1}). PPRC-9609 and PPRC-9614 showed slight differences in LC_{50} but not at LC_{90}. The results revealed the higher potency of *M. anisopliae* as compared with the *B. bassiana* isolates tested. The study suggests that the use of entomopathogenic fungi may hold promise as an alternative method to control pests of stored-product in Ethiopia.

Key words: *Entomopathogenic fungi,* Beauveria bassiana, Beauveria brongniartii, Metarhizium anisopliae*,* Paecilomyces *sp*., Sitophilus zeamais, Prostephanus truncatus*, Efficacy, dose–response, microbial control, Ethiopia*

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INTRODUCTION

The maize weevil, *Sitophilus zeamais* (Motsch.) (Coleoptera: Curculionidae), and the larger grain borer, *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae), are the two most important and destructive pests of stored grain in the tropics (Dobie *et al.,* 1984; Dick, 1988). The maize weevil is regarded as a cosmopolitan pest in tropical countries while the larger grain borer, native to Central America and Mexico, was accidentally introduced into East and West Africa between the 1970s and 1980s and became a serious pest of stored maize and cassava (Dick, 1988). The favourable climatic conditions and poor storage systems in Africa often favours growth and development of these pests, resulting in considerable crop losses. For example, losses as high as 40 % are reported on stored maize due to *P. truncatus* and *S. zeamais* (Meikle *et al.*, 1998). In Africa, where subsistence grain production supports the population, such grain losses may be substantial (Golob & Tyler, 1994). In addition to grain weight loss, pests of stored grain also cause secondary fungal infection, resulting in a reduction in seed vigour, quality and commercial value.

Synthetic chemical insecticides have been widely used for the control of pests of stored grain. This, however, caused problems such as insecticide resistance (Beeman & Wright, 1990), chemical residues in foodstuffs, increasing cost, and environmental pollution (Morallo-Rejesus, 1987). The increased public awareness and concern for environmental safety has directed research to the development of alternative control strategies. Entomopathogenic fungi have been shown to be effective biological control agents against several insect pests. Recently, there is also a growing interest in the use of mycopathogens for the development of biological control strategies for stored product pests. Fungi such as *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces fumosoroseus* have been found to control a number of stored product pests (Adane *et al.,* 1996; Hidalgo *et al.,* 1998; Moino *et al.,* 1998; Smith *et al.,* 1998, Rice & Cogburn, 1999; Bourassa *et al.*, 2001; Dal Bello *et al.*, 2001; Padin *et al.*, 2002). However, several problems remain with regard to the regular use of these entomopathogens. The recent work of Hidalgo *et al.* (1998), Smith *et al.* (1999) and Meikle *et al.* (2001) addressed the problem of formulating the potent isolates of *B. bassiana* for the management of stored-grain insect pests. In granaries of most tropical countries, the occurrence of mixed insect pests infestations are common. Hence, the selection of virulent isolate that can be used effectively against a mixture of stored-grain pests is a prerequisite for the development of a microbial control program for a grain storage system. Here, data are presented relating to the efficacy of isolates of entomopathogenic fungi from Ethiopia against

S. zeamais and *P. truncatus*. The virulence of different strains against these storage pests was tested and the dose-mortality relationship of selected strains on *S. zeamais* assessed.

MATERIALS AND METHODS

Insect rearing

The initial stock cultures of *S. zeamais* and *P. truncatus* were obtained from the Institute for Stored Product Protection, Berlin, and the Institute for Biological Control, Darmstadt, respectively. Maize grains were cleaned and disinfested by storing at –20°C for a week. Insect cultures were maintained in glass jars (1.5 L) filled with 500 g of clean and disinfested maize grain. The jars were covered with a perforated lid and incubated for 30 days at 30°C and 70 % RH. After 30 days, the parental adults were removed by sieving and the maize grains were kept at the same conditions until the emergence of the F_1 generation.

Fungal isolates

Thirteen different isolates of entomopathogenic fungi were obtained from the collection maintained by the Ethiopian Agricultural Research Organisation (EARO) at the Plant Protection Research Center (PPRC), Ambo. Details of these isolates are given in Table 1. None of the isolates were obtained from the pest insects tested here but had been isolated originally from other Coleoptera in different agro-ecological zones of Ethiopia. The initial cultures of all isolates were stored at -5°C and sub-culturing was made as appropriate.

Preparation of conidial suspensions

Cultures of all test isolates were grown on Malt extract agar (MEA) (3 % malt extract, 0.5 % soya-peptone, 1.5 % agar) in darkness at 25°C. Conidia from two week old cultures of each isolate were harvested using sterile deionised water containing Tween 80 (0.1% v/v). The resulting suspensions were vortexed for about 3 min. to break up the conidial chains or clumps. The spore concentration was counted with a Thoma haemacytometer and adjusted to 1×10^7 and 1×10^8 conidia ml^{-1} for *P. truncatus* and *S. zeamais*, respectively. The lower concentration was selected for *P. truncatus* on account of its high degree of susceptibility observed at 1×10^8 conidia ml^{-1} prior to this experiment. Viability of the conidia was assessed by germination test using an antibiotic medium (2 % glucose, 0.5% soya-peptone, 0.5 % yeast extract, 1.5 % agar amended with 30 mg streptomycinsulfate and 50 mg chloramphenicol). For each strain, 100 µl of the suspension containing 1×10^6 conidia ml^{-1}

were spread on plates, which were then placed in a plastic box and incubated at 25°C in darkness for 24 h. Germination was assessed by counting 300 spores after fixing with lactophenol cotton blue. For the *M. anisopliae* isolate, the germination rate ranged from 40 to 70 % while in all other isolates it ranged from 90 to 100 %.

Virulence tests

For *S. zeamais,* eight *B. bassiana* isolates, one *Paecilomyces* sp. isolate and one *M. anisopliae* isolate were tested (Table 1). For *P. truncatus*, two further *B. bassiana* isolates (PPRC-DD and PPRC-FF) and one *B. brongniartii* isolate were tested (Table 1). Either *S. zeamais* (10 per replicate) or *P. truncatus* (15 per replicate) were placed in sterile Petri-dishes (9 cm diameter). Inoculation was made by dipping each group of test insects for 5 sec. in 1 ml suspension containing 1 x 10^7 and 1 x 10^8 conidia ml $^{-1}$ for *P. truncatus* and *S. zeamais*, respectively. The control insects were treated in deionised water with Tween 80 (0.1% v/v). The treated insects and the suspension were subsequently poured into a new plate containing filter paper (9 cm diameter) and sealed with Parafilm to prevent insects from escaping. The filter paper helped

TABLE 1. List of entomopathogenic fungal isolates tested, their host insect, place of origin and altitude.

Isolate No.	Fungal species	Host Insect	Place of origin and altitude (m)
PPRC-9501	*Beauveria bassiana*	*Melolontha* sp. (Coleoptera: Melolonthidae)	West Showa (2500) (Tukurinchinii)
PPRC-9604	*Beauveria bassiana*	*Aceraea acerata* (Lepidoptera: Nymphalidae)	Arbaminch (1925)
PPRC-9609	*Beauveria bassiana*	*Blosyrus rugulosus* (Coleoptera: Curculionidae)	Dila (1875)
PPRC-9614	*Beauveria bassiana*	Ground beetles (Coleoptera)	Awassa (1750)
PPRC-9615	*Beauveria bassiana*	Spider (Arachnida)	Awassa (1750)
PPRC-BB	*Beauveria brongniartii*	Spider (Arachnida)	Tigray (1950)
PPRC-CC	*Beauveria bassiana*	Adult beetle (Coleoptera)	Woldiya (1950)
PPRC-DD	*Beauveria bassiana*	Adult beetle (Coleoptera)	Debremarkos (2030)
PPRC-FF	*Beauveria bassiana*	Adult beetle (Coleoptera)	Ashengae (2400)
PPRC-GG	*Beauveria bassiana*	Adult beetle (Coleoptera)	Ashengae (2400)
PPRC-HH	*Beauveria bassiana*	Adult beetle (Coleoptera)	Ashengae (2450)
PPRC-EE	*Metarhizium anisopliae*	Crustacean (sow pill)	Alemata (1500)
PPRC-AA	*Paeciliomyces* sp.	Adult beetle (Coleoptera)	Korem (2450)

to absorb the excess moisture and increased conidial load in each insect by allowing secondary spore pick up (Adane *et al.*, 1996). The treated insects were kept without food for 24 h at 27°C and 70 % RH. After 24 h, insects in each replicate were transferred into plastic

assay cups (6.5 cm diameter and 4.5 cm height, with a perforated lid) containing cracked maize grains and kept at 27°C and 70 % RH for 11 days. For both pests, the experiment was arranged in a completely randomised design with four replications. Mortality was recorded daily for 11 days. Cadavers from each treatment were washed in 70 % ethanol, rinsed in deionised water three times and kept separately in Petri-dishes. These plates were then incubated in a plastic box with high relative humidity to observe the outgrowth of fungus.

Dose-mortality relationship on Sitophilus zeamais

Four isolates (PPRC-9609, PPRC-9614, PPRC-HH and PPRC-EE) were selected for a further dose-mortality study on *S. zeamais*. Conidia from two week old cultures were harvested using similar methods as described previously. Serial dilutions were made to obtain four different concentrations (1 x 10^4, 10^5, 10^6, and 10^7 conidia ml^{-1}) in sterile deionised water with Tween 80 (0.1 % v/v). Twenty-five mixed age adults of *S. zeamais* were chosen at random from the rearing jar and placed in Petri-dishes (9 cm diameter). Inoculation and handling was carried out as described previously. The control insects were treated with sterile deionised water with Tween 80 (0.1 % v/v). The experiment was repeated four times.

Statistical Analysis

Data for cumulative percentage mortality were corrected for the corresponding control mortality (Abbott, 1925), arcsine transformed and subjected to analysis of variance using the general linear model procedure (PROC GLM) (SAS Institute, 1989). Means were separated using the Waller-Duncan K-ratio t-Test (SAS Institute, 1989). The median survival time (MST) was derived using the Kaplan-Meier estimate. The homogeneity of the survival curves among the treatments was tested using Log-rank test in LIFETEST procedure (SAS Institute, 1989). Probit analysis (PROC PROBIT) was used to estimate both the LC_{50} and LC_{90} of the isolates with 95 % confidence limits (SAS Institute, 1989).

RESULTS

Virulence test

Our studies demonstrated a different degree of susceptibility of *S. zeamais* and *P. truncatus* to various entomopathogenic fungi. Mortality caused by the isolates was confirmed based on sporulating cadavers and all the dead insects supported sporulation of the respective isolates. Control mortalities did not show mycosis. Cumulative mortality of *S. zeamais* 4 days after treatment showed a significant variability among the isolates (F = 32.34, df = 9, $p < 0.001$)

(Table 2). As time progresses, cumulative mortality for all isolates increased but at differing rates. The survival curve analysis also revealed a distinct difference between isolates in speed of kill (χ^2 = 384.68, $p < 0.001$). On *S. zeamais* which had been exposed to PPRC-HH, the earliest death (2.5 %) occurred on day 2 and by day 4 mortality reached 100 %, resulting in a shorter median survival time (MST) (Table 2). PPRC- GG caused 100 % mortality on day 4 (Table 2). For the remaining isolates, mortality started on the fourth day at differing rates. Six

TABLE 2. Corrected percentage mortality 4 days after treatment and median survival time (MST) of *S. zeamais* and *P. truncatus* exposed to different isolates of entomopathogenic fungi. Concentration (conidia ml^{-1}) used include 1 x 10^7 for *P. truncatus* and 1 x 10^8 for *S. zeamais* (means $\pm$ SE; N = 4).

Isolate	*S. zeamais*		*P. truncatus*	
	Percent Mortality	MST (days)	Percent Mortality	MST (days)
B. bassiana				
PPRC-HH	100.00 (0.00) [a]	3.58 (0.08)	100.00 (0.00) [a]	2.85 (0.05)
PPRC-GG	100.00 (0.00) [a]	4.00 (0.00)	98.33 (1.72) [ab]	3.43 (0.11)
PPRC-9614	37.50 (10.31) [c]	4.65 (0.08)	100.00 (0.00) [a]	3.67 (0.07)
PPRC-9609	75.00 (6.46) [b]	4.38 (0.14)	96.67 (1.53) [a-d]	3.93 (0.06)
PPRC-9604	60.63 (7.09) [b]	4.42 (0.08)	93.33 (2.81) [a-d]	3.98 (0.06)
PPRC-9615	37.61 (13.01) [c]	4.74 (0.12)	98.33 (1.72) [ab]	3.78 (0.06)
PPRC-9501	58.89 (7.16) [bc]	4.48 (0.10)	95.00 (3.29) [a-d]	3.80 (0.07)
PPRC- CC	10.00 (4.08) [d]	6.28 (0.41)	85.00 (5.87) [bcd]	4.05 (0.11)
PPRC-BB[a]	-	-	85.00 (4.32) [d]	3.90 (0.09)
PPRC-DD[a]	-	-	90.00 (5.95) [a-d]	4.00 (0.07)
PPRC-FF[a]	-	-	96.67 (3.44) [abc]	3.53 (0.09)
M. anisopliae				
PPRC-EE	62.50 (9.47) [b]	4.65 (0.18)	100.00 (0.00) [a]	3.20 (0.09)
***Paecilomyces* sp.**				
PPRC - AA	2.50 (2.5) [d]	10.38 (0.29)	81,67 (10.26)[cd]	3.98 (0.09)

Means within a column followed by the same letters are not significantly different ($p < 0.001$, Waller-Duncan K-ratio t-Test), [a] Isolate was not tested for *S. zeamais*.

isolates (PPRC-9614, PPRC-9615, PPRC-9501, PPRC-9604, PPRC-EE, and PPRC-9609) caused 38-75 % mortality on day 4 and exceeded 95 % by day 6 and for these isolates, MST ranged from 4.38-4.74 days (Table 2). Two further isolates (PPRC-CC and PPRC-AA) caused less than 15 % mortality on day 4 (Table 2). Mortality caused by PPRC-CC exceeded 60 % by day 5 resulting a relatively longer MST of *S. zeamais* (Table 2). At the end of the experiment, five *B. bassiana* isolates (PPRC-HH, PPRC-GG, PPRC-9609, PPRC-9604 and PPRC-9614) caused 100 % mortality. One *M. anisopliae* (PPRC-EE) and three other *B. bassiana* isolates

(PPRC-9501, PPRC-9615, PPRC-CC) showed 92-97 % mortality. The *Paecilomyces* sp. (PPRC-AA) showed less than 27 % mortality within 11 days post exposure.

For *P. truncatus*, mortality caused by all isolates increased with time but occurs at a higher rate when compared with *S. zeamais*. Differences among the isolates were apparent on cumulative mortalities that were observed on day four after treatment ($F = 2.63$, df = 9, $p < 0.05$) (Table 2). Three isolates showed 100 % mortality on day four. Seven other isolates caused more than 90 % mortality on day four and three further isolates resulted in 82 – 85 % mortality (Table 2). Six days after treatment, mortality for all isolates exceeded 97 % and there was no apparent difference between isolates. Survival curve analysis showed significant differences between isolates in speed of kill ($\chi^2 = 489.95$, $p < 0.001$) (Table 2). Significantly shorter MST was recorded for PPRC-HH followed by PPRC-EE. For the remaining isolates the MST ranged from 3.43 - 4.05 days (Table 2). Despite the low concentration used for *P. truncatus*, the MST was shorter for *P. truncatus* than for *S. zeamais*. Unlike *S. zeamais,* all isolates tested caused the earliest mortality two days post inoculation, indicating the greater susceptibility of *P. truncatus* to non-specific entomopathogenic fungi.

Dose-mortality relationship with S. zeamais

Based on the virulence study, three *B. bassiana* isolates (PPRC-HH, PPRC-9609 and PPRC-9614) and one *M. anisopliae* isolate (PPRC-EE) were considered primary candidates for a dose-mortality relationship study. These isolates showed highest mortality and short MST in the virulence test. In addition to their efficacy, these isolates also revealed higher vegetative growth and spore yield (personal observation). However, the study warrants multiple dose-mortality study for the other isolates using both *S. zeamais* and *P. truncatus*.

The results indicated that the level of mortality and MST of treated insects varied with isolates ($F = 25.24$, df = 3, $p < 0.001$), conidial concentrations ($F = 433.69$, df = 3, $p < 0.001$) and their interactions ($F = 5.31$, df = 9, $p < 0.001$). For all isolates, mortality significantly increased with increased concentrations (Figure 1a-d). *Metarhizium anisopliae* (PPRC-EE) caused 69 and 98 % mortality at the concentration of 1 x 10^6 and 1 x 10^7 conidia ml^{-1}, respectively (Figure 1a) while all *B. bassiana* isolates caused the highest (> 83 %) mortality only at 1 x 10^7 conidia ml^{-1} (Figure 1b-d). The survival curve analysis revealed a significant difference in MST between the various conidial concentrations tested ($p < 0.001$). *Metarhizium anisopliae* (PPRC-EE) at the concentration of 1 x 10^6 and 1 x 10^7 conidia ml^{-1}

caused mortality three days after inoculation and this increased sharply thereafter, resulting in a shorter MST (Figure 1a). At the lower concentrations, mortality occurred on the fourth day and increased gradually at 1 x 10^5 and remained slow at 1 x 10^4 conidia ml^{-1}. In both cases, however, mortality did not reach 50 %. For all *B. bassiana* isolates, the first mortality occurred on day 3 and reached a peak (over 75 %) within four to six days only at higher concentrations (Figure 1b-d). For the remaining concentrations tested, mortality started between five and six days after inoculation and remained lower than 32 % throughout the study period. For these isolates, the MST of the treated insects was shorter only at a higher concentration (Figure 1). Median survival time was not determined for isolate-concentration combinations showing a mortality lower than 5 %.

The parameters of the probit analysis are given in Table 3. The LC_{50} and LC_{90} values were 12 – 14 % lower for *M. anisopliae* PPRC-EE than for the remaining *B. bassiana* isolates tested (Table 3). Among the latter, the lowest LC_{50} and LC_{90} values were obtained with PPRC-HH. PPRC-9609 and PPRC-9614 showed slight differences in LC_{50} but not at LC_{90}.

DISCUSSION

The results obtained in the present study demonstrated the susceptibility of *S. zeamais* and *P. truncatus* to non-specific entomopathogenic fungi indigenous to Ethiopia. The results also revealed differences in susceptibility among the different test insects, P. *truncatus* being more susceptible to all isolates tested than *S. zeamais*. Smith *et al*. (1998) described the higher degree of susceptibility of *P. truncatus* to *B. bassiana* than *S. zeamais*. They stated further that isolates that were most virulent to *S. zeamais* were also highly pathogenic to *P. truncatus*. Our results are consistent with these findings. In addition, our study also demonstrated that isolates that were least virulent to *S. zeamais* were most virulent to *P. truncatus*, indicating the higher degree of susceptibility of *P. truncatus* to mycopathogens. Under the conditions prevailing in the present study isolates that caused 92 –100 % mortality with MST of 3.58-6.2 days are considered potential candidates for further research work. The data presented in here further indicated that future screening programmes should be done with both pests but at lower dosages.

Investigations carried out by Adane *et al.* (1996) on *S. zeamais* indicated that very virulent isolates could cause high mortality at lower concentrations. In our study, the selection of isolates was based on exposure to high inoculum densities using a total immersion bioassay

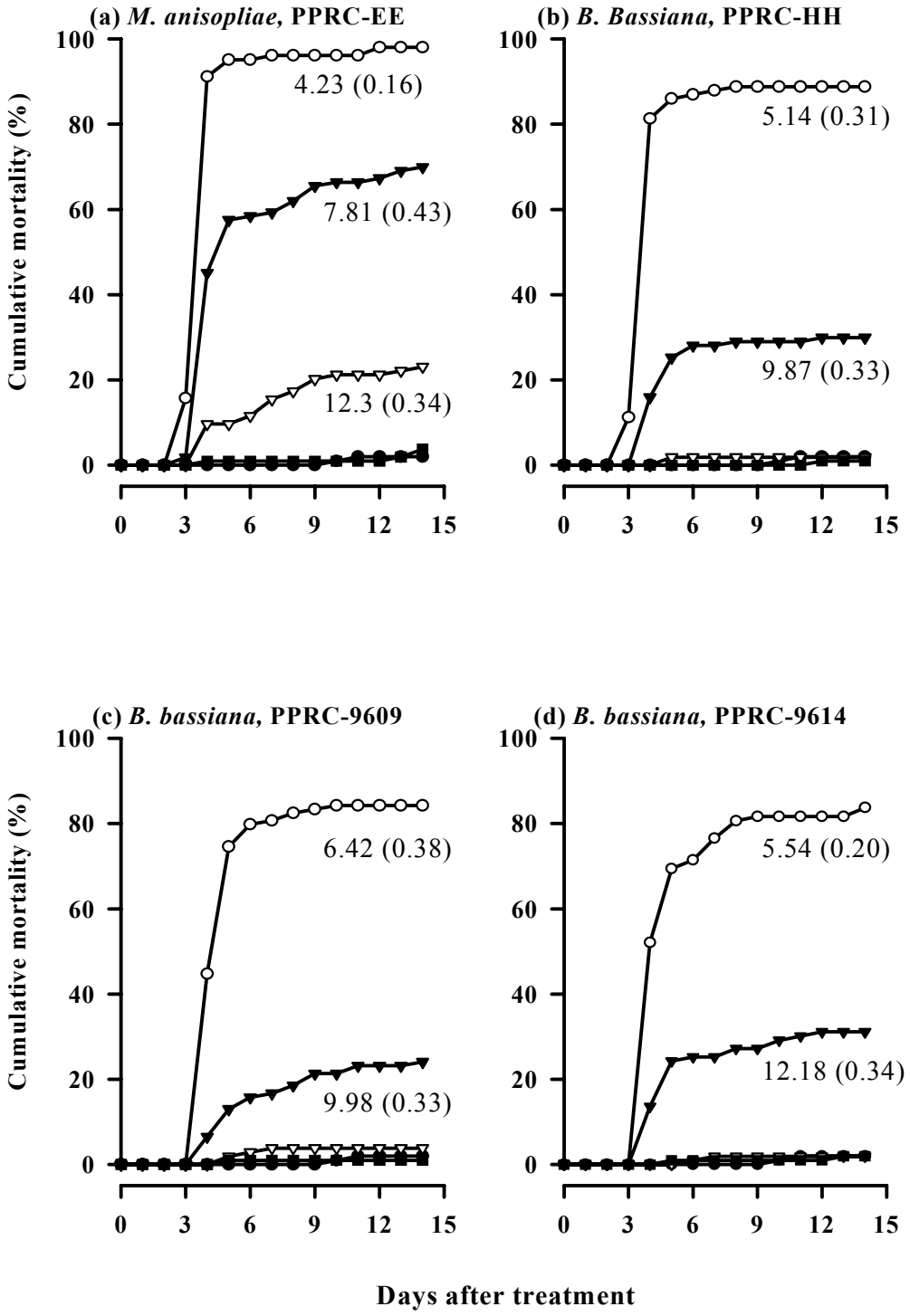

FIGURE 1. Cumulative mortality (%) of *S. zeamais* treated with different conidial concentrations of *M. anisopliae* and *B. bassiana* isolates. (●): Control, (■): 1 x 10^4, (▽): 1 x 10^5, (▼): 1 x 10^6 and (○): 1 x 10^7 conidia ml^{-1} (A) *M. anisopliae* isolate PPRC–EE, (B) *B. bassiana* isolate PPRC-HH, (C) *B. bassiana* isolate PPRC-9609 and (D) *B. bassiana* isolate PPRC-9614. Values indicated under ach curve represents the median survival time (± SE) for the corresponding curve.

method along with secondary pick-up from the substrate. Isolates with low performance (i.e. *Paecilomyces* sp. isolate PPRC-AA, for *S. zeamais*) would thus not be worth evaluating further. However, isolate PPRC-AA showed same level of virulence with some of the *B. bassiana* isolates against *P. truncatus*. The results obtained in the dose-mortality relationship studies further indicated differences among the four potent isolates tested. *Metarhizium anisopliae* (PPRC-EE) and *B. bassiana* (PPRC-HH) appeared to be highly virulent to *S. zeamais* at lower concentrations. Other authors for example (Rodrigues & Pratissoli, 1990; Lourençao *et al*., 1993; Moino *et al*., 1998) observed higher mortalities of stored-grain pests inoculated with *B. bassiana* than with *M. anisopliae*. The work of Lourenção *et al*. (1993) indicated that *M. anisopliae* infected and killed insects more slowly than *B. bassiana*. According to Moino *et al*. (1998), *Rhizopertha dominica* is more susceptible to *M. anisopliae* than *S. zeamais* and *S. oryzae*. In contrast to these findings, the present study clearly indicated that when either *B. bassiana* or *M. anisopliae* were applied to *S. zeamais* at a lower concentration (1 x 10^6 conidia ml^{-1}), the latter caused a significantly higher mortality and shorter median survival time. On malt extract agar (MEA), the *M. anisopliae* isolate showed 40 to 70 % germination rate after 24 h incubation at 25ºC. In a separate experiment, the same isolate took 19 and 52 h to reach 50 and 95 % germination, respectively (Kassa *et al.*, unpublished), which leads to the conclusion that it has a slow germination rate compared to the remaining *B. bassiana* isolates.

During our investigation, we did not adjust the concentration in terms of viable conidia. Despite the slow germination rate observed, PPRC-EE caused higher mortality and short median survival time of the test insects. It has been reported that low viability does not affect the virulence of *B. bassiana* against silver leaf whitefly, *Bemisia argentifolii* (James and

TABLE 3. Parameters for the regressions of probit-transformed percentage mortality data of *S. zeamais* treated with *M. anisopliae* and *B. bassiana* isolates (LC_{50} and LC_{90} values are based on $\log_{10}$ of the concentration) (CL = confidence limts).

Species	Intercept (± SE)	Slope (± SE)	n	χ^2	*P*	LC_{50}	95 % CL	LC_{90}	95 % CL
M. anisopliae									
PPRC-EE	-7.98 ± 0.83	1.44 ± 0.14	514	99.47	< 0.001	5.53	5.38-5.66	6.42	6.23-6.65
B. bassiana									
PPRC-HH	-11.58 ± 1.24	1.84 ± 0.19	523	90.10	< 0.001	6.31	6.19-6.42	7.01	6.86-7.21
PPRC-9609	-10.12 ±1.08	1.58 ± 0.16	507	87.93	< 0.001	6.37	6.24-6.49	7.17	6.99-7.43
PPRC-9614	-10.79 ± 1.37	1.68 ± 0.20	530	64.34	< 0.001	6.42	6.29-6.54	7.19	7.02-7.44

Jaronski, 2000). These results could be due to live conidia retaining full infection capacity, or could be a result of the occurrence of high exotoxins levels in lots with low viability (James and Jaronski, 2000). Our results concurs with the above findings, indicating the higher adaptation of this isolate to infect *S. zeamais* and *P. truncatus*. *Beauveria bassiana* appeared to be virulent for *S. zeamais* only at concentrations higher than 1 x 10^7 conidia ml^{-1} and variability within the different *B. bassiana* isolates were apparent.

In Benin, application of *B. bassiana* conidia in an oil formulation significantly reduced *P. truncatus* populations but did not prevent grain losses (Meikle *et al.*, 2001). This suggested that future development of an effective stored-product bio-pesticide should look for more effective isolates and appropriate formulations (Meikle *et al.*, 2001). Further more, selection of virulent isolates for a range of stored-grain pests plays an essential part in the development of microbial control programmes. Based on the present study, *M. anisopliae* (PPRC-EE) and *B. bassiana* (PPRC-HH) can be recommended as primary candidates for further research in order to develop a mycoinsecticide for stored-grain pests in Ethiopia. *B. bassiana* isolates (PPRC-9609 and PPRC-9614) could be considered as alternatives. Taking into account further aspects, such as mass production, formulation, storage, spectrum of activity to broad range of stored-grain pests and safety to non-target organisms will help focus on a single isolate for product development. In their studies, Moino *et al*. (1998) reported the importance of two-phase isolate selection processes. The results presented in this study also indicated the advantage of multi-stage screening procedure to obtain the most virulent isolates. The first phase considers rapid bioassays to allow screening of several fungal species and isolates at doses lower than that considered in our screening study. The second phase involves studying the dose-mortality relationship using a range of concentrations and a reduced number of virulent isolates promoted from the first phase. The third step should concentrate on mass production, formulations, storage, host range, and safety studies. The results presented in here are interesting, a range of non-specific isolates showed pathogenicity to *S. zeamais* and *P. truncatus*, indicating isolates virulent to wide range of stored-grain pests may be found through systemic search amongst the indigenous strains of entomopathogens. Further the study suggested that the use of entomopathogenic fungi may hold promise as an alternative method to control pests of stored-product.

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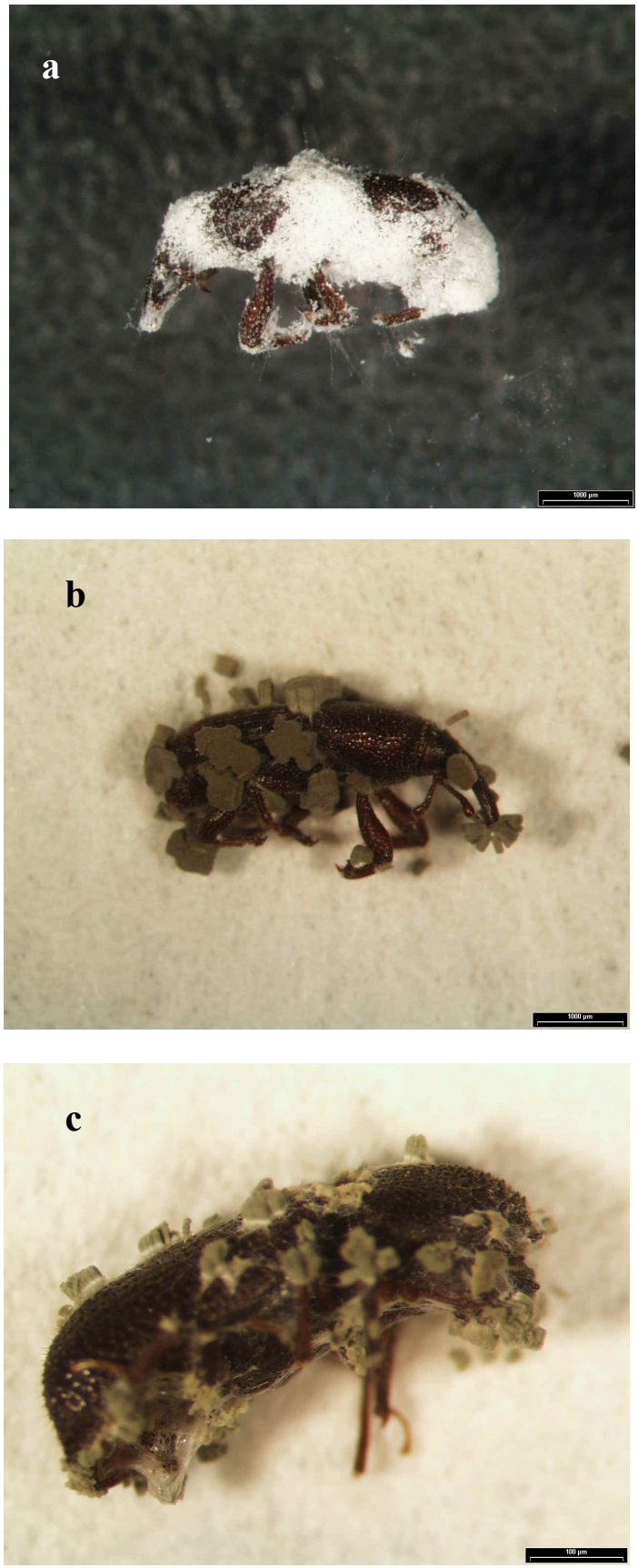

Plate 1. Sporulation of *B. bassiana* (a) and *M. anisopliae* (b and c) on infected *S. zeamais* (a and b) and *P.truncatus* (c).

CHAPTER 7

Production and formulation of *Metarhizium anisopliae* and *Beauveria bassiana* conidia and submerged spores for control of *Sitophilus zeamais* Motsch. (Coleoptera: Curculionidae)

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We evaluated the use of *Beauveria bassiana* (Bals.) Vuillemin and *Metarhizium anisopliae* (Metch.) Sorokin conidia and submerged spores/conidia for the control of *Sitophilus zeamais* (Motsch.). Therefore we examined three *B. bassiana* isolates (PPRC-HH, PPRC-9609, PPRC-9614) and one *M. anisopliae* isolate (PPRC-EE) for their productivity in liquid culture, and formulation and efficacy aspects. Yield and quality of the spores varied between isolates and type of liquid medium used. Isolate PPRC-HH and PPRC-9609 produced the highest yield. These two isolates also produced submerged conidia in TKI medium. The results of the immersion bioassay showed that both fresh and formulated submerged spores/conidia of the isolates were active against *S. zeamais* but the LC_{50} and LC_{90} value varied depending on the isolate, liquid medium, the drying method as well as type of formulation used. Admixture of different dry powder formulations to maize grain resulted in an effective dose transfer to the test insects. Conidia and talcum-based dry powder formulations of PPRC-HH and PPRC-EE provided a substantial control efficacy against *S. zeamais* (LD_{50} = 7.2 – 7.8 x 10^6 conidia g^{-1}). Aerial conidia of the two isolates formulated with talcum, commercial H-milk and molasses as well as all submerged conidia-based formulations of PPRC-HH were less effective and did only perform better at a higher application rate (LD_{50} = 1.7 – 3.9 x 10^8 spores g^{-1}). Air-dried formulations maintained a higher efficacy when compared to spray or freeze dried formulations.

Key words: aerial conidia, *B. bassiana*, DP formulations, efficacy, liquid culture *M.anisopliae*, production, submerged spores, submerged conidia,

Introduction

The maize weevil, *Sitophilus zeamais* (Motsch.) (Coleoptera: Curculionidae) is the most important and destructive pest of stored grain in the tropics causing considerable quantitative as well as qualitative losses (McFarlane, 1988; Mbata, 1992). Synthetic chemical insecticides have been used for the control of pests of stored grain for many years. Recently, concerns have emerged regarding the use of protectant insecticides as a result of environmental issues and resistance development (Zettler and Cuperus, 1990; Arthur, 1992). This has fueled research and development of alternative management strategies. One management strategy could be biological control using entomopathogenic fungi (Ferron, 1985), underlined by many recent advances in the use of entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* for the control of stored product insect pests (Fernades *et al.*, 1983; Frydocva *et al.*, 1989; Lourenção *et al.*, 1993; Hidalgo *et al.*, 1998; Smith *et al.*, 1999). However, commercial products for use in storage pest control are not yet available in Africa. For the development of a successful commercial bioinsecticides, the possibility for large scale production, appropriate formulation, performance of the formulation and the price of the final product in a competitive market are all prerequisites (Malsam *et al.*, 1997, Butt *et al*., 2001). Several techniques for mass production of entomopathogenic fungi are now available (Wraight *et al*., 2001). For example, industrial production of aerial conidia involves either a surface cultivation or a two-stage production process. These methods of cultivation are widely used for commercial production of mycoinsecticides. On the other hand, in liquid culture both blastospores (Bidochka *et al.*, 1987; Kleespies *et al.*, 1992) and submerged conidia (Thomas *et al.*, 1987; Rombach *et al.*, 1988; Hegedus *et al.*, 1990) have been produced in *Beauveria* and *Metarhizium* species. Submerged production techniques may have the advantage that fungi can be produced rapidly using conventional deep-tank fermenters, and scaling-up of the process is relatively easy (Bradley *et al.*, 1992).

Kassa *et al.* (2002), using Ethiopian isolates of entomopathogenic fungi, identified a number of highly virulent *B. bassiana* and *M. anisopliae* strains for control of *Sitophilus zeamais* and *Prostephanus truncatus*. In their experiments they used water suspensions of aerial conidia produced on malt extract agar. However, under practical circumstances grain in stores must remain dry to suppress the growth of other microorganisms and to avoid grain deterioration, emphazising the need to develop applicable production and formulation strategies. Furthermore, studies on the potential use of submerged spores/conidia of *M. anisopliae* and *B. bassiana* for control of storage pests are limited. Therefore, in the present study, using

selected isolates of *B. bassiana* and *M. anisopliae* we evaluated different liquid media for submerged spores/conidia production. Furthermore, the efficacy of unformulated submerged spores/conidia against *Sitophilus zeamais* was assessed. Moreover, different dustable powder (DP) formulations based on aerial conidia and submerged spores/conidia of virulent *B. bassiana* and *M. anisopliae* isolates were developed and their efficacy towards *S. zeamais* was assessed.

Materials and Methods

Insect rearing

Sitophilus zeamais beetles were obtained from a continuous rearing culture established at the Federal Biological Research Center for Agriculture and Forestry (BBA), Institute for Biological Control, Darmstadt. They were reared on maize grain at 30°C and 60-70% RH, following the methodology described by Kassa *et al.*, (2002).

Fungal strains

Three *B. bassiana* isolates (PPRC-HH, PPRC-9609, PPRC-9614) and one *M. anisopliae* isolate (PPRC-EE) were obtained from the Plant Protection Research Center (PPRC), Ambo, Ethiopia. Previous studies have established that these four isolates are highly virulent against *S. zeamais* and *P. truncatus* (Kassa *et al.*, 2002). Primary stock and working cultures of these isolates were maintained on Sabouraud dextrose agar at -20°C and 5°C, respectively.

Submerged spore production

Five different media were used to study the submerged spore/conidia production of the four isolates (Table 1). The fungi were grown in liquid media in shake flasks. Each medium was poured in 50-mL quantities in 250-mL Erlenmeyer flasks and autoclaved for 20 min at 121°C. Each flask was then inoculated with 5 x 10^7 submerged spores (= 1 x 10^6 mL^{-1}) from a previous subculture. The inoculated flasks were incubated for 72 h at 30°C on a rotary shaker (New Brunswick Scientific, Germany) at a speed of 180 rpm. For TKI medium, the fermentation period was extended to 7 days. The entire experiment was repeated twice and there were four replications of all treatments.

Determination of spore yield and dry weight

In each sample, spore yield was counted in a suitably diluted suspension by using a Thoma haemocytometer. For dry weight determination an aliquot of the culture (25-mL) was taken

Table 1. Liquid media and their composition used for submerged spores/conidia production of *M. anisopliae* and *B. bassiana* isolates.

Medium	Compositions	Source
Adámek standard (AS)	3 % cornsteep, 4 % yeast extract, 4 % glucose and 0.4 % Tween 80®)	Adámek (1963)
Adámek half (AH)	Half concentration of the standard medium (see above)	Modified
Samšináková (SM)	Glucose 2,5 %; Solubel starch 2,5 %; Corn-step 2 %; NaCl 0.5 %; $CaCO_3$ 0,2 %	Samšináková (1966)
Catroux (CM)	Corn-step 2 %; Saccharose 3 %; KH_2PO_4 0,68 %; $MgSO_4$-$7H_2O$ 0.01% ; $CaCO_2$ 0,2 %; KNO_3 0.5%	Catroux *et al.* (1970)
TKI	Glucose 50g; KNO_3, 10g; KH_2PO_4, 5g; $MgSO_4$, 2g; $CaCl_2.2H_2O$, 50mg; $FeCl_3.6H_2O$, 12mg; $MnSO_4.H_2O$, 2.5mg; $Co(NO_3).6H_2O$, 0.25mg; $Na_2MoO_4.2H_2O$, 0.2 mg; $ZnSO_4.7H_2O$, 2.5mg; and $CuSO_4.5H_2O$, 5mg	Thomas *et al.* (1987)

from each culture and centrifuged at 9630 g for 5 min at 4 °C (Couiter Bioresearch, Germany). Fresh biomass weight was measured and 2 g biomass samples were dried at 105°C using a Sartorius moisture balance (Sartorius AG, Germany). In addition, for TKI medium, glucose concentration was measured in the supernatants every 24 h for 7 days.

Germination of submerged spores

The rate of germination of submerged spores/conidia was estimated using water agar (1.5 % agar amended with 30-mgL^{-1} of streptomycin sulphate, 50-mgL^{-1} chloramphenicol and 0.005 % Benomyl). Submerged conidia from the different treatments were suspended in deionised water containing Tween 80® (0.01 % by vol.) and the concentration was adjusted to 1 x 10^6 mL^{-1}. Several drops of a 2-µl spore suspension were dropped onto the agar plate and incubated at 25°C. Viability was assessed after 8 h, 16 h and 24 h incubation period by counting 300 spores in each sample (3 samples/replicate) using a Zeiss Axioplan microscope (Germany). The criterion for germination was that the spores must have formed a germ tube of a length twice as the diameter of the spore.

Following the shake flask experiment, isolates PPRC-EE and PPRC-HH were selected for further production and formulation studies. Submerged spores of PPRC-EE and submerged conidia of PPRC-HH were produced in standard Adámek and TKI liquid medium, respectively (Table 1). In both cases, 500-mL of sterile medium was inoculated with 5 x 10^8

submerged spores/conidia and incubated for 72 h at 30°C on a rotary shaker at a speed of 100 rpm. The resulting slurry was centrifuged at 9630 g for 5 min at 4 °C and the pellet was used for formulation development. The pellets were re-suspended in 200-mL deionised water and homogenised using a magnetic stirrer at low speed. Volume, fresh weight, dry weight and spore yield of the fresh biomass were determined before the formulation and drying process.

Production of aerial conidia

Conidia of PPRC-EE were produced by the use of a standard two-stage solid production system described by Jenkins *et al*. (1998) with modifications. The initial submerged spore inoculums were produced in standard Adámek liquid medium following a similar methodology described previously. A 75-ml spore suspension was transferred into a mushroom bag containing 500-g parboiled and autoclaved (121°C, 20 min) rice substrate for growth and sporulation. The inoculated bags were incubated at 30°C for 15 days. After 15 days, the bags were reopened and placed in an air drying cabin, Hygrex-laboratory dryer CH 2500 (Hellmann Hygrex, Hamburg) for two days. The drying temperature and the final moisture content were adjusted at 30°C and 10 %, respectively. Dried spores were separated from the grain by sieving using 120-µm mesh size and kept in a desiccator until the moisture content dropped below 10 %.

At the time of this investigation, the standard two-stage solid production system was not optimized for isolate PPRC-HH. Therefore, the aerial conidia of PPRC-HH were produced on a TKI broth solidified with 1.5 % agar on Petri-dishes (Thomas *et al*., 1987). The plates were inoculated with 100-µl of the spore suspension and incubated for 2 weeks at 30°C in darkness. The spores were harvested by scrapping and placed inside a desiccator until the moisture content dropped below 10 %. In both cases the dry weight and spore yield of the biomass were determined before the formulation process.

Formulation

Aerial conidia or submerged spores/conidia of PPRC-EE and PPRC-HH were mixed separately with different carriers and, by using air, freeze or spray drying techniques, eight different formulations were developed. A list of formulations, their components and their main characteristics are presented (Table 2).

Table 2. Formulations developed and their properties (MC: Moisture content, CFU: Colony forming unit).

Formulation (code)	Description of the formulation	MC (%)	Spore conc. (x 10^{10} /g)	CFU (x 10^{10} /g)	Germination (%) after 16 h at 25°C
F1	PPRC-EE, air dried aerial conidia: Talcum (1:2)	3.37	0.40 ± 0.03	127 ± 0.05	< 50
F2	PPRC-EE, air dried aerial conidia: H-milk: Talcum: Molasses (1:1:2:0.08)	3.78	1.23± 0.12	638 ± 0.20	< 50
F3	PPRC-HH, air dried aerial conidia: Talcum (1:2)	3.53	4.09 ± 0.51	3.50 ± 0.54	94.43 ± 0.61
F4	PPRC-HH, air dried aerial conidia: H-milk: Talcum: Molasses (1:1:2:0,08)	3.36	3.19 ± 0.02	0. 83 ± 0.17	84.83 ± 0.25
F5	PPRC-HH spray dried submerged conidia :skim milk powder: Molasses (1:1:0,08)	4.13	3.51 ± 0.28	1.65 ± 0.82	95.23 ± 0.41
F6	PPRC-HH, freeze dried submerged conidia: skimmed milk powder: glycerol (1:1:0,08)	5.76	3.67 ± 0.32	1.35 ± 0.76	74.4 ± 0.99
F7	PPRC-HH, air dried submerged conidia: H-milk: talcum: molasses (1:1:2:0,08)	3.37	3.57 ± 0.34	0.40 ± 0.16	94.83 ± 0.27

Aerial conidia of the respective isolates were formulated in two different ways. The first set (F1 and F3) was formulated with talcum carrier while the second set (F2 and F4) was formulated with a mixture of talcum, H-milk and molasses. In both cases, after mixing the spores with the carriers, the formulations were air-dried on a clean bench with a continuous air flow at room temperature until the moisture content was below 5 %. Once the product had been dried to the desired moisture level, it was crashed gently with the help of a mortar and pestle. The content was sieved through 120-µm mesh and the resulting dustable powder (DP) was stored in a glass bottle at 4°C.

Submerged conidia of PPRC-HH were formulated with skimmed milk powder, molasses, or glycerol carriers. The final formulations were either spray-dried (F5) or freeze-dried (F6) following the methodology described by Stephan and Zimmermann (1998; 2001) and stored

as described formerly. Molasses was used for spray-drying whereas glycerol was added for freeze-drying of submerged conidia. Spray and freeze-drying were not used for submerged spores of PPRC-EE. Submerged conidia of PPRC-HH (F7) and submerged spores of PPRC-EE (F8) were mixed separately with talcum, H-milk and molasses. The mixture was air-dried and processed following similar procedures described previously. For all formulations, the moisture content, spore yield and viability were assessed.

Efficacy of unformulated submerged spores and aerial conidia

In order to get pure submerged spore/conidia suspensions, samples of the fermentation product were filtered through a double lining cloth mesh. For each isolate-media combination, a 10-mL stock suspension containing 1 x 10^8 submerged spore mL^{-1} were prepared. From this, serial dilutions were made to obtain 5 different concentrations (1 x 10^4, 10^5, 10^6, 10^7 and 10^8 submerged spore mL^{-1}) in sterile deionised water with Tween 80 (0.01 % by vol). Twenty-five mixed age adults of *S. zeamais* were chosen at random from the rearing jar and placed in Petri-dishes (9-cm diameter). Inoculation and handling was carried out as described by Kassa *et al.*, (2002) and incubated at 30 °C and 60-70% RH. The control insects were treated with sterile deionised water with Tween 80 (0.01 % by vol). The experiment was replicated four times. Mortality was recorded daily for 11 days and cadavers were washed three times with sterile deionised water and placed into a humidity chamber and incubated at 25°C for the outgrowth of the fungus. The efficacy of unformulated aerial conidia of the two isolates (PPRC-HH and PPRC-EE) was also assessed as described.

Efficacy of formulated submerged spores and aerial conidia

Bioassays were carried out using a water suspension of the formulated product. From each formulation 0.1g of the product was suspended in 10-mL of deionised water with Tween 80 (0.01 % by vol). Serial dilutions were made to obtain five different concentrations (1 x 10^4, 10^5, 10^6, 10^7 and 10^8 spores mL^{-1}). The control insects were treated as described previously. Twenty mixed age adults of *S. zeamais* were used for each replicate and there were four replications of all treatments. Inoculation, handling and assessment were performed as described previously.

Dose-response study with DP formulations

Small Plexi-glass containers (2 cm^3) were filled with a gram of cleaned and disinfested crumbled maize grain. Samples of DP formulations corresponding to the rates of 2 x 10^7, 1 x

10^8, 3 x 10^8 and 5 x 10^8 spores g^{-1} of maize seeds were applied into each container and tumbled to thoroughly mix the spores with the grains. There were three groups of controls. The first two include blank formulations of talcum: H-Milk: molasses (10 g kg^{-1}) and talcum alone (5 g kg^{-1}). The third one represents the untreated control. For F3, assessments were also made with additional doses of 4 x 10^7, 6 x 10^7, 8 x 10^7 spores g^{-1} of maize seeds, including control groups treated with the respective doses of the blank formulation as well as the untreated control. Ten mixed age adults of *S. zeamais* were released into each cup containing the treated grain. A part of the lids of the cups was replaced with fine wire-mesh to allow adequate ventilation. Samples were maintained for 20 days at 30°C and 60–70% RH. There were four replications of all treatments. The adult insects were examined for mortality at intervals of 5 days and dead insects were removed and handled as described previously.

Data analysis

In order to homogenise the variances, all data were transformed into either log or arcsine scale. When appropriate, cumulative percentage mortality data were corrected for the corresponding control mortality (Abbott, 1925). Data were analyzed following the general linear model procedure (PROC GLM, SAS Institute, 1989). Repeated-measures ANOVA were used to assess the effect of DP formulations, time of exposure and their interaction on mortality of test insects (SAS Institute, 1989). When appropriate, the treatment means were separated using the Student-Newman-Keuls (SNK) test (SAS, 1989). Probit analysis (PROC PROBIT) was used to estimate both the LC_{50} and LC_{90} of the isolates with 95 % confidence limits (SAS Institute, 1989).

Results

Production of submerged spores/conidia

The submerged spore production was highly dependent on isolates (F = 42.83, $D.F.$ = 3, P < 0.001), the media tested (F = 23.42, $D.F.$ = 3, P < 0.001), and their interaction (F = 14.12, $D.F.$ = 9, P < 0.001). PPRC-HH and PPRC-9609 produced high spore yields in most of the media tested when compared to PPRC-EE and PPRC-9614, which resulted in a low level of spore yield in most media (Figure 1a). PPRC-9614 produced relatively more spores in the two Adámek media tested whereas PPRC-EE showed a comparatively higher spore yield only in the standard Adámek medium (Figure 1a). In TKI medium, only PPRC-HH and PPRC-9609 were able to produce submerged spores and the yield was higher for the former isolate. After 72 h fermentation period, the submerged spore yields in the Adámek,

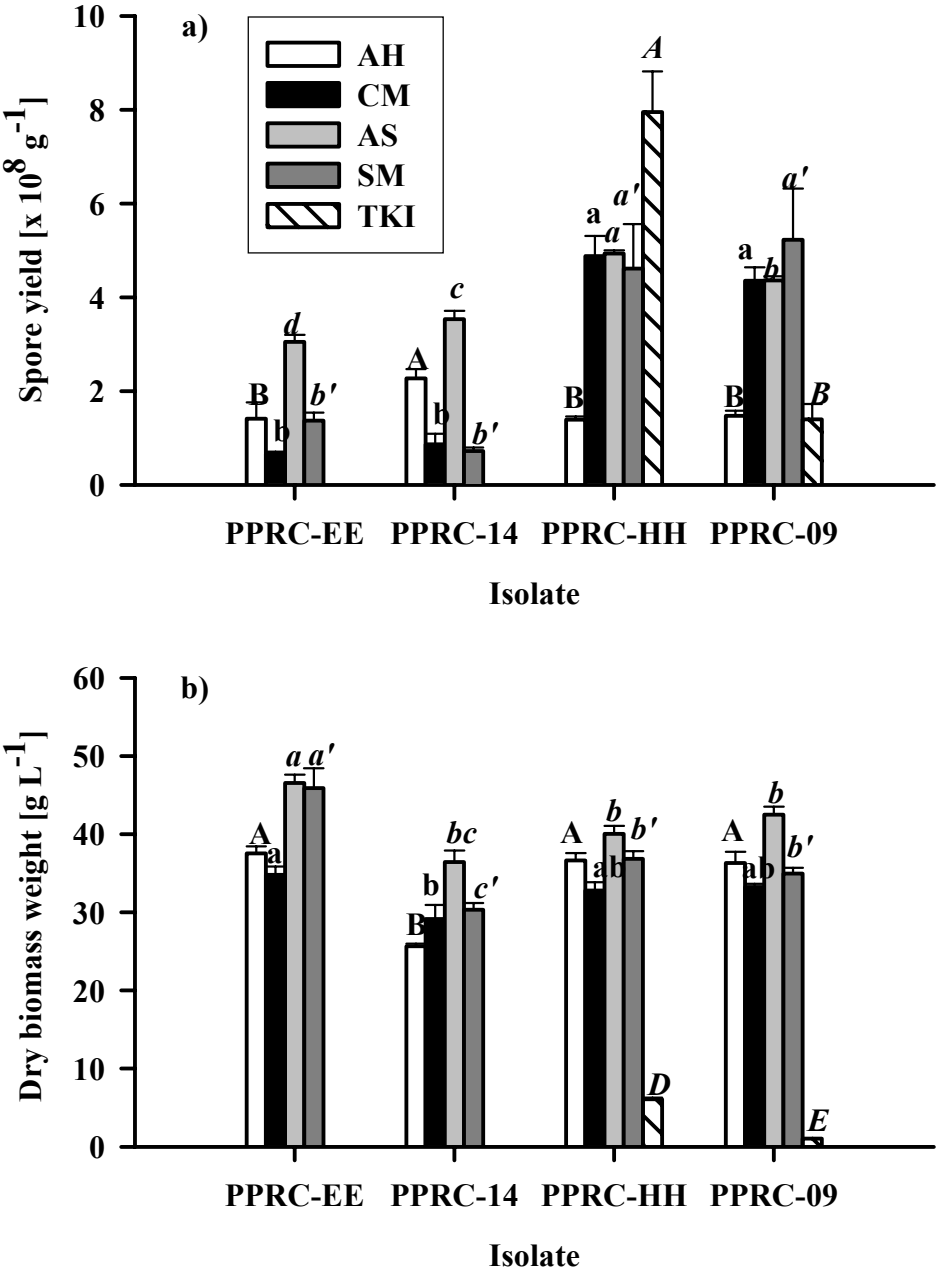

Figure 1. Mean spore yield (a) and biomass dry-weight (b) of *B. bassiana* and *M. anisopliae* isolates grown in different liquid media for 72 h at 30°C (Mean ± SE, N = 8). In the same medium means followed by the same letter are not significantly different following SNK test ($p < 0.05$). Liquid media: AH: Adamék-half concentrated, CM: Catroux medium, AS: Adamék standard, SM: Samšináková medium, TKI: TKI medium.

Samšináková and Catroux medium were dramatically reduced for all isolates, and the mycelium biomass increased (Data not shown). In contrast to this, in the TKI medium the spore yield in both isolates reached a peak level after 144 h and started to decline thereafter (Figure 2). Furthermore these two isolates produced a higher proportion (> 90 %) of submerged conidia in TKI medium and underwent microcyclic conidiation (Plate 1). During the initial stages of the fungal growth the amount of glucose in the TKI medium gradually decreased (Figure 2). The maximum utilization of glucose occured from the 3rd to the 7th day, when sporulation was higher. Thereafter no glucose remained in the medium. Aerial conidia production was made only for the development of the formulations, yields were therefore not compared statistically with those of submerged spores.

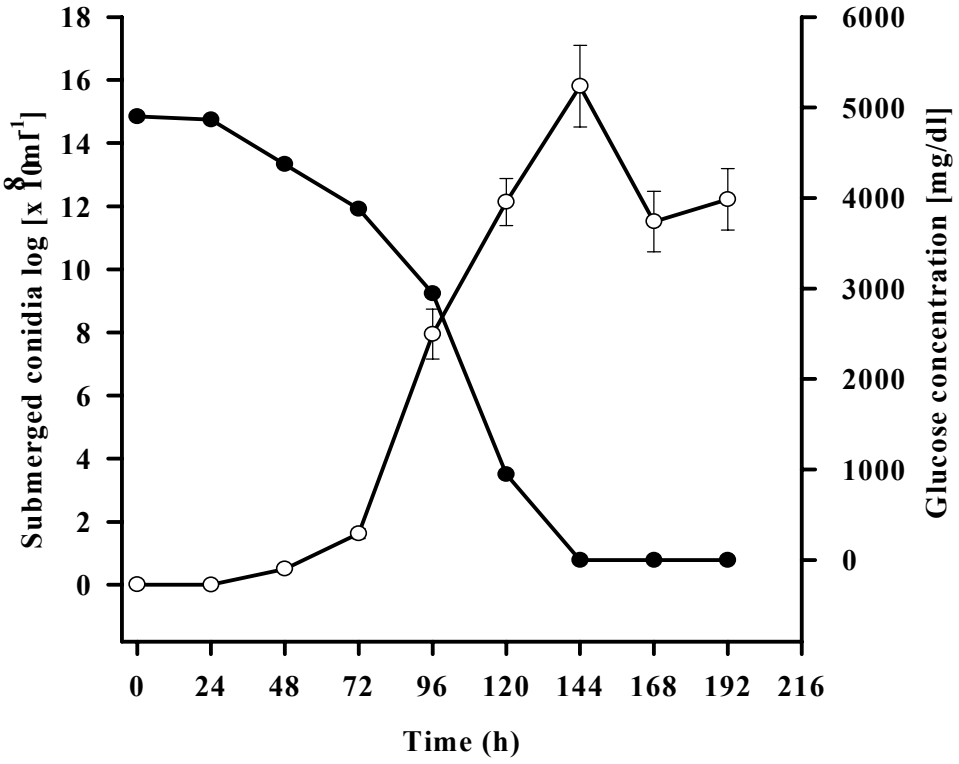

Figure 2. Mean submerged conidia yield of isolate PPRC-HH in TKI liquid medium and reduction in glucose concentration of the medium with time (○) spore yield and (●) Glucose concentration.

Total biomass production

Biomass production differed significantly between isolates ($F = 40.73$, $D.F. = 3$, $P < 0.001$), media ($F = 13.74$, $D.F. = 3$, $P < 0.001$) and their interaction ($F = 5.59$, $D.F. = 9$, $P < 0.001$) ($F =$, $D.F. =$, $P < 0.001$). Isolate PPRC-EE produced high amounts of biomass in all tested media and they were much higher in the Samšináková and Adámek medium (Figure 1b). PPRC-

9614 showed low biomass production in most of the tested media whereas PPRC-9609 and PPRC-HH resulted in an intermediate biomass production in the first four media and a significantly lower biomass yield in TKI medium (Figure 1b). In most media the isolates tested produced a high mycelium content, which caused difficulties during spore separation.

Germination rate of submerged spores

Germination rates of the different isolates after 8 h incubation at 25°C were significantly different among the isolates ($F = 15.59$, $D.F. = 3$, $P < 0.001$), media ($F = 57.90$, $D.F. = 2$, $P < 0.001$) and their interaction ($F = 2.48$, $D.F. = 6$, $P = 0.0295$)(Table 3). Regarding the isolates, PPRC-EE showed faster germination when compared to the remaining *B. bassiana* isolates (Table 3). All isolates tested showed a higher germination rate in Adámek medium followed by the Catroux, TKI and Samšináková medium in that order. However, after 16 h and 24 h incubation period, germination in all isolate-medium combinations exceeded 95 % and there were no significant differences among the treatments ($P = 0.05$).

Table 3. Mean germination (%) of fresh submerged spores of *Beauveria bassiana* and *Metarhizium anisopliae* isolates produced in different liquid media and incubated at 25 °C for 8 h (Mean ± SE, N = 8).

	Percent germination after 8 h[a]				
Isolate No.	Samšináková	TKI[b]	Catroux	Adámek half	Adámek standard
PPRC-14	69.20 ± 0.99 b	Ns	79.83 ± 5.54 a	84.85 ± 0.65 a	86.85 ± 0.72 a
PPRC-09	67.23 ± 0.78 c	65.53 ± 0.80 c	80.58 ± 5.06 b	94.66 ± 0.54 a	95.60 ± 0.50 a
PPRC-HH	68.20 ± 2.03 c	64.51 ± 0.51 c	76.81 ± 4.75 b	94.70 ± 0.37 a	95.50 ± 0.40 a
PPRC-EE	86.45 ± 1.18 b	Ns	90.16 ± 2.60 b	97.21 ± 0.38 a	97.00 ± 0.40 a

[a] Analysis was made on arcsine √percentage transformed data; TKI medium was not included for the two-way ANOVA.
[b] Ns: No spore production.
Means within a row followed by the same letter are not significantly different (One-way ANOVA, SNK test, $P = 0.05$). Two- way ANOVA: Isolate ($F = 15.59$, df = 3, $P = 0.0001$), Media ($F = 57.90$, df = 3, $P < 0.0001$), isolate * interaction ($F = 2.48$, df = 12, $P = 0.0295$).

Efficacy of submerged spores

The three-way interaction table for mortality of treated *S. zeamais* is indicated in Table 4. Virulence of submerged spores differed significantly among the isolates, media and the concentration tested as well as their interaction. At a lower concentration (1×10^6 spores mL^{-1}), submerged spores of PPRC-EE cultivated in Adámek and Catroux medium caused 75%

and 97% mortality, respectively. In contrast, at the same concentration the other three *B. bassiana* isolates resulted in a mortality ranging from 40 – 55% and 30 to 50% in Adámek and Catroux medium, respectively. On the other hand, submerged spores of all isolates produced in Samšináková and TKI medium showed a lower than 40 % mortality at the concentration of 1 x 10^6 spores mL^{-1}. In all tested media at 1 x 10^7 and 1 x 10^8 spores mL^{-1}, all isolates caused 57–100% and 93–100% mortality, respectively. The isolates also varied in LC_{50} and LC_{90} values depending on the type of liquid medium used for submerged spore production (Table 5). PPRC-EE and PPRC-HH expressed the lowest LC_{50} and LC_{90} values when they were produced in Catroux and Adámek medium. On the other hand, for PPRC-9609 and PPRC-9614 lower LC_{50} and LC_{90} values were observed when submerged spores were produced in Adámek medium (Table 5). The LC_{50} values observed in the Samšináková and TKI medium were high for all isolates. Furthermore, in the TKI medium an increased fermentation time during the production process from 3 to 7 days also increased the LC_{50} and LC_{90} value of PPRC-HH by 13% in both cases (Table 5).

Table 4. Three-way interaction table for mortality of *S. zeamais* treated with various doses of submerged spores of different isolates produced in different liquid culture (Analysis was made on arcsine √percentage transformed data) ($P = 0.05$, $R^2 = 0.93$, C. V. = 27.65 %, Root MSE = 0.183).

Source of variation	DF	Mean square of Error	*F*-value	*P*-value
Isolates	3	0.105	3.15	0.0265
Media	2	0.816	24.45	0.0001
Dose	4	18.135	543.21	0.0001
Isolates x media	6	0.238	7.12	0.0001
Isolates x doses	12	0.111	3.32	0.0002
Media x doses	8	0.144	4,32	0.0001
Isolates x media x doses	24	0.086	2.53	0.0003

Efficacy of formulated aerial conidia and submerged spores

Formulated and both dried aerial conidia and submerged spores of PPRC-HH and PPRC-EE were able to infect *S. zeamais,* when they were applied as water suspension. However, mortality of *S. zeamais* differed significantly among the formulations ($F = 12.29$, df = 6, $P < 0.0001$), Doses ($F = 213.60$, df = 4, $P < 0.0001$) and their interaction ($F = 2.48$, df = 24, $P = 0.0008$) (Table 6). At concentrations of 1 x 10^4 and 1 x 10^8 spores mL^{-1}, there was no difference between the unformulated and formulated spores tested. In the remaining three

other concentrations, the unformulated conidia of both isolates showed a higher mortality than the formulated aerial and submerged conidia (Table 6). Among the formulated products, F3 caused a relatively higher mortality at the application rate of 1 x 10^7 spores mL^{-1}. The LC_{50} and LC_{90} values are shown in Table 7. The unformulated spores of both isolates experienced a reduced value as compared to the formulated product. Regarding the formulated product, the F3 and F7 revealed a lower LC_{50} and LC_{90} when they were applied as water suspension than the remaining other formulations (Table 7).

Table 5. Parameters for the regression of probit-transformed percentage mortality of *S. zeamais* treated with *M. anisopliae* and *B. bassiana* submerged spores/conidia produced in different liquid media (N = 478-600, PROC PROBIT, $P < 0.001$).

Isolate	Medium[a]	Slope ± SE	Chi-square	Log LD_{50}	95 % CL[b]	Log LD_{90}	95 % CL[b]
M. anisolpliae							
PPRC-EE	AS	0.96 ± 0.08	154.81	6.01	5.84-6.18	7.35	7.13-7.63
	CM	1.79 ± 0.26	48.87	5.32	5.14-5.47	6.03	5.88-6.27
	SM	1.18 ± 0.19	36.55	6.80	6.53-6.99	7.89	7.65-8.29
B. bassiana							
PPRC-HH	AS	1.38 ± 0.14	101.40	6.19	6.03-6.33	7.11	6.94-7.35
	CM	1.14 ± 0.21	30.31	5.88	5.47-6.11	7.00	6.79-7.37
	SM	1.52 ± 0.21	54.29	6.42	6.23-6.57	7.27	7.09-7.52
	TKI[c]	1.32 ± 0.14	84.23	6.62	6.44-6.77	7.59	7.39-7.87
	TKI[d]	1.12 ± 0.17	46.08	7.54	7.35-7.73	8.68	8.37-9.19
PPRC-9609	AS	1.06 ± 0.12	77.17	6.05	5.82-6.24	7.26	7.04-7.55
	CM	0.97 ± 0.11	79.74	6.26	6.02-6.45	7.58	7.34-7.91
	SM	1.39 ± 0.15	84.68	6.49	6.32-6.64	7.41	7.23-7.66
	TKI[c]	0.77 ± 0.09	68.01	7.19	6.96-7.44	8.85	8.44-9.45
PPRC-9614	AS	1.05 ± 0.10	102.79	5.98	5.78-6.15	7.20	6.99-7.48
	CM	2.35 ± 0.49	22.16	6.08	5.91-6.19	6.62	6.47-695
	SM	1.26 ± 0.14	82.18	6.24	6.05-6.40	7.26	7.07-7.53

[a] AS: Adámek standard medium (1963), CM: Catroux medium, SM: Samšináková medium, TKI: TKI medium (Thomas 1987).
[b] CL: Confidence limits.
[c], [d] represents submerged spores used after 3 and 7 days fermentation, respectively.

Dose-response studies with DP formulations

Mortality of *S. zeamais* differed significantly between the formulations ($F = 61.39$; df = 6; $P < 0.0001$), doses ($F = 20.46$; df = 2; $P < 0.0001$), time (Roy's Greatest Root = 5.65; $F = 109.28$; df = 3, 58; $P < 0.0001$), time x formulation (Roy's Greatest Root = 1.33; $F = 13.33$; df = 6, 60; $P < 0.0001$), and time x doses (Roy's Greatest Root = 0.21; $F = 4.18$; df = 3, 59; P

Table 6. Mean mortality of *S. zeamais* 10 days after exposure to various concentrations of water suspensions of different DP formulations of *M. anisopliae* and *Beauveria bassiana* aerial and submerged conidia (Mean ± (SE), N = 4).

Formulation[b]	Corrected percent mortality at different doses[a]				
	1x 10^4	1x 10^5	1x 10^6	1x 10^7	1x 10^8
F.Conidia HH	1.35 (2.55) a	10.74 (5.63) a	16.15 (5.63) b	91.88 (3.49) a	95.94 (1.35) a
F. Conidia EE	3.85 (2.62) a	11.03 (5.69) a	48.61 (17.46) a	89.18 (7.65) a	98.65 (1.35) a
F1	1.28 (4.05) a	4.06 (2.61) b	3.98 (5.58) c	50.67 (7.99) bc	85.12 (13.14) a
F3	2.63 (4.94) a	3.98 (3.41) b	5.33 (5.18) c	67.54 (10.12) ab	81.07 (11.15) a
F5	1.43 (2.59) a	4.06 (1.38) b	3.98 (7.77) c	55.94 (8.52) bc	82.42 (8.07) a
F6	1.43 (2.59) a	4.13 (1.35) b	4.13 (2.59) c	20.35 (9.64) bc	77.01 (8.94) a
F7	2.78 (5.41) a	5.49 (2.71) b	6.83 (6.74) c	50.75 (6.49) bc	97.29 (1.56) a
F-value	0.85	3.21	4.46	10.40	1.21
P– value	0.5483	0.0253	0.0062	<.0001	0.3448

[a] Analysis was made on arcsine √ percentage transformed data. For description of the formulations see Table 1 in Materials and Methods. [b] F. conidia stands for fresh conidia

Means within the same column followed by the same letters are not significantly different (one- way ANOVA, SNK test, PROC GLM procedure)

Two-way ANOVA (Formulation: $F = 12.29$, df = 6, $P = < 0.0001$; Doses: $F = 213.60$, df = 4, $P < 0.0001$; Formulation* Doses: $F = 2.48$, df = 24, $P = 0.0008$).

= 0.0095). The time x formulation **x** dose interaction however, was not significant (Roy's Greatest Root = 0.33; $F = 1.68$; df = 12, 60; $P < 0.0935$), suggesting the need to analyse the mortality data observed at each dose separately. Similarly, the repeated measures ANOVA performed for each dose revealed a highly significant difference between formulations, time and their interaction ($P < 0.0001$) (Figure 3a-c). For all doses tested, mortality occur faster (MST < 5 days) and it was significantly higher on maize grains that were treated with F3 and F1 formulations (Figure 3a-c). For these two formulations differences in mortality were observed at 5 days after exposure at 1 x 10^8 and 3 x 10^8 spores g^{-1} of maize grains (Figure 3a-b). At a higher dose, differences were not observed (Figure 3c). Mortality in F3 and F1 treated grains exceeded 90% 10 days after exposure and reached at peak at 15 and 20 days after exposure (Figure 3a-c). For the remaining formulations, 20 days after exposure the observed mortality at the application dose of 1 x 10^8 spores g^{-1} of maize grains ranged from 32–62%, occurred at a lower rate and they were not statistically different from each other (Figure 3a). At the same dose, the median survival time ranged from 14–16 days for F2 and F4 and from 16–17 days for F5, F6, and F7. For these groups of formulations, the mortality increased with

Table 7. Parameters for the regression of probit-transformed percentage mortality of *S. zeamais* treated with water suspension of different DP formulations of *M. anisopliae* and *B. bassiana* submerged spores and aerial conidia (N = 713–717, PROC PROBIT, $P < 0.0001$, CL: Confidence limits).

Formulation	Slope ± SE	Chi-square	Log LD_{50}	95 % CL	Log LD_{90}	95 % CL
PPRC-HH unformulated conidia	1.48 ± 0.22	39.83	6.42	6.20 – 6.59	7.29	7.10 – 7.59
PPRC-EE unformulated conidia	1.18 ± 0.14	66.76	6.02	5.81 – 6.19	7.10	6.88 – 7.41
F1	1.28 ± 0.15	70,91	7.09	6.92 – 7.26	8.09	7.87 – 8.43
F3	1.08 ± 0.14	58.34	6.96	6.75 – 7.14	8.15	7.88 – 8.56
F5	1.19 ± 0.13	76.86	7.07	6.89 – 7.24	8.13	7.89 – 8.48
F6	1.60 ± 0.22	54.18	7.52	7.36 – 7.67	8.32	8.11 – 8.65
F7	1.68 ± 0.23	51.77	6.94	6.76 – 7.08	7.69	7.52 – 7.97

For description of the formulations, see Table 1 in Materials and Methods.

increasing dose (Figure 3b-c). The observed median survival time for these formulations ranged from 10-16 days, the lowest values being for F2 and F4 at 5 x 10^8 spores g^{-1} of maize grains.

At the lower dose (2 x 10^7 spores g^{-1} of maize grains), mortality was only observed on F1, F2 and F3 treated grains. The mortality exceeded 50% 10 days after treatments, resulting in a median survival time of < 10 days for F1 and F3 whereas on F2 treated grain, the mortality reached 56% after 20 days, resulting in a higher median survival time (16 ± 0.74 days) (data not shown in Figure 3). At the same dose, after 20 days, the mortality reached 97 ± 2.94% and 62 ± 8.85% on F1 and F3 treated grains, respectively. In contrast to these, F4, F5, F6 and F7 showed no mortality attributable to fungal infection at the application rate of 2 x 10^7 spores g^{-1} of maize grains. Because of these data, this dose was excluded from the repeated measures ANOVA. Similarly, F3 at 4 x 10^7, 6 x 10^7, 8 x 10^7 spores g^{-1} of maize grains caused a mortality ranging from 70–85 % and 90–98% after 5 and 20 days of exposure period and these data were used for the estimation of the LC_{50} and LC_{90} values of F3 (not shown in Figure 3). For all control groups, the mortality remained less than 15% and did not significantly differ between each other ($P = 0.05$). Therefore, all data in the control groups were combined for the analysis.

The corresponding LC_{50} and LC_{90} values are presented in Table 8. For all formulations, the LC_{50} and LC_{90} increased with increasing exposure period from 10 to 15 days. At both assessment days, F3 and F1 were more active and showed lower LC_{50} and LC_{90} followed by F2 and F4. Among the remaining other formulations, F6 resulted in a higher LC_{50} and LC_{90} when calculated at 10 days after exposure followed by F7, F5 and F4. However, after 15 days the LC_{50} and LC_{90} value in all of these formulations ranged from 8.2–8.6 and 9.2–10.2, respectively and there were no significant differences among the formulations (Table 8).

Table 8. Parameters for the regression of probit-transformed percentage mortality of *S. zeamais* treated with different DP formulations of *M. anisopliae* and *B. bassiana* submerged spores and aerial conidia (N = 240-291, PROC PROBIT, $P < 0.01$, CL: Confidence limits).

	10 DAT				15 DAT			
Formulation	LD_{50}	CI	LD_{90}	CL	LD_{50}	CI	LD_{90}	CI
F1	7.92	7.64– 8.13	9.19	8.84– 9.93	6.86	5.87–7.19	7.75	7.49–8.09
F2	7.94	7.61–8.19	9.48	9.01–10.64	7.85	7.41–8.13	9.43	8.94–10.76
F3	6.89	2.64–7.71	8.02	6.58–8.89	6.88	5.43–7.23	7.94	7.77–8.44
F4	8.34	7.97–8.57	9.43	8.98–11.78	8.23	7.89–8.40	9.04	8.78–9.98
F5	8.80	8.54–1.52	10.09	9.30–22.21	8.59	8.30–9.79	9.82	9.15–19.17
F6	13.18	–	21.18	–	8.23	4.25–8.54	9.66	9.02–35.75
F7	9.22	8.82–19.17	10.39	9.45–35.75	8.41	8.23–8.55	9.06	8.83–9.64

For description of the formulations see Table 1 in Materials and Methods.

Discussion

In this study, we compared for the first time aerial conidia and submerged spores/conidia of *B. bassiana* and *M. anisopliae* for management of stored product insect pests. The results demonstrate that both aerial conidia and submerged spores/conidia of the two fungal entomopathogenes can be used in different formulations for the control of storage pests. However, productivity and efficacy varied among the different strains as well as the formulations tested.

All four isolates tested are capable of producing submerged spores in different liquid media. However, the yield and quality of the spores varied between isolates and the type of liquid medium used. The two *B. bassiana* isolates (PPRC-HH and PPRC-9609) produced the highest yield in most of the media tested compared to *M. anisopliae* (PPRC-EE) and *B. bassiana*

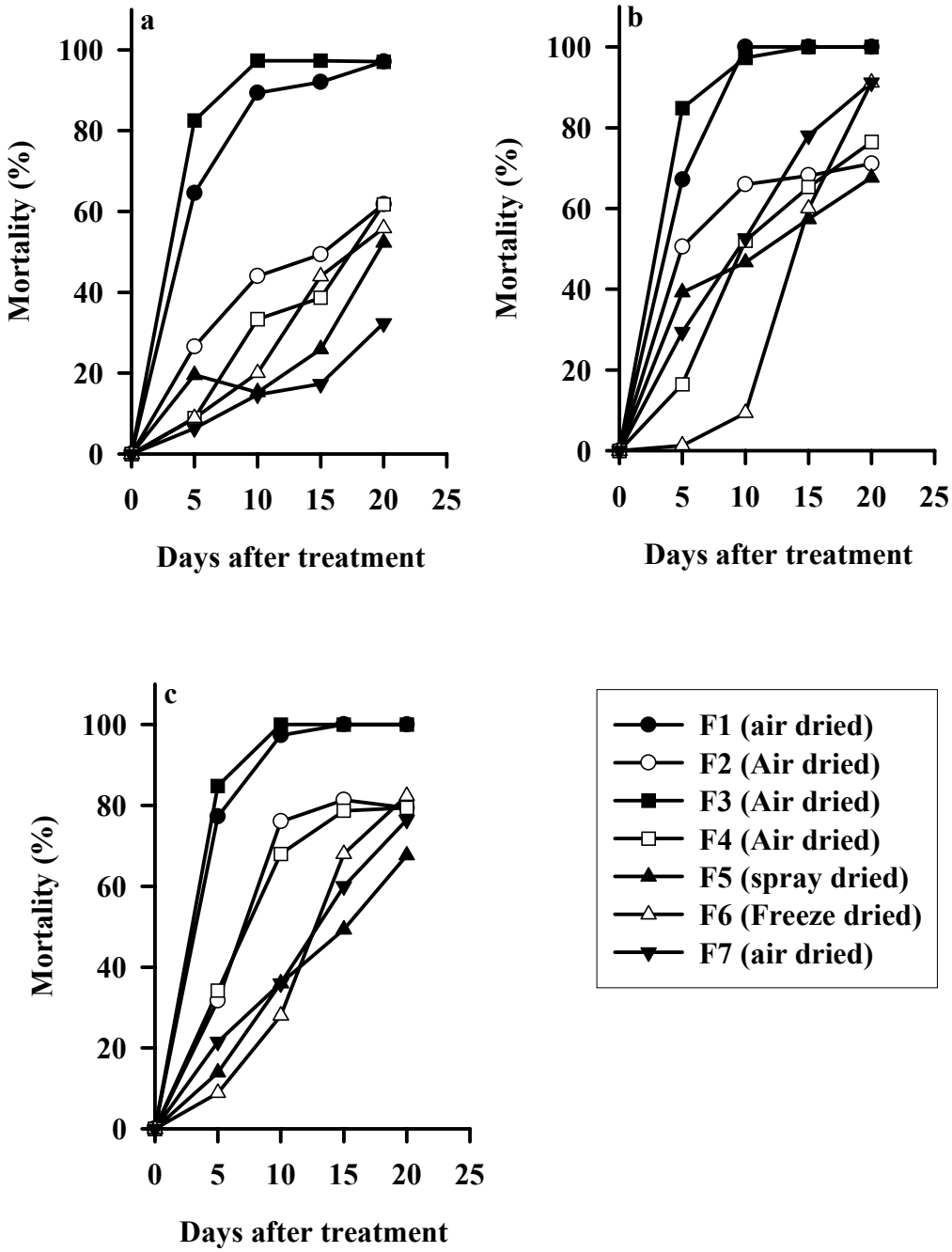

Figure 3. Mean corrected mortality (%) of *S. zeamais* exposed to maize grains treated with various DP formulations. Spores g^{-1} of maize seeds: a) 1 x 10^8, b) 3 x 10^8 and c) 5 x 10^8. Greenhouse-Geisser Epsilon = 0.76, $P < 0.0001$). In all formulations, the infection rate ranged from 98-100%.

(PPRC-9614), which even could not be cultivated in TKI medium. In a nutrient rich medium, most of the isolates produced a higher proportion of submerged spores and mycelial biomass (Bidochka *et al.*, 1987). In Adámek medium, for example, all isolates produced submerged

spores with a high amount of mycelium. On the other hand, in the Catroux and Samšináková medium, all isolates produced submerged spores and submerged conidia as well as mycelium biomass. Furthermore in these two media the proportion of submerged conidia was much lower than that of the submerged spores. Various studies have demonstrated that it is possible by thorough manipulation of nutritional and environmental conditions to produce conidia during submerged growth of certain strains (Thomas *et al.*, 1987; Hegedus *et al.*, 1990; Bosch and Yantorno, 1999). Furthermore, the abundance and type of spore produced (submerged conidia or submerged spores i.e. blastospores) depend on the nature of the carbon source, the C: N ratio as well as the strain used (Thomas *et al.*, 1987). The results obtained in our study corroborate these findings. Among the isolates tested, PPRC-HH was able to produce the highest yield of submerged conidia in TKI medium followed by PPRC-9609. Furthermore, these isolates also produced low mycelium biomass, which makes the formulation process easier. Microcycle conidiation in *B. bassiana* growing in a TKI medium was reported previously (Thomas *et al.*, 1987; Hegedus *et al.*, 1990; Bidochka *et al.*, 1995; Bosch and Yantorno, 1999). Similar results were obtained with our local isolates of *B. bassiana* (PPRC-HH and PPRC-9609) grown in TKI medium where microcycle conidiation was observed in submerged conidia in the presence of normal vegetative growth.

Regarding the speed of germination, isolates showed variability only at an early stage (8 h) after incubation. After 16 h and 24 h incubation at 25°C, isolates as well as media had no influence on the germination rate. Kassa *et al.* (2002) already pointed out that aerial conidia of PPRC-EE have a low (40–70%) germination rate when cultured in MEA for 24 h at 25°C. In contrast to this, the submerged spores of PPRC-EE germinated considerably faster and at a higher rate (> 95% after 16 h) on water agar. On the other hand, this study reveals that the virulence of fresh submerged spores/conidia was influenced by the isolate as well as the type of liquid medium used for production. In general submerged spores/conidia of the four isolates were active against *S. zeamais* when applied as a water suspension. The virulence of aerial conidia of these four isolates was tested previously (Kassa *et al.*, 2002), reporting high activity of PPRC-EE and PPRC-HH against the same test insect. The results obtained by using submerged spores/conidia of these two isolates corroborate the above findings. However, for the same isolates, the observed LC_{50} values may or may not out way the aerial conidia and is entirely dependent on the type of liquid media used for the production of submerged spores/conidia. In his study, Kmitowa (1979) also reported differences in virulence among conidia of entomopathogenic fungi produced in different liquid media.

One *M. anisopliae* isolate (PPRC-EE) and one *B. bassiana* isolate (PPRC-HH) were selected and various DP formulations were developed for further tests. The results reveal that all the tested formulations were active, (especially at higher concentration) when they were applied as a water suspension against *S. zeamais*. However, at a lower concentration, they showed a slightly reduced activity when compared to the unformulated spores. Moreover, the dose-response study demonstrated that admixture of DP formulations to maize grains resulted in an effective dose transfer to the test insects. However, efficacy assessed by mortality and median survival time varied among the different DP formulations and doses applied. Under the conditions of the present study, DP formulations of *B. bassiana* and *M. anisopliae* based on a 1:2 mixture of aerial conidia and talcum (F3 and F1) provided substantial control efficacy against *S. zeamais* at all doses tested. The corresponding LD_{50} value after 15 days of exposure were 7.6 x 10^6 and 7.2 x 10^6 conidia g^{-1} of maize seeds for F3 and F1, respectively. The results obtained with these two formulations are similar to those obtained by Hidalgo *et al.*, (1998) who observed 78% and 90% mortality of *S. zeamais* 15 days after application of DP formulation of *B. bassiana* to maize seeds at 5 x 10^9 and 2 x 10^{10} conidia kg^{-1} of maize seeds, respectively. Similarly, Rice and Cogburn (1999) using *Beauveria bassiana* conidial powder (4–5 x 10^6 conidia g^{-1} of rice grain) observed 80-100% mortality and 83–99% reduction in progeny emergence on *S. oryzae* (L.) and *Rhyzopertha dominica* (F.). On the other hand aerial conidia of the two isolates formulated with talcum, commercial H-milk and molasses (F2 and F4) as well as all submerged conidia-based formulations of *B. bassiana* (F5, F6 and F7) were less effective and perform better at a higher (> 3 x 10^8 spores g^{-1} of maize grain) application rate, resulting in a higher LD_{50} when compared to F3 and F1. This indicates that components of the formulations and the drying process may have contributed to reduced performance of these formulations. Air-dried formulations maintained a higher efficacy than spray or freeze dried products. One reason for these findings could be that formulations based on the latter two drying systems are hygroscopic and absorb moisture in the surroundings which contribute to uneven distribution of the spores within the grain system as well as for a quick decline of the activity of the spores. Furthermore, adding commercial H-milk and molasses to the formulations may also reduce the inherent biological activity of aerial and submerged conidia by affecting the adhesion to the cuticle, the spore distribution, an alteration of enzymatic production or by supporting the growth and development of other bacteria and yeasts which may interfere with the activity of the entomopathogenic fungus.

In general, the results obtained in this study indicate that it is possible to achieve a high level of control of storage pests using formulated *B. bassiana* and *M. anisopliae* aerial and submerged conidia. However, further improvements in the formulations and applications are indispensable in order to make use of the product in an IPM system against stored-product insect pests. Furthermore, the formulations need to be evaluated in large scale simulated laboratory or semi-field experiments in order to assess the impact of the formulations on a storage pest complex, the progeny production, persistence, grain damage and weight loss as well as on the storability of the formulations.

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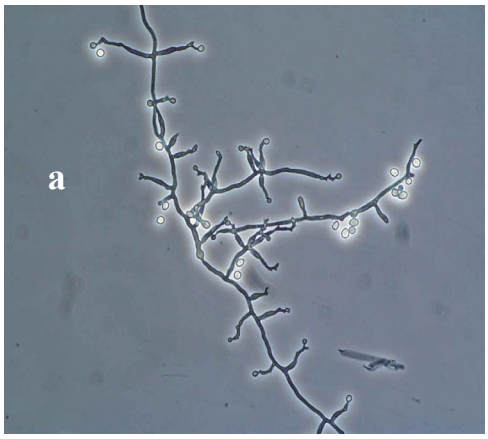


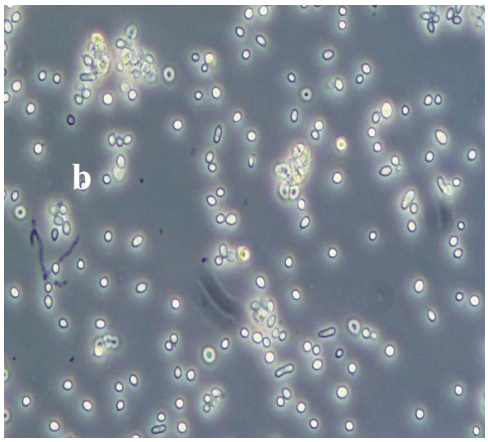
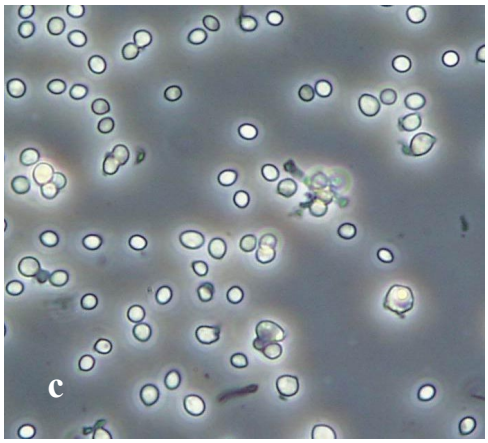


Plate 1. Submerged conidia of *B. bassiana* produced in TKI liquid medium. a) After 72 h and microcyclic conidiation and (b) conidia after 96 h fermentation (Bar = 60µm).



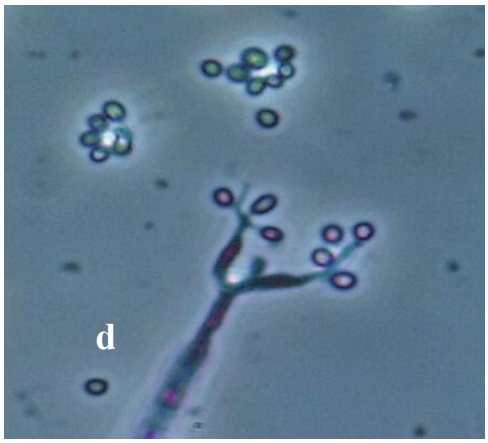


Plate 2. Aerial conidia of *B. bassiana* (PPRC-HH) produced (c) in solidified TKI medium and (d) in Malt extract agar (Bar = 20µm).

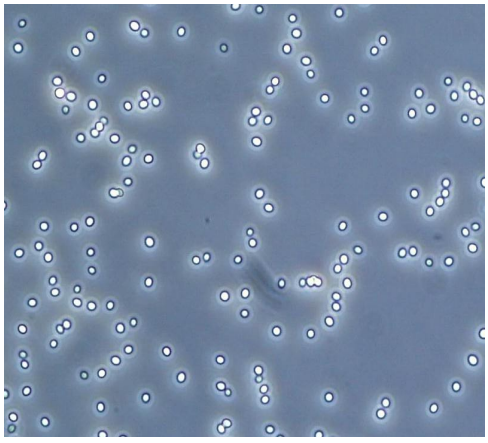


Plate 3. Aerial conidia of *B. bassiana* (PPRC-HH) grown on dead *Sitophilus zeamais* (Bar =50µm).

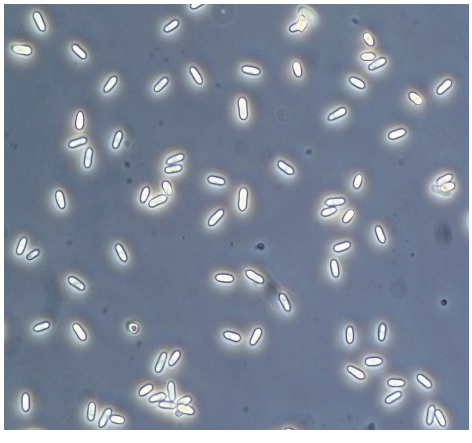


Plate 4. Aerial conidia of *M. anisopliae* (PPRC-EE) produced on rice (Bar = 20µm).

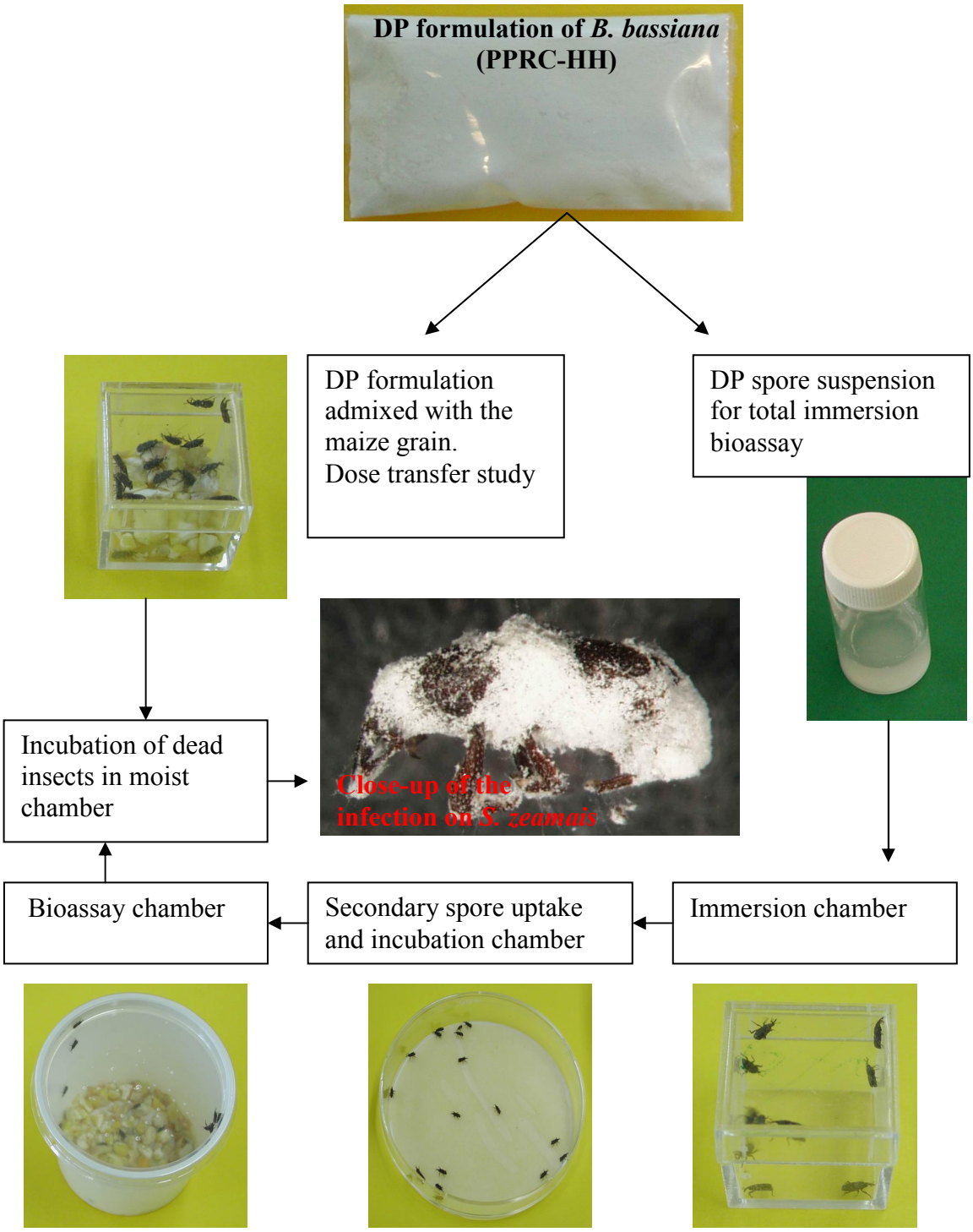


Plate 5. Total immersion bioassay and dry powder dose transfer study procedure for storage pests.

CHAPTER 8

Efficacy, persistence and storability of conidia and submerged spore formulations of *Beauveria bassiana* and *Metarhizium anisopliae* for use against Coleopteran storage pests

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We assessed the efficacy of eight different mycoinsecticide formulations against *Sitophilus zeamais* (Motschulsky) and *Prostephanus truncatus* (Horn). The formulations were prepared using aerial conidia, submerged spores and submerged conidia of virulent isolates of *Beauveria bassiana* (PPRC-HH) and *Metarhizium anisopliae* (PPRC-EE) obtained from Ethiopia. The efficacy test was conducted on maize grains stored at 30°C and 60–70% RH in the laboratory. Moreover, we assessed the persistence of the formulations after application and their storability at 4°C and 30°C. The results revealed that talcum and conidia-based dustable powder formulations of PPRC-HH and PPRC-EE were highly effective (efficacy = 52–100 %) against both test insects. For these formulations, mortality was 40–99 % at 5 days after treatment. Compared to the control, emergence of progeny was reduced by 63–96%, damage by 43–65% and weight loss by 57–85% for these treatments. Conidia: talcum: milk: molasses-based formulation of PPRC-EE also showed efficacy in the range of 44–81%. In contrast, formulations based on submerged spores/conidia of both strains showed low efficacy (< 40%). All formulations persisted for up to 5 months after application at varying levels of efficacy. Furthermore, conidia: talcum-based formulation of PPRC-HH when stored at both 4°C and 30°C maintained a high level of viability and efficacy (80–100%) for a period of 5 months . Storage of the remaining formulations was possible at both temperatures for up to four months. However, the decline in viability and efficacy occurred at a higher rate when stored at 30°C. Further field trails using the most effective formulation are suggested.

Key words: conidia, formulation, entomopathogenic fungi, storage pest, submerged conidia, submerged spores

Introduction

After harvest, cereal crops and commodities from cereals frequently host insect pests that can cause severe economic damage (Storey 1983, Dick 1988, Sinha and Sinha 1992). Of these, *Sitophilus zeamais* (Motsch.) (Coleoptera: Curculionidae) and *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) are the most important and destructive storage pests in the tropics, causing both high quantitative and qualitative damage to cereals (Demianyk and Sinha 1987).

The prevailing control measures for stored product insect pests rely heavily on the use of synthetic insecticides (Rice and Cogburn 1990, Wildey et al. 2002). The development of insecticide-based techniques for protecting grains in small traditional farm stores in Africa has however, been only partially successful on account of the high costs involved or the unavailability of insecticides, and their misuse (Obeng-Ofori and Reichmuth 1997). Moreover, the awareness of the consequences of environmental pollution, possible effects on non-target organisms, insecticide residues in food products and the increasing problem of insect resistance have fostered the development of alternative pest management strategies (Rice and Cogburn 1999, Perez-Mendoza 1999, Rigaux et al. 2001, Crespo et al. 2002, Casaco et al. 2002). Biological control methods are attractive for two reasons: they do not result in chemical residues in the commodity and they are safe and harmless for the environment. Among others, one promising biological control strategy is the use of entomopathogenic fungi and other microbial control agents (Padín et al. 1997). The potential of using fungal pathogens to control insects has been well documented (Müller-Kögler 1965, Burges 1981), and the use of entomopathogenic fungi as control agents against storage pests has been proved in several studies (Searle and Doberski 1984, Adane 1996, Hluchy and Samšináková 1989, Hidalgo et al. 1998, Moino et al. 1998, Rice and Cogburn 1999, Sheeba et al. 2001, Padín et al. 2002, Kassa et al. 2002, Crespo et al. 2002).

The development of effective mycopesticides is dependent on the selection of virulent strains, an adequate mass production and appropriate formulations (Burges 1998). In previous studies, the incorporation of dry aerial conidia powder of *Metarhizium anisopliae* (Metsch.) Sorokin and *Beauveria bassiana* (Bals.) Vuill. has resulted in a satisfactory control of *Acanthoscelides obtectus* (Say), *Sitophilus zeamais* (Motsch.), *Sitophilus granarius* (L.), *Sitophilus oryzae* (L.) *Rhyzopertha dominica* (F.), *Tribolium castaneum* (Herbst) and *Sitotroga cerealella* (Rodríguez and Pratissoli 1990, Adane 1996, Hluchy and Samsinakova. 1989, Rice and Cogburn 1999, Ekesi 2000). The use of dry conidia alone, however, may not

be economically feasible and could potentially cause allergenic hazards (Smith et al. 1999). Therefore, the development of alternative cost effective formulations is necessary. More recently, fat pellets, oil and dustable powder formulations of *B. bassiana* aerial conidia have been tested on *S. zeamais* and *P. truncatus* (Hidalgo et al. 1998, Smith et al. 1999). However, studies on the potential use of submerged spores of *M. anisopliae* and *B. bassiana* for control of storage pests are still rare. Therefore, in the research at hand different dustable powder (**DP**) formulations based on aerial conidia and submerged spores/conidia of virulent *B. bassiana* and *M. anisopliae* isolates obtained from Ethiopia were developed. Furthermore, their efficacy towards *S. zeamais* and *P. truncatus*, as well as their persistence after application and their storage properties were assessed.

Materials and Methods

Insect Rearing

The storage pest species *S. zeamais* and *P. truncatus* were obtained from a continuous rearing culture established at the Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control (BBA), Darmstadt. The two insect strains were reared on maize grains at 30°C and 60-70% RH, following the methodology described in Kassa et al. (2002).

Fungal strains

Beauveria bassiana (isolate PPRC-HH) and *Metarhizium anisopliae* (isolate PPRC-EE) were obtained from the Plant Protection Research Center (PPRC), Ambo, Ethiopia. Previous studies have demonstrated that these two isolates are highly virulent against *S. zeamais* and *P. truncatus* (Kassa et al. 2002). Primary stock and working cultures of the isolates were maintained on Sabouraud dextrose agar slant at -20°C and 5°C, respectively.

Production

Aerial conidia of PPRC-HH were produced using jellified TKI medium as described by Thomas et al. (1987) whereas conidia of PPRC-EE were produced using the standard two-stage solid production system described by Jenkins and Prior (1998). Submerged spores of PPRC-EE were produced in Adámek liquid medium (Adámek 1963) following the methodology described by Stephan and Zimmermann (1998, 2001) and the submerged conidia of PPRC-HH were produced in TKI broth as described by Thomas et al. (1987).

Formulation

Aerial conidia and submerged spores/conidia of PPRC-EE and PPRC-HH were mixed separately with different carriers. A total of eight different formulations were developed and dried either by air, freeze-or spray-drying techniques (Stephan and Zimmermann 1998, 2001). The formulations used, their respective components and their main characteristics are presented in Table 1.

Table 1. List of formulations, their main properties and the amounts used for the efficacy tests (Values in parentheses are SEM).

Formulation (code)	Description of the formulation	MC[a] (%)	Spore conc. (x 10^{10} /g)	CFU[b] (x 10^{10} /g)	Viability (%) after 16 h	Amount used g / kg of maize seeds
F1	PPRC-EE, air dried aerial conidia: Talcum (1:2)	3.37	0.40 (0.03)	127 (0.05)	< 50	5
F2	PPRC-EE, air dried aerial conidia: H-milk: Talcum: Molasses (1:1:2:0.08)	3.78	1.23 (0.12)	638 (0.20)	< 50	26
F3	PPRC-HH, air dried aerial conidia: Talcum (1:2)	3.53	4.09 (0.51)	3.50 (0.54)	94.43 (0.61)	8
F4	PPRC-HH, air dried aerial conidia: H-milk: Talcum: Molasses (1:1:2:0,08)	3.36	3.19 (0.02)	0. 83 (0.17)	84.83 (0.25)	10
F5	PPRC-HH spray dried submerged conidia :skim milk powder: Molasses (1:1:0,08)	4.13	3.51 (0.28)	1.65 (0.82)	95.23 (0.41)	10
F6	PPRC-HH, freeze dried submerged conidia: skimmed milk powder: glycerol (1:1:0,08)	5.76	3.67 (0.32)	1.35 (0.76)	74.4 (0.99)	10
F7	PPRC-HH, air dried submerged conidia: H-milk: Talcum: molasses (1:1:2:0,08)	3.37	3.57 (0.34)	0.40 (0.16)	94.83 (0.27)	10
F8	PPRC-EE, air dried submerged spores: H-milk: Talcum: molasses (1:1:2:0,08)	3.65	0.42 (0.26)	1.86 (0.93)	96.23 (0.43)	76
Control-1	Blank formulation, H-milk: skimmed milk powder: molasses) (1:2:0.08)	-	-	-	-	10
Control-2	Blank formulation (talcum alone)	-	-	-	-	5
Control-3	Untreated control	-	-	-	-	0

Except F1, which was applied at 2 x 10^7/g of maize seeds, all the other formulations were used at 3.2 x 10^8/g of maize seeds. The amount applied per kg of maize seeds varies depending on the spore concentration of the formulation.

[a] MC, Moisture content;

[b] CFU, Colony forming unit.

Efficacy test

Untreated yellow maize grains (variety unknown) were used as a test substrate for rearing *S. zeamais* and *P. truncatus*. The moisture content of the grains was adjusted to 12.5% and 30 g

of cleaned and disinfected maize grains were dispensed into a glass jar (250-ml capacity). A total of 40 glass jars were prepared and maintained for 24 h at 27 ± 2°C for acclimatization. Thereafter, the different formulations were applied at a rate of 3 x 10^8 spores per gram of maize grains. F1 was used at 2 x 10^7 spores per gram of grains on account of a lack of conidia. The treated maize grains were tumbled for 5 min by hand and stored for 24 h at 27 ± 2°C. Unsexed adults of *S. zeamais* (20 per replicate) and of *P. truncatus* (10 per replicate) were then released into each jar containing the treated maize grains. All test insects were of mixed age (1–3 weeks of age). Samples were maintained at 30°C and 60–70% RH which is the optimum for the growth and development of the test insects. Three groups of controls were set up (Table 1), and all treatments were run with four replications.

The adult insects were examined for mortality at intervals of 5 days. Dead insects were removed at each assessment date and all remaining adult insects were removed after 25 days. Cadavers were washed at least three times with sterile deionized water and placed in a humidity chamber and incubated at 25°C until outgrowth of the fungus. Grains were maintained for 60 days under the conditions described previously for F_1 progeny emergence, which were counted at 45 and 60 days after the first infestation time. Grains were sieved to remove insect debris, grain powder, and components of the formulation. Damaged and undamaged grains were separated, counted and weighed. From this data, percent damaged seeds and percent weight losses were derived. Percent weight loss (L) due to insect feeding was calculated using the count and weight method described as $L = [(N_d W_a – N_a W_d)/ W_a(N_d + N_a)] x100$ (Boxall 1986), where N_d is the number of damaged grains, N_a the number of undamaged grains, W_a the weight of undamaged grains, and W_d the weight of damaged grains, respectively.

Persistence

The persistence of the formulations after mixing with the grains was assessed in a separate experiment. One gram of crumbled maize seeds was placed in a small Plexi-glass container (2 cm^3) and treated with 3 x 10^8 spores of each formulation. Untreated crumbled maize seeds were used as controls. Samples were stored at 30°C and 60–70% RH. This experiment was replicated four times for all treatments. Four treated samples were taken from each treatment at intervals of 30 days during a period of 5 months and each was infested with 10 adults of *S. zeamais*. Handling and assessment of mortality was carried out following the methodology described previously.

Storability

To assess the storability, 0.5 g of each formulation was placed in 10 small plastic cups (1.5 ml capacity). The cups were divided into two groups (each contained 5 cups) and maintained in oxygen deprived glass jars (Schott, Germany, Duran 100 ml), containing silica gel. The glass jars were then stored at 4°C and at 30°C. Samples of the different formulations were collected for each storage temperature regime during a period of 5 months at intervals of 30 days, and the viability and efficacy of the formulations against *S. zeamais* were assessed. The efficacy test was set up following the methods described in the persistence study. All treatments were replicated three times. The viability was determined by suspending 0.1 g of each product in 10ml sterile demineralised water. A dilution of 1:1000 was prepared and 36 µl of the suspension was plated on malt extract-peptone-agar using spiral plating equipment (Spiral systems Inc., USA). The number of colony forming units was counted three days after incubation at 25°C. The experiment was replicated three times.

Data analysis

In order to homogenize the variances, all data was transformed into either log or arcsine scales. One-way and repeated-measures ANOVA were used to assess the effect of formulations, time of exposure and their interaction on the mortality of the insects tested (PROC GLM, SAS 1989). Data as to progeny emergence, percentage damage, weight loss and viability was analyzed using the one-way ANOVA procedure (PROC GLM, SAS 1989). When appropriate, the treatment means were separated using either the Student-Newman-Keuls (SNK) test or the Waller-Duncan K-ratio *t* Test (SAS, 1989).

Results

Efficacy

The mortality of *S. zeamais* differed significantly between formulations ($F = 74.43$; df = 8; $P < 0.0001$), time (Roy's Greatest Root = 3.68; $F = 26.71$; df = 4, 29; $P < 0.0001$), and formulation by time interaction (Roy's Greatest Root = 0.76; $F = 3.03$; df = 8, 32; $P = 0.0119$). The formulation F3 achieved the highest (99%) mortality 5 days after exposure followed by F1 and F2 (Table 2). All other formulations achieved a mortality of less than 12%, 5 days after exposure and this remained less than 50% even 25 days after exposure. Moreover, these results did not significantly differ from the control (Table 2). For all formulations, the mortality of *S. zeamais* increased with increasing exposure time, and this

effect was higher for F3 followed by F1 and F2. The resulting median survival time was less than 5 days for F3, 15 ± 1.03 days for F1 and 17 ± 1.00 days for F2. Due to the low mortality (< 50%), the median survival time was not calculated for the other formulations.

Table 2. Mean mortality (%) of *S. zeamais* exposed to maize grains treated with different *B. bassiana* and *M. anisopliae* based dustable powder (DP) formulations at different time intervals (Means ± SEM; N = 4).

Formulation	Percent mortality days after exposure				
	5	10	15	20	25
F1	39.79 (4.52)b	44.87 (2.87)b	49.99 (1.77)b	60.14 (2.79)b	62.52 (1.05)b
F2	24.34 (1.14)c	42.57 (6.91)b	46.45 (7.77)b	50.39 (10.3)b	55.46 (8.37)bc
F3	98.75 (1.25)a	100.00(0.00)a	100.00(0.00)a	100.00 (0.00)a	100.00 (0.00)a
F4	7.32 (3.09)de	11.01 (5.03)c	19.64 (3.79)c	23.93 (2.09)c	30.83 (3.63)cd
F5	2.63 (1.52)de	6.38 (1.21)c	6,38 (3.13)c	6.38 (3.13)d	19.28 (7.21)d
F6	11.69 (5.14)d	11.69 (5.14)c	20.44 (8.25)c	28.16 (6.22)c	36.10 (4.79)cd
F7	9.89 (2.75)d	14.96 (1.87)c	21.28 (6.67)c	25.16 (4.82)c	33.86 (6.76)cd
F8	2.71 (1.57)de	5.34 (2.27)c	9.29 (3.45)c	10.61 (3.84)cd	14.50 (3.56)d
Control	0.86 (0.57)e	4.72 (1.35)c	12.38 (3.03)c	18.66 (4.12)cd	25.26 (4.99)d
F-Value	75.57	50.01	40.30	36.47	27.60
P- value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Analyses were made on arcsine √ percentage transformed data.
Means within the same column followed by different letters are significantly different (SNK test, One-way ANOVA, PROC GLM). Repeated measure analysis of variance: formulations (F = 74.43; df = 8; $P < 0.0001$); time (Roy's Greatest Root = 3.68; F = 26.71; df = 4, 29; $P < 0.0001$) and formulation by time interaction (Roy's Greatest Root = 0.76; F = 3.03; df = 8, 32; P = 0.0119).

The mortality of *P. truncatus* followed patterns that were comparable to *S. zeamais* (Table 3), significantly differing between formulations (F = 19.47; df = 8; $P < 0.0001$), time (Roy's Greatest Root = 3.47; F = 20.80; df = 4, 24; $P < 0.0001$), and formulation by time interactions (Roy's Greatest Root = 0.85; F = 2.88; df = 8, 27; P = 0.0187). The highest mortality of *P. truncatus* occurred 5 days after exposure in F3 treated grains and reached 100% after 10 days (Table 3), resulting in a median survival time of 6 ± 0.30 days. The mortality of *P. truncatus* in F1 and F2 treatments ranged from 56 to 63% on day 5 and increased with increasing exposure time without significant differences. After 25 days of exposure, F1 and F2 achieved a mortality level comparable to F3 (Table 3). For F1 and F2, the median survival time of *P. truncatus* was 8 ± 0.72 and 12 ± 1.56 days, respectively. In all other formulations, the mortality of *P. truncatus* increased with increasing exposure time but occurred at a lower rate; not exceeding 50% after 25 days and not differing significantly from the control (Table 3).

Regardless of the formulations tested, the observed mortality was higher and faster in *P. truncatus* than in *S. zeamais*.

Table 3. Mean mortality (%) of *P. truncatus* exposed to maize grain treated with different *B. bassiana* and *M. anisopliae* based DP formulations at different time intervals (Means ± SEM; N = 4).

	Percent mortality days after exposure				
Formulation	5	10	15	20	25
F1	62.78 (7.36)b	70.56 (10.08)b	78.61 (9.9)b	78.61 (9.87)b	78.61 (9.87)a
F2	55.69 (10.02)b	58.47 (9.34)bc	67.15 (11.83)cb	69.93 (12.94)cb	83.54 (6.98)a
F3	82.50 (11.09)a	100.00 (0.00)a	100.00 (0.00)a	100.00 (0.00)a	100.00 (0.00)a
F4	8.33 (5.32)c	17.36 (7.38)ed	31.94 (13.07)cd	31.94 (13.07)cd	37.50 (11.16)b
F5	14.19 (6.15)c	21.34 (4.61)ed	30.98 (6.89)cd	43.13 (10.88)cd	48.75 (9.67)b
F6	11.31 (3.81)c	11.31 (3.81)ed	14.88 (5.86)d	14.88 (5.86)d	22.02 (9.09)b
F7	18.13 (4.49)c	31.25 (5.16)cd	33.75 (6.89)cd	33.75 (6.89)cd	38.75 (9.66)b
F8	4.17 (4.17)c	9.72 (3.49)ed	30.16 (4.98)cd	35.71 (5.13)cd	43.45 (7.54)b
Control	3.18 (3.17)c	4.76 (4.75)e	12.06 (4.40)d	15.08 (4.65)d	20.79 (5.51)b
F-Value	17.21	22.44	15.62	12.79	11.28
P- value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Analysis was made on arcsine √ percentage transformed data
Means within the same column followed by different letters are significantly different (SNK test, One-way ANOVA, PROC GLM). Repeated measure analysis of variance: formulations (F = 19.47; df = 8; P < 0.0001); time (Roy's Greatest Root = 3.47; F = 20.80; df = 4, 24; P < 0.0001); formulation by time interaction (Roy's Greatest Root = 0.85; F = 2.88; df = 8, 27; P < 0.0187).

Progeny emergence

Both, *S. zeamais* and *P. truncatus* produced progeny on untreated and treated maize grains. However, progeny production of each pest species varied among the different treatments (Table 4). The progeny data of *S. zeamais* revealed significant differences among the formulations tested (F = 7.16; df = 8; P < 0.0001). A significantly lower number of progeny emerged on F3 and F1 treated maize grains. A higher number of *S. zeamais* progeny emerged on F5 followed by F4 treated maize grains. The progeny production on the remaining dustable powder formulations ranged from 29 to 41 in number and did thus not significantly differ from the control (Table 4). The progeny of *P. truncatus* also varied among the formulations (F = 4.21; df = 8; P = 0.0016) (Table 4). A significantly lower number of progeny emerged on F3 and F2 treated grains followed by maize grains treated with the F1 formulation. The progeny production in all other formulations did not significantly differ from the control (Table 4).

Grain damage

Grain damage and weight loss caused by the combined effect of *S. zeamais* and *P. truncatus* varied among the formulations ($F = 4.99$; df = 8; $P = 0.0006$; for grain damage and $F = 3.22$; df = 8; $P = 0.0096$; for weight loss) (Table 4). Significantly less grain damage and weight loss were observed in maize grains that were treated with F3 (Table 4), followed by the F1 and F2 treatments. Maize grains treated with F5 showed a significantly higher damage and weight loss compared to the control. For the other treatments, grain damage and weight loss ranged from 28 to 47% and 10 to 13%, respectively, and did not significantly differ from the control (Table 4).

Table 4. Mean number of F_1 progeny emerged, grain damage (%) and weight loss (%) in maize grains treated with different *B. bassiana* and *M. anisopliae* based DP formulations (Means ± SEM; N = 4).

Formulation	Number of F_1 progeny emerged[1]			
	S. zeamais	*P. truncatus*	Grain damage (%)	Weight loss (%)
F1	12.50 (3.62)d	7.50(7.17)bc	20.35 (2.79)de	5.10 (1.90)bc
F2	30.00 (13.42)cd	3.00 (0.71)c	28.14 (8.02)cde	6.99 (2.15)bc
F3	0.25 (0.25)e	0.75 (0.48)c	12.32 (2.94)e	1.73 (0.83)c
F4	58.00 (15.79)ab	11.25 (4.57)abc	47.08 (10.04)ab	10.55 (2.88)ab
F5	65.50 (5.52)a	30.50 (8.66)a	60.15 (5.48)a	18.97 (2.98)a
F6	40.50 (12.23)abc	26.75 (10.90)a	41.63 (5.19)abc	12.62 (1.55)ab
F7	28.75 (4.05)bcd	21.00 (7.52)ab	32.61 (2.63)bcd	9.30 (1.35)ab
F8	32.50 (6.96)bc	29.00 (9.41)a	35.95 (5.94)bcd	11.51 (4.35)ab
Control	33.50 (4.69)bc	17.42 (2.02)ab	39.59 (4.49)bc	11.80 (2.12)ab

[1] Analyses were made on square-root transformed data.
Means within the same column followed by different letters are significantly different (Waller-Duncan K-ratio t Test, One-way ANOVA, PROC GLM).
Progeny emergence: S. zeamais ($F = 7.16$; df = 8; $P < 0.0001$); *P. truncatus* ($F = 4.21$; df = 8; $P = 0.0016$). Damage ($F = 4.99$; df = 8; $P = 0.0006$) and weight loss ($F = 3.22$; df = 8; $P < 0.0096$).

Persistence

The persistence of the formulations on treated maize grains reduced significantly with increasing storage duration at 30°C and 60-70 % RH (Fig. 1). The mortality of *S. zeamais* varied significantly among the formulations tested ($F = 12.02$; df = 7; $P < 0.0001$), the storage duration after application ($F = 57.17$; df = 5; $P < 0.0001$) and their interaction ($F = 5.46$; df = 35; $P < 0.0001$). The formulations also significantly differed in their speed of action (Roy's

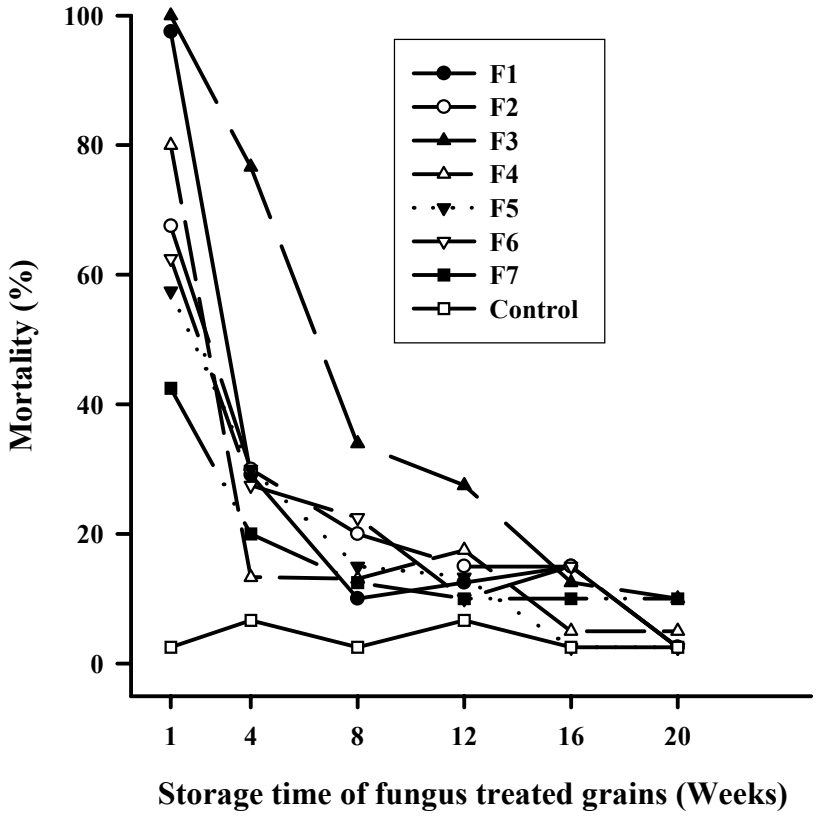

Fig. 1. Mean mortality (%) of *S. zeamais* exposed to grains treated with different DP formulations of *B. bassiana* and *M. anisopliae* after storage at 30°C and 60–70% RH.

Greatest Root = 2.39; $F = 106.74$; df = 3, 134; $P < 0.0001$), the formulation x speed of action (Roy's Greatest Root = 0.19; $F = 3.87$; df = 7, 136; $P < 0.0007$), the speed of action by storage duration (Roy's Greatest Root = 0.99; $F = 26.96$; df = 5, 136; $P < 0.0001$) and by the speed of action x formulation x storage duration (Roy's Greatest Root = 0.57; $F = 2.21$; df = 35, 136; $P = 0.0007$). In all formulations, mortality increased with increased exposure period from 5 days to 20 days. For the first 12 weeks of storage after treatment, F3 exhibited high and fast mortality, whereas the same formulation resulted in a reduced mortality and slow activity after 16 weeks of storage at 30°C. All other formulations resulted in a higher mortality after the first week of storage of treated grains. . Although variations among these formulations are significant, the mortality observed after 4, 8 and 12 weeks of storage was reduced to less than 40% (Fig. 1). After 16 and 20 weeks storage of treated grains at 30°C, all formulations showed a similar and reduced efficacy. The mortality in the untreated control remained lower than 7% throughout the study period.

Storability

The viability of all formulations was significantly affected by increasing storage duration for both temperatures tested (Figs. 2a-b). The CFU g^{-1} observed after different storage durations differed significantly among formulations (F = 147.76; df = 6; P <.0001), storage duration (F = 342.91; df = 4; P <.0001), temperature (F = 67.51; df = 1; P <.0001), formulation x storage duration interaction (F = 7.67; df = 24; P <.0001), formulation x temperature interaction (F= 8.08; df = 6; P <.0001) and formulation x storage duration x temperature interaction (F = 5.20; df = 17; P <.0001). However, the interaction effect between storage duration and temperature was not significant (F= 1.34; df = 3; P = 0.2632). Significant reductions in the viability of conidia and submerged spore formulations occurred two months after storage at 30°C, whereas at 4°C, a significant reduction in the viability occurred after three months of storage (Figs. 2a-b). After 4 months (120 days) at 4°C, F3 and F5 maintained a significantly higher number of CFU g^{-1}, followed by F6, F7, and F1. F2 and F4 suffered high losses of viability throughout the storage period (Fig. 2a). A similar trend was observed after 4 months of storage at 30°C; however, differences among F5, F6, F7 and F1 were not significant (Fig. 2b). F2 and F4 showed a higher loss of viability (> 95%) than that experienced at 4°C storage (82-86%) (Fig. 2b). At a higher temperature, increasing the storage duration to 5 months (150 days) caused a significant loss in the viability of most of the formulations (Fig. 2b). A significantly higher survival rate after 5 months of storage was observed for F3 (Fig. 2b).

The mortality of *S. zeamais* differed significantly among the formulations, storage temperatures, storage durations and time of assessments (Table 5). With the exception of the time of assessment by temperature interaction, most of the other interactions also revealed significant differences in the mortality of *S. zeamais* (Table 5). F3 maintained a significantly higher efficacy during storage at both 4°C and 30°C for 4 to 5 months (Figs. 2c-d), followed by F1, F2 and F4. However, the mortality in these three formulations reduced significantly with increasing age of the formulations as well as with increasing storage temperatures (Figs. 2c-d). The other formulations caused a mortality of less than 40% one month after storage, which then decreased significantly with increasing storage time and temperature (Figs. 2c-d). The time at which mortality of *S. zeamais* occurred also differed among the formulations and storage duration. For F3, median survival time was < 5 days for the first three months which increased to 5-10 days after a 4 to 5 months storage period at both 4°C and 30°C. In contrast F1 showed a median survival time of 5-10 days for the first 1 to 2 months compared to > 15

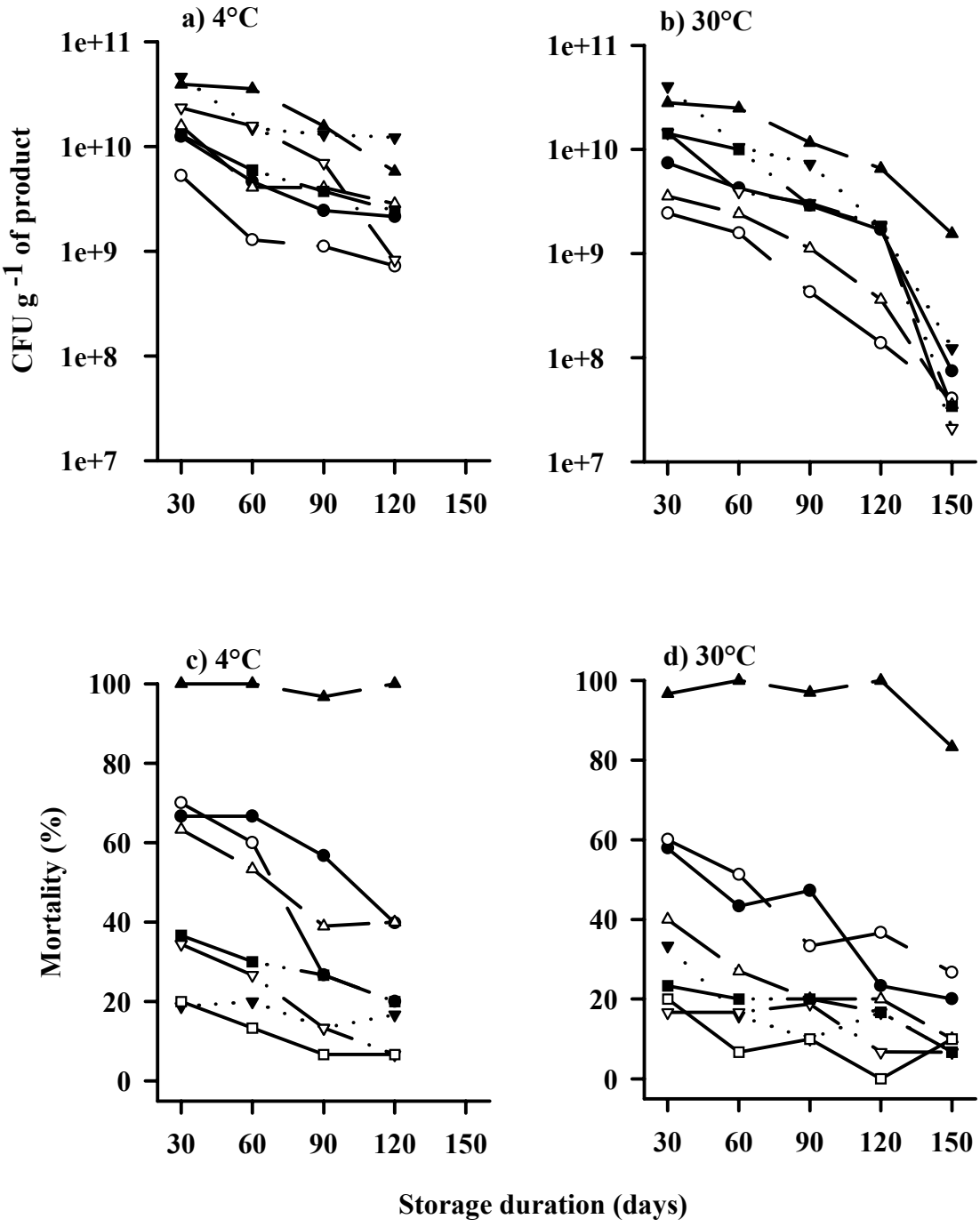

Fig. 2. **Mean viability and efficacy of different DP formulations after storage at 4°C and 30°C. (a) and (b) viable CFU g^{-1} at 4°C and 30°C, respectively; (c) and (d) mean mortality (%) of *S. zeamais* after storage of formulations at 4°C and 30°C, respectively (□) Control (●)F1, (○)F2, (▲) F3, (△) F4 , (▼) F5 , (▽) F6 and (■) F7.**

days to the time afterwards at both storage temperatures. The other remaining formulations which had been stored at 4°C and at 30°C caused either a mortality of less than 50% or the median survival time exceeded 15 days for both storage temperatures.

Table 5. Output for repeated measures ANOVA for mortality of *S. zeamais* treated with different DP formulations stored at different temperatures for 5 months.

Source	DF (Num, Den)	RGR	*F*- value	*P*-value
DP Formulation (DPF)	7	-	151.28	< 0.0001
Temperature (T)	1	-	5.81	0.0172
Storage duration (SD)	4	-	21.47	< 0.0001
Time of assessment (TA)	2,143	3.13	223.82	< 0.0001
DPF * T	7	-	1.10	0.366
TA *DPF	7,144	0.66	13.66	< 0.0001
TA* T	2,143	0.03	2.27	0.1068
TA* SD	4,144	0.19	7.12	< 0.0001
TA* DPF*T	7,144	0.13	2.64	0.0134
TA* DPF*SD	28,144	0.94	4.82	< 0.0001
TA*T*SD	3,144	0.03	1.32	0.2697
TA*DPF*T*SD	21,144	0.29	1.97	0.0108

Greenhouse-Geisser Epsilon = 0.8494, Num:- Numerator and Den:- Denominator , RGR:- Roy's Greatest Root

Discussion

Mycopesticide formulations based on *Beauveria bassiana* and *Metarhizium anisopliae* can be used effectively for the management of storage pests. Their efficacy however which was assessed by determining mortality rate, progeny build-up, grain damage and weight loss, varied greatly, depending on the type of formulation and the type of spores used. Our research data demonstrated that talcum-based dustable powder (DP) formulations of conidia of *Beauveria bassiana* (F3) and *Metarhizium anisopliae* (F1) are highly effective against both storage pest insects. The results gained in our research using Ethiopian fungal strains corroborated with those of similar studies carried out on various stored product insect pests (Adane et al. 1996, Padín et al. 1997, Hidalgo et al. 1998, Rice and Cogburn 1999, Ekesi et al. 2000, Padín et al. 2002). The two formulations (F1 and F3) showed superior performance throughout the study period. However, the observed efficacy was relatively lower; and the corresponding progeny emergence, damage and weight loss were relatively higher for F1 than F3. These differences were attributed to the amount of spores/g of substrate used initially rather than due to differences in the efficacy of the two formulations. This has been substantiated by a separate experiment (unpublished data). Aerial conidia of *M. anisopliae* formulated in talcum, H-milk and molasses based carriers (F2) also showed better efficacy

when compared to the same formulation of *B. bassiana* (F4) and all the remaining submerged spore/conidia based formulations. Generally, aerial conidia formulations were more effective as dry powder formulations than the corresponding submerged spore formulations.

With the exception of F2, all aerial and submerged conidia based formulations, which contained H-milk, molasses and /or glycerol resulted in a low efficacy when applied as a dry powder on stored maize grain at 30°C. On the other hand, when the same formulations were applied as a water suspension to *S. zeamais*, they caused 77-95 % mortality within 15 days (unpublished data). This indicates that the components of a given formulation significantly influence the inherent biological efficacy of aerial and submerged conidia. The reason might be that some components of the formulation have an impact on the adhesion of the spores to the cuticle, the spore distribution, and alterations in the enzymes produced by the fungus, such as chitinase, chyemolestase, chymotrypsin, and estrase, which degrade the cuticle of the host insect during penetration. These enzymes are considered to be an essential prerequisite for successful fungal infection (Samšináková et al. 1971, Charnley 1984, Gupta et al. 1984, St. Leger et al. 1988). Furthermore the drying process also affects efficacy. Formulations based on air-drying systems maintained a higher efficacy when compared to spray or freeze dried products. Moreover, additives like milk or molasses may also stimulate the growth and multiplication of other microorganisms, like bacteria or yeasts, which may interfere with the entomopathogenic fungus. An increase in progeny, damage and weight loss was observed in some of the formulations tested, thus indicating that the formulation additives are nutrients for the beetles.

Apart from its efficacy, a mycopesticide formulation should maintain its persistence for some time after application (Burges 1998). All formulations described in our study persisted for up to 5 months and caused not only mortality and but also infected adults of *S. zeamais* to a different degrees. Aerial conidia of *B. bassiana* formulated with talcum alone (F3) maintained good persistence and efficacy up to three months whereas the other formulations showed an efficacy of less than 20% three months after application. We have observed that with increasing age of the treated maize kernels (4 to 5 months), the persistence and speed of activity of the formulations reduced quite substantially. However, one of the advantages of microbial control systems is that disease cycling occurs, and that infected, dead insects increase the amount of available inoculums after sporulation, thus also effectively increasing the persistence of a mycopesticide, which then results in a long-term effect (Thomas et al.

1995, Wood and Thomas 1996, Hidalgo et al. 1998). During the course of our study, dead insects were removed from the grain systems, and we did therefore not observe a secondary recycling process. However, 95-100% of dead insects observed in the different formulations expressed infections through sporulation after incubation in a moist chamber. In all cases the outgrowth of the fungus appeared 24-48 h after incubation, and after 96 h dead insects were covered with masses of spores, indicating the existence of an adequate dose transfer from the treated maize grain. However, storage environments typically maintain dry conditions, and an outgrowth of the fungus from killed insects followed by a sporulation has yet to be examined under these conditions.

An effective mycopesticide formulation should possess adequate storage characteristics (Burges 1998, Smith et al. 1999). Couch and Ignoffo (1981) suggested a minimum shelf life of at least 12-18 months for microbial pesticides. With regard to locusts and grasshoppers, Moore & Caudwell (1997) suggested a shelf life of 3 to 6 months for mycoinsecticides. In our study, conidia of *B. bassiana* in talcum-based DP formulation (F3) maintained a high level of viability (1 x 10^{10} CFU g^{-1}) and efficacy (> 80-100%) for a storage period of 4 to 5 months at both 4°C and 30°C. For this formulation, a reduction in the viability and efficacy was observed only after 5 months of storage at 30°C. Hidalog et al. (1998) also reported 83 % and 91% viability of DP formulation of *B. bassiana* conidia after 45 days of storage at 4°C and 25°C, respectively. These storage characteristics will be perfectly met under African conditions, where in most cases temperatures in pesticide stores exceed 30°C. Moreover, our results also demonstrate that for dry powder formulations of submerged spores a long term storage (up to 4 months) is possible at both 4°C and 30°C, thus corroborating with the findings of Stephan and Zimmermann (1998, 2001). However, we observed a decline in viability and therefore of efficacy at higher rates when formulations were stored at 30°C.
The results also revealed differences in susceptibility among the different test insects, *P. truncatus* being more susceptible to all formulations than *S. zeamais*. Moreover, in most of the formulations, the mortality was higher and faster in *P. truncutus* than in *S. zeamais*. Similar results have been reported previously (Smith et al. 1998, Kassa et al. 2002).

In general, the results obtained in our study indicate that it is possible to achieve a successful level of control for *S. zeamais* and *P. truncatus* on stored and infested maize using DP formulations of conidia of *B. bassiana* and *M. anisopliae*. Recently, the use of mycoinsecticides to control insect pests has become popular and formulations that are stable

and cost-competitive with conventional insecticides are now available (Shah and Goettel 1999). Mycopesticides pose minimal risks to humans, domestic animals and non-target invertebrates compared to chemical insecticides (Siegel and Shadduck 1990, Saik et al. 1990, Ekesi et al. 2000). This is an advantage, especially in developing countries, where the risk of pesticide misuse is a major problem. Thus, our study suggests that the most effective formulations reported here could be a starting point for field application studies and for a further improvement in application strategies. As submerged spore formulations also have a good persistence, it is worth testing them further as possible suspensions or emulsions against other pest insects. The rapid action of some of the formulations indicates that it would be possible to use these formulations as components of an integrated control program against stored product insect pests. The short infection time could enable infected insects to increase the inoculums and secondary recycling. Furthermore *B. bassiana* and *M. anisopliae* are easily amenable to large-scale production without a high input of technology (Ekesi et al. 1998, Prior 1988), implying the feasibility of microbial control in the context of subsistence agriculture in developing countries such as Ethiopia.

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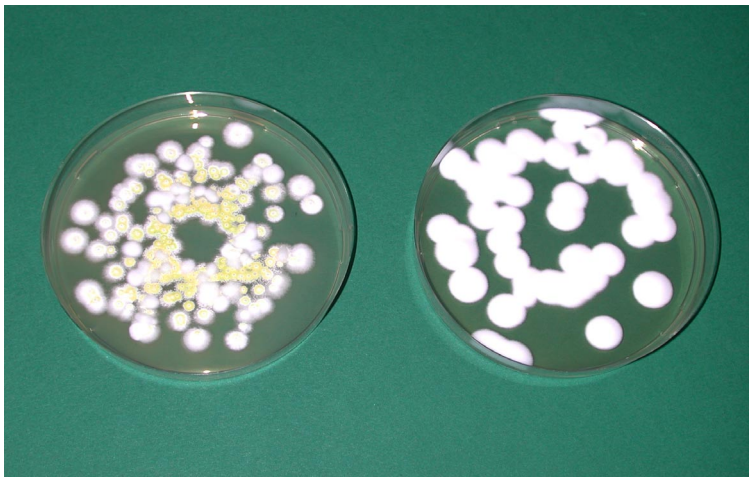


Plate 1. Contaminant free colonies of F1 and F3 DP formulation on MEA.

Plate 2. Maize grain damaged by *S. zeamais* and *P. truncatus*.

Plate 3. Microcosm used for the efficacy test of mycoinsecticide formulations against storage pests.

CHAPTER 9

SUMMARY

Microbial control has recently been recognized as an important component of integrated pest management strategies for field crops and stored product commodities. The successful development of a mycoinsecticide ultimately depends on the availability of a virulent strain, an optimized and economic production system and a suitable formulation to optimise its application, efficacy and storage characteristics as well as the persistence after application. It is clear from analysis and synthesis of the present studies that considerable efforts have been made towards the selection of virulent isolates, the optimisation of the production process, the development of new formulations and testing their efficacy against the intended target insect pests, their persistence and storability under different environmental conditions. Accordingly, it was made possible to use BCAs for two distinct groups of agriculturally important insect pests, namely, field pests and storage insect pests. The field pest group was represented by locusts and grasshoppers (*Locusta migratoria*, *Hieroglyphus daganensis* and *Cryptocatantops haemorrhoidalis*), whereas the storage insect pests were represented by *Sitophilus zeamais* and *Prostephanus truncatus*. Due to the diverse nature of the test insects and their habitat, the methods followed to develop appropriate formulations are different for the two groups, for which the major findings are summarized as follows.

The production and processing study indicates that submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189) can be mass produced in a BH medium (3% biomalt and 1% yeast extract) under specific culturing conditions. Moreover, submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189) can be effectively dried using the additives skimmed milk powder, molasses or glycerol either by freeze-drying or spray-drying technique. Storage at low temperatures (< 10°C) is possible for over 11 months without significant loss in viability. The initial viability of spores remains higher for spray-dried submerged spores. Furthermore, the viability and the efficacy of freeze-dried submerged conidia are significantly reduced when the product was ground to fine powder through a 120 µm mesh.

When aerial conidia, submerged spores (mostly blastospores) and submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189) were tested in different formulations in the laboratory and under field conditions, aerial conidia formulated in diesel oil displayed a higher efficacy against *L. migratoria*, *H. daganensis* and *C. haemorrhoidalis* at a lower application rate of 5 x

10^{12} conidia ha^{-1} than all submerged conidia and submerged spore formulations, which were applied at 1 x 10^{13} conidia ha^{-1}. Freeze or spray dried submerged conidia of *M. anisopliae* var. *acridum* (IMI 330189) formulated in an oil flowable concentrate formulation are equally infectious to *L. migratoria* under laboratory conditions at a higher rate of 1 x 10^{13} spores ha^{-1}.

Submerged spores/conidia of *M. anisopliae* var. *acridum* (IMI 330189) were formulated as emulsions, as an oil flowable concentrate or as water-based formulations, out of which an oil flowable concentrate formulation revealed good biocontrol potential under laboratory and field conditions followed by emulsions. Under the conditions of the present research work and under the harsh environmental conditions that occur in the Sahel, water-based formulations of submerged spores are not suitable for grasshopper control using ULV applications. It was also observed that insects varied in their susceptibility to mycoinsecticide formulations, *C. haemorrhoidalis* being more susceptible to the mycopathogen than *L. migratoria*. Overall, the study provides evidence on the potential use of submerged conidia of *M. anisopliae* var. *acridum* under Sahelian environmental conditions. However, further improvements on the production process along with the quality of the formulation are indispensable.

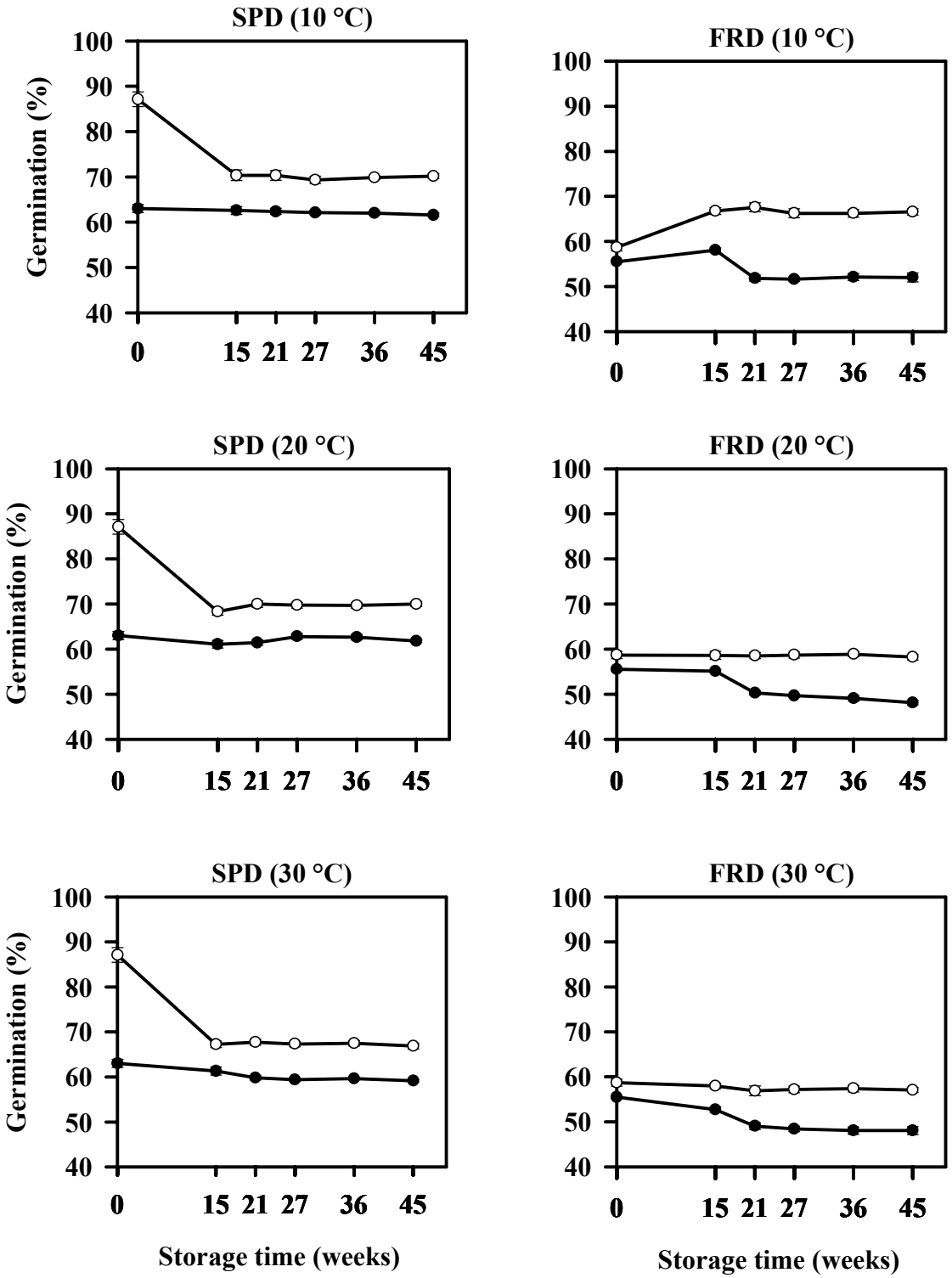
Strain selection according to thermal tolerance may be warranted when choosing an isolate for development as a microbial control agent. The results presented in this study confirm that there is a considerable variability in germination and growth at different temperatures among isolates of *M. anisopliae*, *B. bassiana, B. brongniartii* and *Paecilomyces* sp. The present study further demonstrated the susceptibility of *S. zeamais* and *P. truncatus* to non-specific entomopathogenic fungi indigenous to Ethiopia at varying degrees, *P. truncatus* being more susceptible to all isolates tested than *S. zeamais*. Furthermore, among the tested isolates, conidia and submerged spores/conidia of *M. anisopliae* (PPRC-EE) and *B. bassiana* (PPRC-HH,) appeared to be highly virulent to *S. zeamais* and *P. truncatus*. However, for the same isolates, the observed efficacy of submerged spores/conidia may or may not out way the aerial conidia and is entirely dependent on the type of liquid medium used for the production. The present study also identified one *B. bassiana* isolate (PPRC-HH), which produced an exceptionally higher yield of submerged conidia in TKI medium. Subsequently, *M. anisopliae* isolate (PPRC-EE) and *B. bassiana* isolate (PPRC-HH) were selected and various dustable powder (DP) formulations were developed and tested against stored product insect pests. The results revealed that all the tested formulations were active, (especially at higher concentration of 1 x 10^{8} spores ml^{-1}) when they were applied as a water suspension against *S. zeamais*.

However, they show differences when they were incorporated on maize grains as a dry powder. Generally, DP formulations of aerial conidia were more effective against the two test insects than the corresponding submerged spore/conidia-based formulations. Moreover, aerial conidia-based formulations were more persistent, and could be stored 5 months at 4°C and 30°C than the corresponding submerged spore/conidia formulations. These storage characteristics could perfectly met under African conditions, where in most cases temperature in pesticide stores does not seem to exceed 30°C. Moreover, this study demonstrated that for dry powder formulations of submerged spores, a long term storage (up to 4 months) is possible at both 4°C and 30°C, however, a decline in viability and efficacy was observed at higher rates when the formulations were stored at 30°C for 5 months. This study also indicates the importance of inert carriers for improved efficacy, persistence and storability of DP formulations of *B. bassiana* and *M. anisopliae* spores.

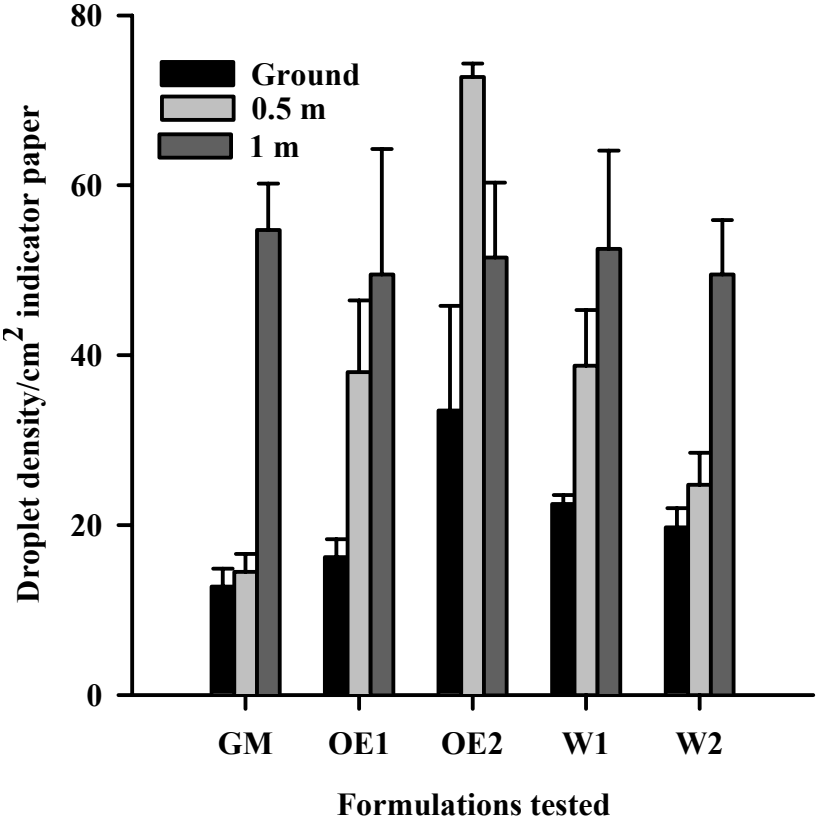
In general, the results of this study magnificently demonstrate that it is possible to achieve a successful level of control for *S. zeamais* and *P. truncatus* on stored and infested cereals using DP formulations of conidia of *B. bassiana* and *M. anisopliae* isolates. This would apparently be advantageous, especially in developing countries, where the risk of pesticide misuse is a major problem. Thus, the most effective formulations reported here could serve as a spring-board for further improvement of field application strategies. Furthermore, the fact that *B. bassiana* and *M. anisopliae* are easily amenable to large-scale production without a high input of technology essentially imply the feasibility of microbial control in the context of subsistence agriculture in developing countries such as Ethiopia. It can be inferred that developments being made now on the horizon would presumably make biological control of insect pests attractive from the practical point of view and also from an economic perspective in Ethiopia. This work could, therefore, be regarded as an important addition towards achieving this goal, as it would certainly shade-light to the sustainable development of more potent BCAs from the untapped beneficial microbial resources of the country.

CHAPTER 10

APPENDIX



Appendix 1. Viability and speed of germination of spray-dried (SPD) and freeze-dried (FRD) submerged spores of *M. anisopliae* var. *acridum* (IMI330189) stored at different temperatures. Speed of germination (●) 8 h and (○) 16 h after incubation at 25°C in darkness. (Bar ± SEM). Supplement for chapter 2.



Appendix 2. Mean number of spray droplets counted on the indicator paper placed at different height from the ground during treatment of *Hieroglyphus daganensis* under field condition in Niger ($p < 0.001$, SNK, Bar ± SEM). Supplement for chapter 3.

Appendix 3 Median survival time of *Locusta migratoria* exposed to the spray residues of different formulations of mycoinsecticides in the laboratory. Supplement for chapter 3.

	Median survival time (Days)			
Formulation	4 DAE**	7 DAE**	10 DAE ns	17 DAE ns
Control	16.76 (0.80)a	16.50 (1.05)a	10.11 (1.66)	17.80 (0.27)
OE1	10.86 (1.54)b	12.12 (1.09)b	12.60 (1.64)	13.80 (1.31)
OE2	11.05 (1.29)b	12.71 (1.15)b	11.82 (2.12)	14.90 (1.97)
AC	9.71 (1.58)c	10.67 (1.29)b	12.33 (1.24)	15.40 (1.72)

** Significant (Log rank test, $p < 0.0001$), ns non-significant (Log rank test, $p = 0.05$,
DAE: Days after exposure

Appendix 4 Mean cumulative mortalities on day 21 among *Hieroglyphus daganensis* collected at 10, 15, and 20 days after treatment of a ha plots with different formulations of *M.anisopliae* var. *acridum* isolate IMI 330189 in East Niger (Mean ± SEM, N = 4). Supplement for chapter 3.

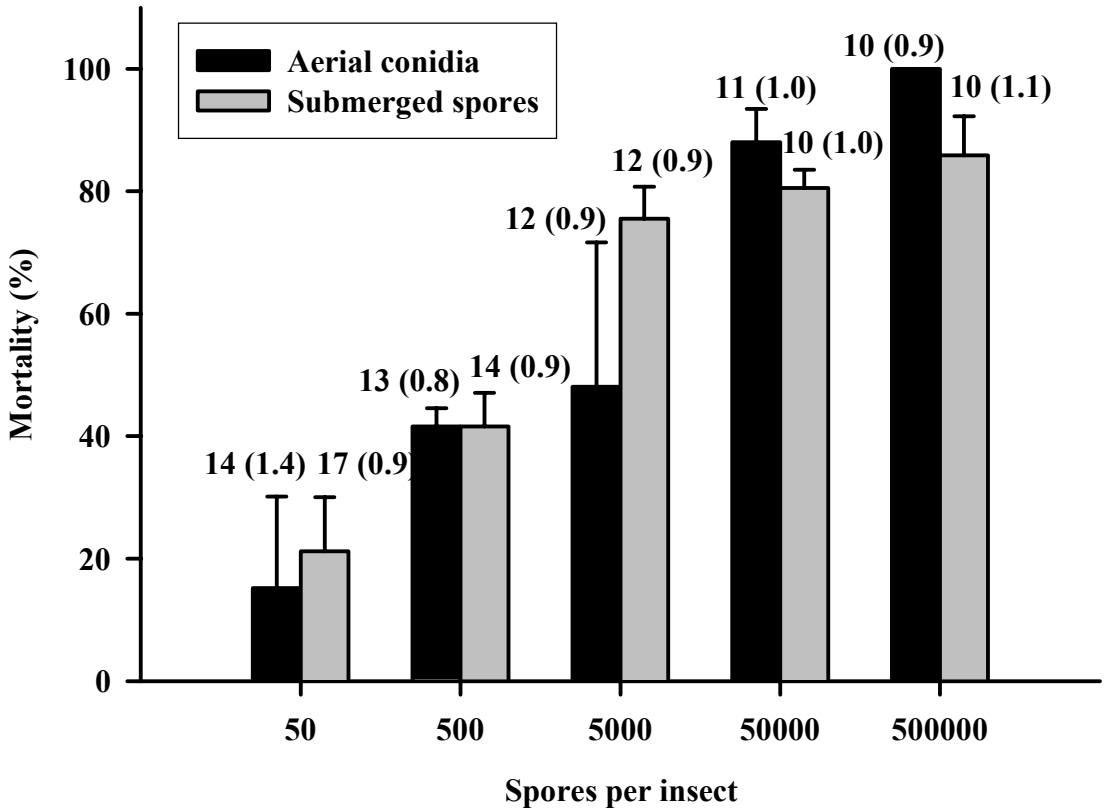
Formulation	Percentage mortality at days after treatment		
	10	15	20
GM	100.00 (1.57) a	100.00 (1.57) a	99.04 (1.52) a
OE1	92.75 (1.39) a	99.14 (1.52) a	98.08 (1.50) a
OE2	96.15 (1.47) a	98.75 (1.51) a	99.12 (1.52) a
W1	93.76 (1.36) a	100.00 (1.57) a	99.04 (1.52) a
W2	91.43 (1.36) a	100.00 (1.57) a	98.81 (1.52) a
Untreated	68.89 (0.98) b	68.89 (0.98) b	68.89 (0.98) b
Orthogonal contrast			
Control vs others Pr >F	0.0001	0.0001	0.0001
GM vs Ss *	0.0829	0.5932	0.9300
Water vs oils **	0.3414	0.679	0.9660
Mineral oil vs veg. oils***	0.1953	0.3347	0.9014
OE1 vs OE2	0.5017	0.8733	0.7986
W1 vs W2	0.982	1.00	0.9494
CV (%)	14.45	7.16	10.07
Type Pr > F	0.0007	0.0001	0.0001

Values followed by the same letter are not significantly different (P = 0.05, Waller grouping).
* Green muscel and the rest formulations of submerged spores.
** Water = W1 and W2; Oil= GM, OE1 and OE2.
*** Mineral oil= GM; Veg.oil= OE1 and OE2.

Appendix 5. Mean cummulative mortality (%) of *Hieroglyphus daganensis* exposed to treated vegetation 7 and 10 days after treatment of a ha plots with different formulations of *M.anisopliae* var. *acridum* isolate IMI 330189 in East Niger (Mean ± SEM, N = 4). Supplement for chapter 3.

Formulation	Percent mortality at days after exposure	
	7	10
GM	99.00 (1.52)	99.00 (1.52)
OE1	96.25 (1.47)	99.12 (1.52)
OE2	99.00 (1.52)	99.00 (1.52)
W1	97.32 (1.49)	96.07 (1.43)
W2	96.67 (1.48)	97.00 (1.45)
Orthogonal contrast		
GM vs others Pr >F	0.7111	0.5961
Water based vs oil based *	0.9453	0.4114
Mineral oil vs veg. oils**	0.7907	0.9860
OE1 vs OE2	0.6467	0.9757
W1 vs W2	0.9247	0.8723
CV (%)	9.87	8.57
Type Pr > F	0.9797	0.7509

Values followed by the same letter are not significantly different (P = 0.05, Waller grouping).
* Water = W1 and W2; Oil= GM, OE1 and OE2. ** Mineral oil= GM, Veg.oil= OE1 and OE2.

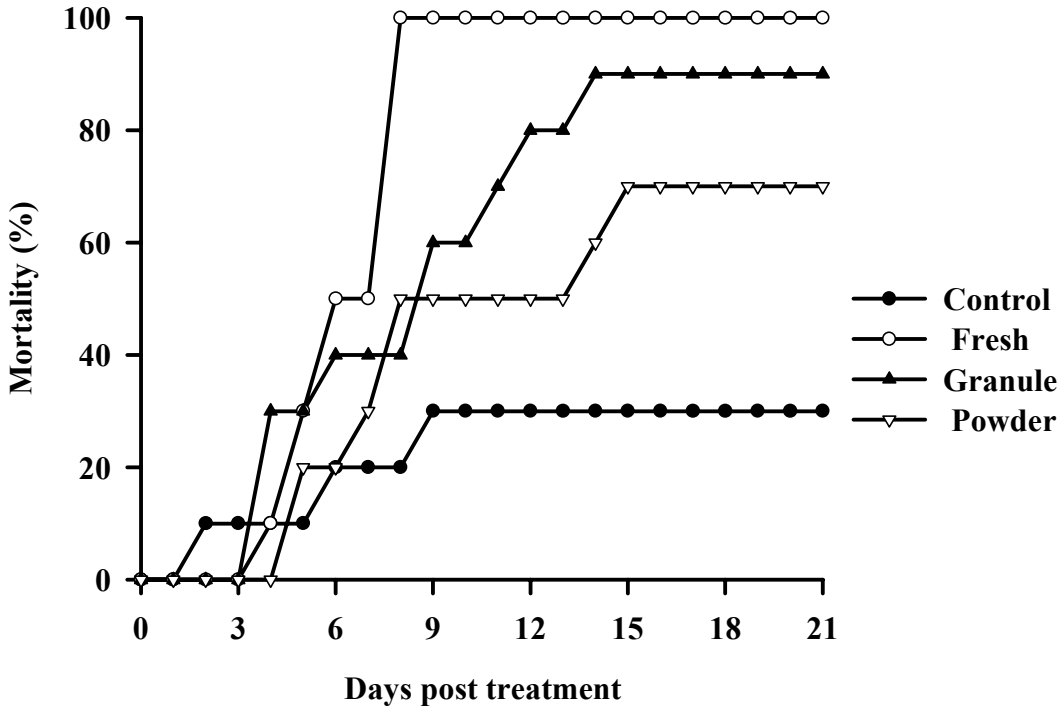


Appendix 6. Mean corrected mortality (%) of *L. migratoria* treated with different concentration of submerged spores and aerial conidia (values on each bar represent MST ± SE). Supplement for chapter 4.

Appendix 7. Efficacy of aerial conidia (Green muscle) and freeze dried submerged spores of *M. anisopliae* var. *acridum* (IMI330189) against third instar larval stages of *Locusta migratoria*. Supplement for chapter 4.

Product	Intercept	Slope	n	$\chi 2$	Log LD_{50}	95 % CL	Log LD_{90}	95 %CL
Submerged spore (Freeze dried)	-1.35 (0.6)	0.5 (0.2)	168	9.6	2.97	0.64-3.89	5.79	4.76-9.21
Green Muscle	-2.75 (1.2)	0.8 (0.3)	164	8.4	3.36	1.24-4.01	4.93	4.30- 6.70

P = 0.01, CL, Confident limits, LD lethal dose



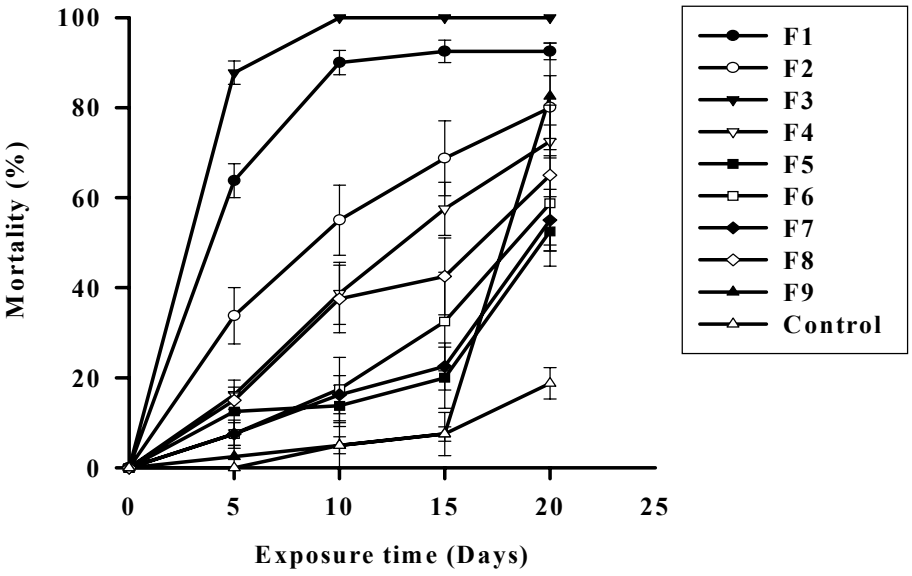
Appendix 8. Cumulative mortality (%) of third instar larvae of *Locusta migratoria* **after treatment with different products of submerged spores of** *M. anisopliae* var. *acridum* **(IMI 330189) in the laboratory. (Log-rank test, χ^2= 16.04, DF = 3 p < 0.001). Supplement for chapter 4.**

Appendix 9. Mean number of spray droplets counted on the indicator paper placed inside and outside of the cages during laboratory spray experiments on *Locusta migratoria*. Supplement for chapter 4.

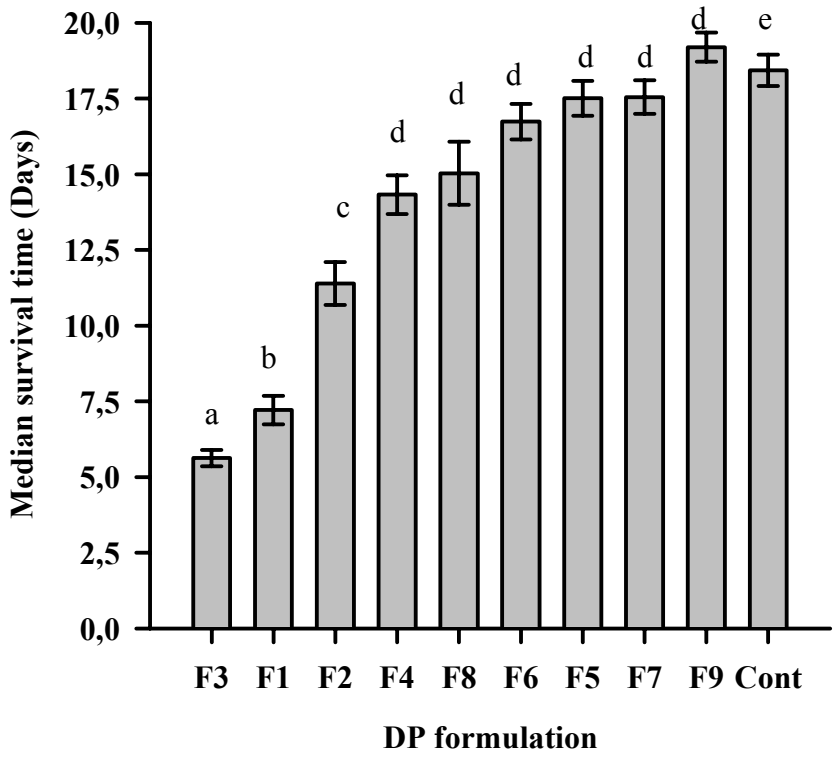
Formulation	Number of spray droplets /cm^2 indicator paper	
	Inside cage	Out side cage
Green Muscle	150 (24.0)	153 (6.0)
SD-OFC2	150 (16.0)	140 (4.0)
SD-OFC3	100 (12.0)	117 (6.0)
FD-OFC2	151 (12.0)	127 (10.0)

Apendix 10. Mean number of spray droplets counted on the indicator paper placed inside and outside of the cages during spray experiments on *Locusta migratoria* and *Cryptocatantops haemorrhoidalis* in Niger. (Mean ± SEM). Supplement for chapter 4.

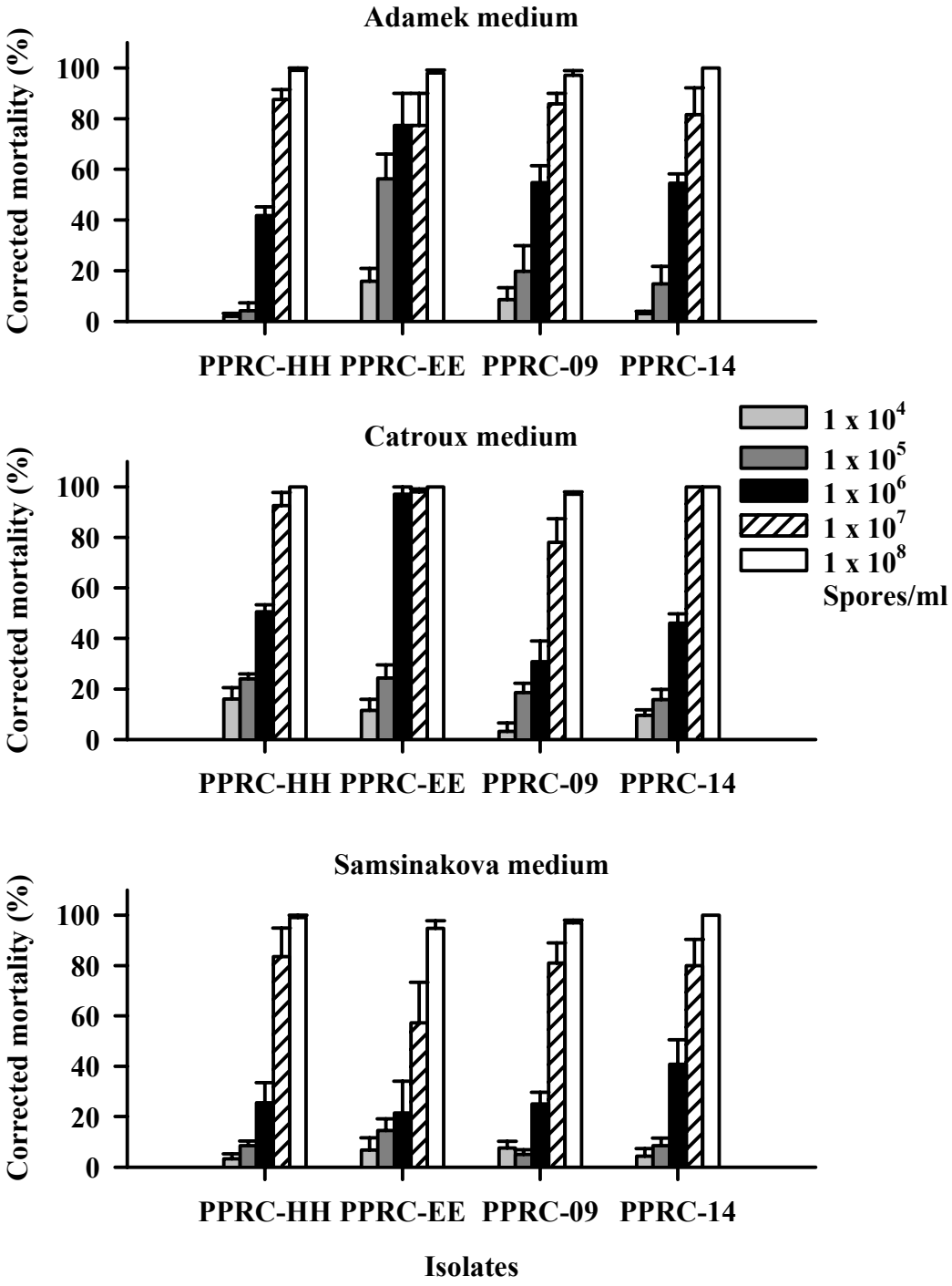
Formulation	Number of spray droplets /cm^2 indicator paper			
	C. haemorrhoidalis		*L. Migratoria*	
	Inside cage	Plant	Inside cage	Plant
GM	113 (4.0)	185 (18.0)	114 (3.0)	97 (9.0)
OFC1	84 (11.0)	90 (9.0)	73 (2.0)	92 (5.0)
OFC2	130 (8.0)	133 (10.0)	121 (6.0)	127 (7.0)
EO4	104 (13.0)	180 (11.0)	136 (8.0)	180 (13.0)
CD	112 (12.0)	192 (11.0)	124 (5.0)	185 (12.0)
OE2	113 (6.0)	186 (11.0)	118 (15.0)	185 (8.0)



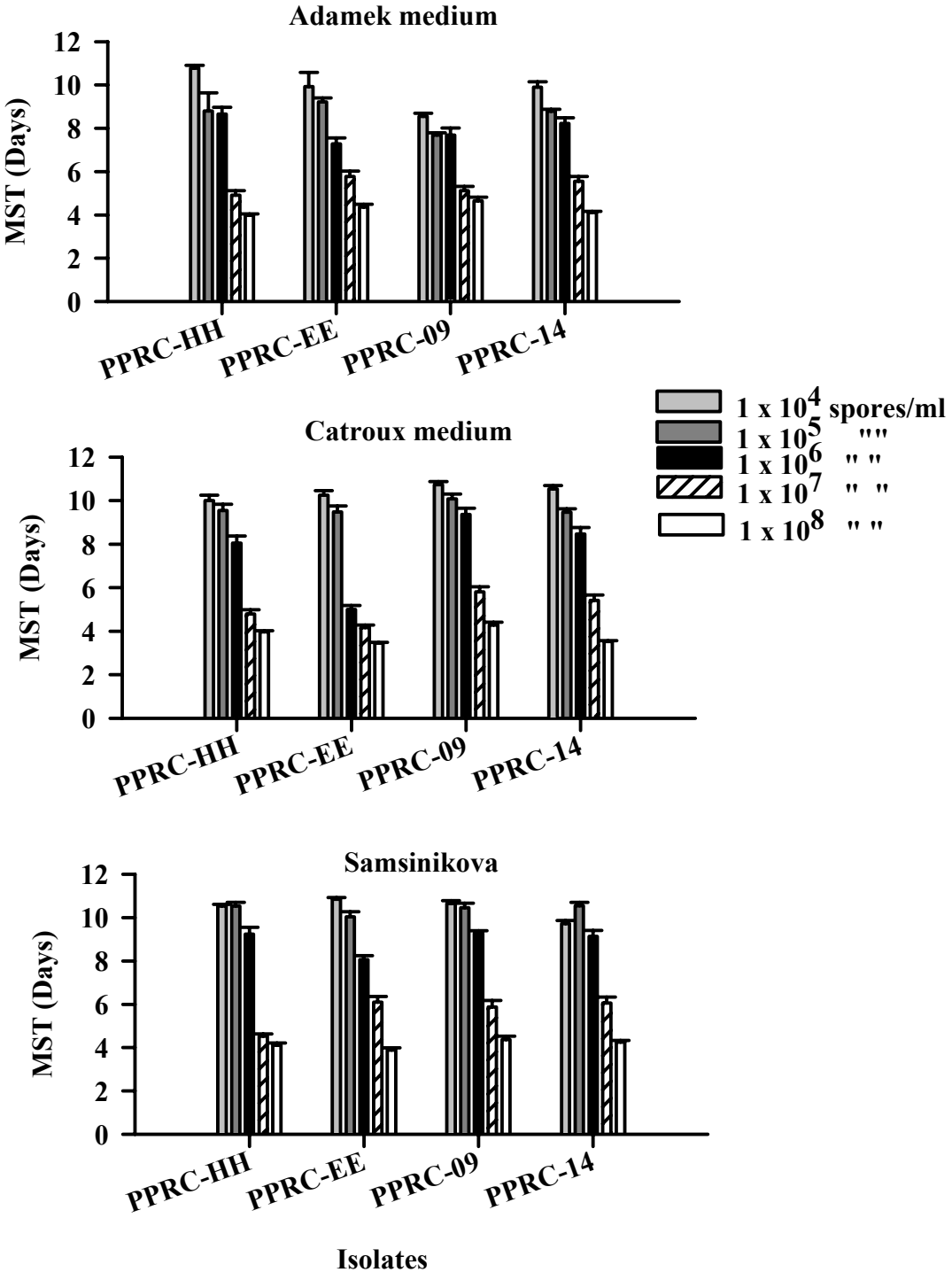
Appendix 11. Mean mortality (%) of *Sitophilus zeamais* exposed to maize grain treated with different DP formulations of *B. bassiana* and *M. anisopliae*. Supplement for chapter 7.



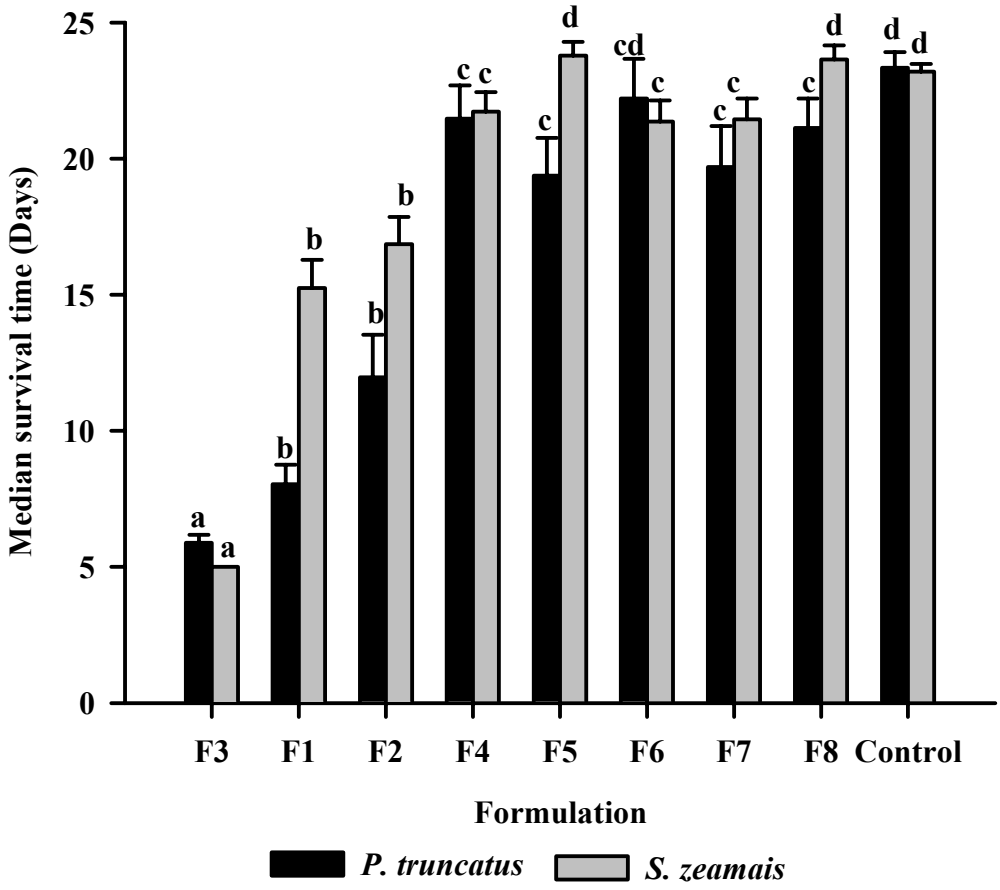
Appendix 12. Median survival time of *Sitophilus zeamais* **treated wiht different** *B. bassiana* **and** *M. anisopliae* **based DP formulations.Bars followed by the same letters are not different in survival curve (Log rank test: $\chi^2 = 344.24$, Df = 9, $p < 0.0001$). Cont: Control. Supplement for chapter 7.**



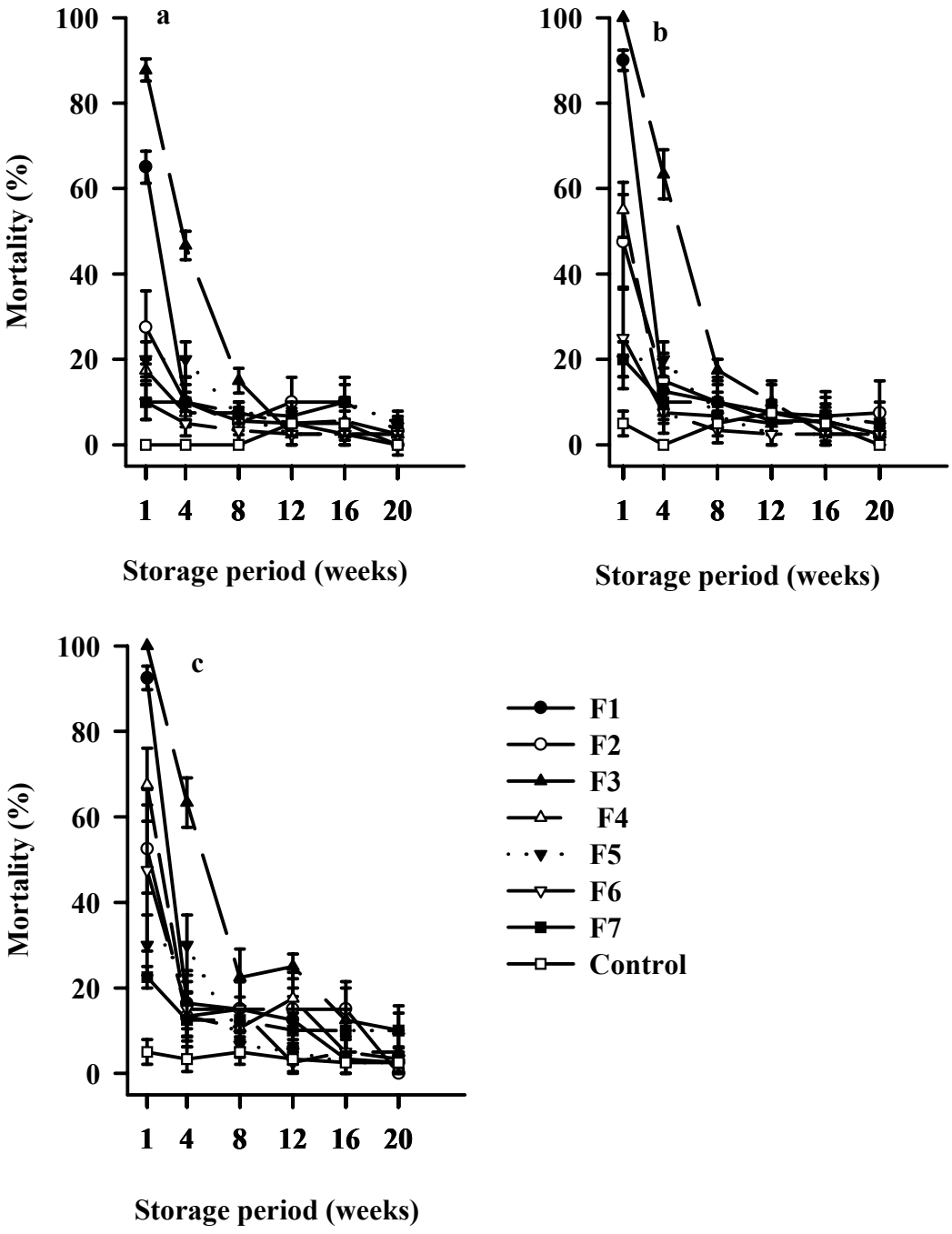
Appendix 13. Corrected cummulative mortality (%) of *Sitophilus zeamais* treated with submerged spores of different isolates produced in different liquid medium.(Bar = SE). Supplement for chapter 7.



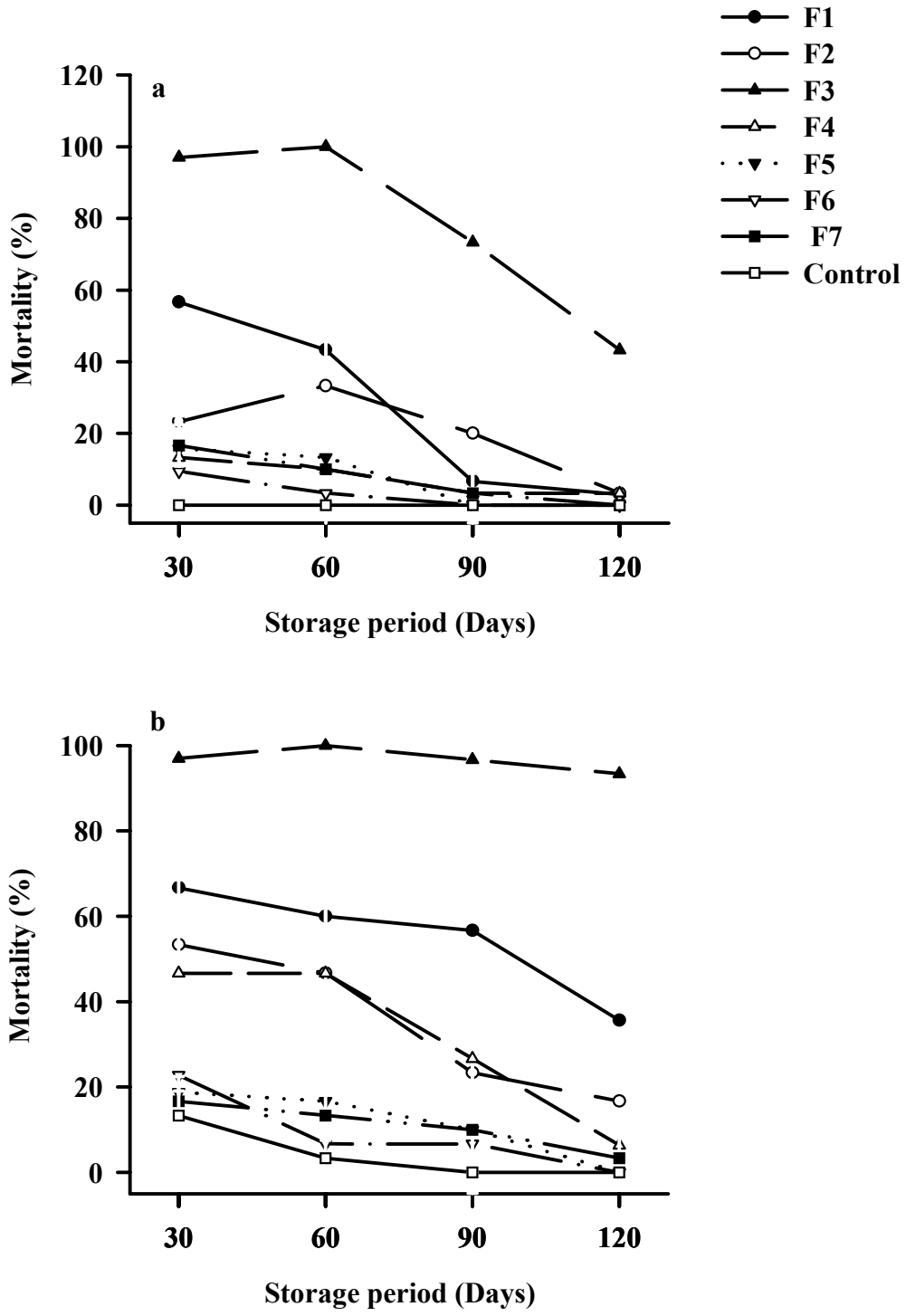
Appendix 14. Median Survival time (MST) of *Sitophilus zeamais* exposed to submerged spores of *B. bassiana* and *M. anisopliae* produced in different liquid media.(Bar = SE). Supplement for chapter 7.



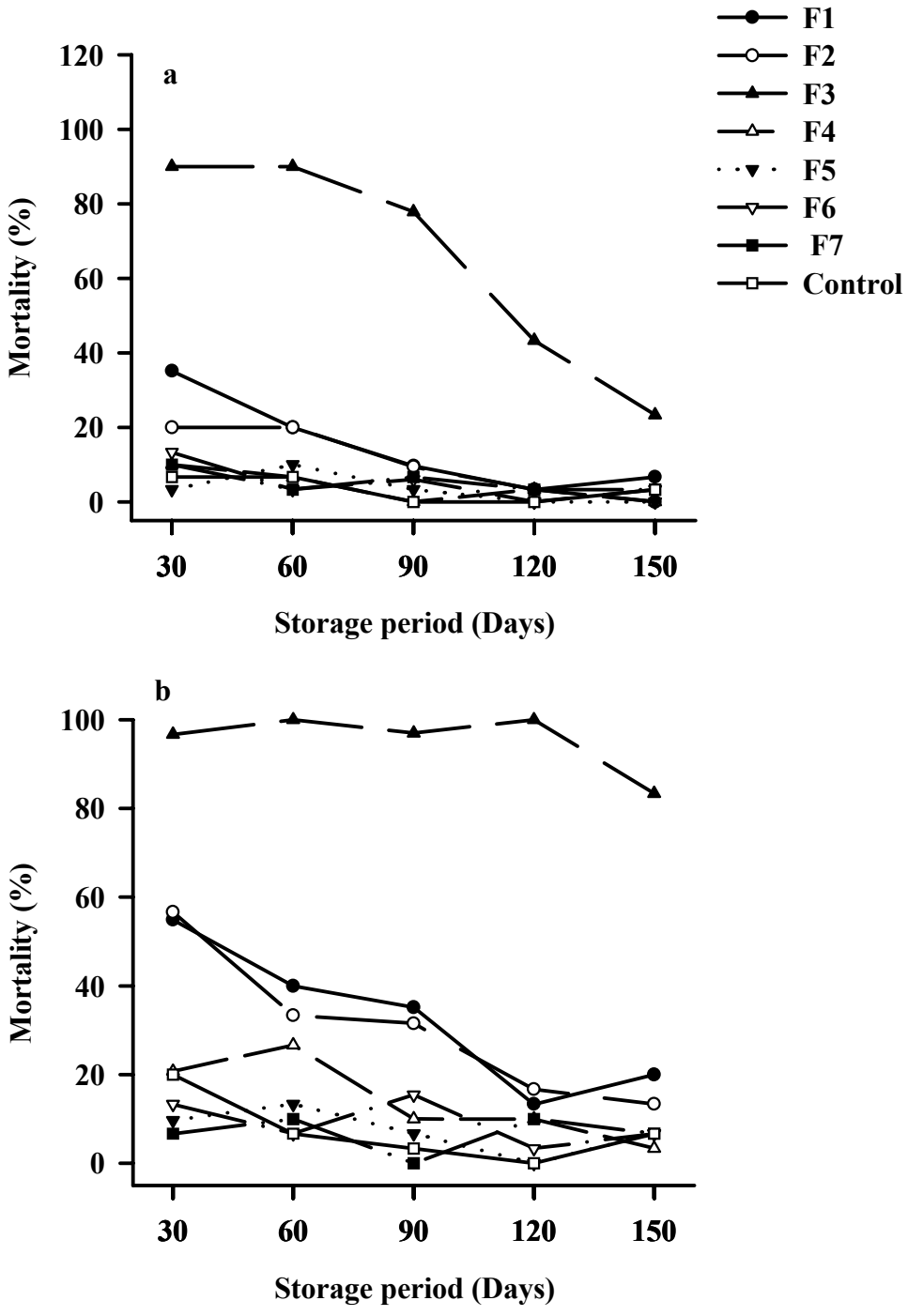
Appendix 15. Median Survival time (MST) of *Sitophilus zeamais* **and** *Prostephanus truncatus* **exposed to different DP formulations of** *B. bassiana* **and** *M. anisopliae* **at the concentration of 1 x 10^8 spores/g of maize grain. (Bar = SE). Supplement for chapter 8.**



Appendix 16. Mean mortality (%) of *Sitophilus zeamais* **and** *Prostephanus truncatus* **exposed to grains treated with different DP formulations of** *B. bassiana* **and** *M. anisopliae* **after storage at 30°C and 60–70% RH. (a) 5 days after exposure, (b) 10 days after exposure and (c) 15 days after exposure. Supplement for chapter 8.**



Appendix 17. Mean mortality (%) of *Sitophilus zeamais* after treatment with different DP formulations that were stored at 4°C for various time intervals. a) 5 and b) 10 days after treatment. Supplement for chapter 8.



Appendix 18. Mean mortality (%) of *Sitophilus zeamais* after treatment with different DP formulations that were stored at 30°C for various time intervals. a) 5 and b) 10 days after treatment. Supplement for chapter 8.

Appendix 19. Efficacy of selected DP formulations against *Sitophilus zeamais* after 12 months storage at 4°C and applied as water suspension at 1 x 10^8 spores ml^{-1}. Supplement for chapter 8.

Formulation	Spore germination (%)	Mortality[1] %	Infection (%)
F3	91	93	93
F5	92	100	100
F6	86	87	62
F7	85	100	67
Control	-	20	0

[1] Treatments were not replicated, number of insects used = 15/treatment.

Biography

PERSONAL DETAILS

Name	Adane Kassa
Nationality	Ethiopian
Date of birth	12 July 1966
Place of Birth	Gondar, Ethiopia

EDUCATION

02/00-07/03	Ph.D studies in agricultural science. Georg-August- University Göttingen, Germany.
09/93-09/94	M. Sc. and diploma in plant pathology and nematology. Imperial College of Science, Technology and Medicine (IC), UK.
09/84-06/88	B. Sc. in agricultural science (major plant science). Alemaya University of Agriculture, Ethiopia.
10/78-08/84	High school. Gondar comp.sec.school. Gondar, Ethiopia
10/72-9/78	Elementary school. Mesert elementary school. Gondar, Ethiopia

OTHER TRAINING

05/98-10/98	Biological control of insect pests using entomopathogenic fungi. Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control, BBA, Germany.
10/98	Rotational advanced studies program in Phytomedicine and Plant Protection in International Agriculture. Rheinische Friedrich-Wilhelms-Universitat, Bonn, Germnay.
06/94	Biological control of arthropod pests and weeds. International Institute for Biological Control (IIBC), UK.
08/93	Disease and insect pests of maize and their control methods.Institute of Agricultural Research, Addis Ababa, Ethiopia.
12/89	General crop protection training course. Institute of Agricultural Research, Addis Ababa, Ethiopia.
06/88	Scientific paper writing. Institute of Agricultural Research, Addis Ababa, Ethiopia.

WORK EXPERIENCE

09/02 –31/03	Research associates (Plant Pathologist). Forschungsanstalt Geisenheim, Germany. Project: Copper replacement on organic viticulture by means of bacterial antagonists.
07/99-12/01	Research associates (insect pathologist/entomologist).Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control (BBA), Darmstadt, Germany. Project: Biological control of locusts and grasshoppers.
10/88-06/99	Junior and Assistant Researcher (entomologist). Ethiopian Agricultural Research Organization (EARO).

Göttingen, 07/05/2003