Studies on the pathogenicity of an oil-based formulation of metarhizium flavoviride for the desert locust, Schistocerca gregaria

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Studies on the pathogenicity of an oil-based formulation of *Metarhizium flavoviride* for the desert locust, *Schistocerca gregaria* (Forskal)

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ABSTRACT

Adult desert locusts , *Schistocerca gregaria*, were inoculated with an oil based formulation of the entomopathogenic fungus *Metarhizium flavoviride* using a track sprayer with an ultra low volume (ulv) spray head. Infection was achieved when inoculated insects were maintained at low RH (35%) and 30°C. The dose required to give 90% kill was considerably lower when the inoculum was applied in a ulv spray (200 conidia/insect) than when applied in a 1μl drop under the pronotum (90,000 conidia/insect). Trials in Benin, West Africa, established protocols for the ulv application of *M. flavoviride* against locusts in the field.

Fungal infection brought about a significant reduction in feeding and tethered flight performance of adult locusts >72h post inoculation. Mycosed insects had a significantly lower haemolymph plasma concentration of lipid and carbohydrate. This was not due to reduced food intake by the infected locusts since 3d of complete starvation had no effect on plasma lipid or carbohydrate. Plasma carbohydrate declined significantly during tethered flight of control locusts, but not of mycosed locusts. It was concluded that a low titre of blood sugar may be, at least in part, responsible for the poor flight performance of mycosed insects. Consistent with this an

injected supplement of 6mg of trehalose improved significantly the flight performance of mycosed male locusts, while saline did not. Flight of female locusts was not significantly affected by mycosis possibly because of larger size and enhanced energy reserves. Therefore trehalose supplement had no effect on female flight performance.

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Chapter I: General Introduction

1.1. The Desert Locust Problem.

The desert locust belongs to the super family *Acridoidea* and to the family *Acrididae*. Locusts are essentially grasshoppers which have the capacity to change their habits and behaviour when they occur in large numbers, becoming gregarious and forming dense bands or swarms.

Locusts have probably been an enemy of man ever since he began to grow crops. However, some 200 grasshopper and locust species still remain as agricultural pests in Africa. Among these the desert locust causes the majority of concern as it is probably the most important locust species for it has a vast invasion area of some 29 million km^2, affecting 57 countries. This is more than 20% of the total land surface of the world. Therefore, it is an international threat in a belt from the Indian-Sub-Continent to the Atlantic coast of West Africa (Steedman, 1990). The recent spectacular feat of crossing the Atlantic Ocean to arrive in the West Indies covering some 500 km distance in about five days time has proved that the pest still remains as a potent threat for agriculture of many countries (David and Mark, 1989).

The return of normal rains to much of Africa in 1985, after a number of relatively dry years, resulted in the simultaneous development of increased population of four species of migratory locust in Eastern, Central and

Southern Africa for the first time in 50 years. The upsurges of the Brown locust, the Red locust and the African Migratory locust stayed rather limited in space and time, while the desert locust, *Schistocerca gregaria,* attained plague proportions affecting many countries in Africa and in the middle east. This plague ended in 1989. This simultaneous upsurge of grasshoppers and locusts was the largest in at least 23 countries for which private and public donors spent about $275 million to control the pests. This doesn't include the amount spent by some African countries from their own scarce funds (Anon, 1990).

1.2: Conventional Chemical Control Method of the Desert Locust

Early chemical control methods included the application of dusts containing Arsenic compounds. Ground and then aerial spraying of persistent organochlorine insecticides (dieldrin and BHC) became the preferred control methods in the 1950s. Dieldrin was banned in the United States and then in Europe because of its environmental and health hazards. Fenitrothion and Malathion were the major chemicals used in the recent upsurges (Anon., 1990).

For adult locust control there is a need for rapid acting pesticides that do not need to be very persistent. For many years the pesticides used after dieldrin were either organophosphates or carbamates; more recently the

synthetic pyrethroids have been added. The control of nymphs (hoppers) requires a different strategy as hoppers move in bands, the technique developed was barrier spraying with dieldrin.

The persistent pesticides like dieldrin which had been successfully used to control attacks in the past were not available when the last plague developed in 1985. Evidence suggests that the chemical pesticides employed against locusts in Africa between 1986-1989 were not very effective (Brader, 1988). Concerns over the effects of wide spread spraying of pesticides on the environment and the poor control exerted by available chemical pesticides have led to a world wide search for alternative biologically based alternatives.

1.3. Biological (Classical) Control Method as an Option for the Control of the Desert Locust.

Samways (1981) defined biological control as the use of natural enemies to reduce a plant or animal population that is inimical to man. Some other authors include in this term various other non-chemical forms of control that are biology based: (a) development of strains of crops that are resistant, or tolerant, to pests or diseases; (b) modification of cultural practices in a way that avoids or reduces infestation; (c) release of sterile males (Fernandez Garcia, 1990).

Jutsum (1988) under the heading "biological control" included a broad range of approaches from the use of obligate parasites and pathogens, to facultative parasites and pathogens, to competitors, to toxin-producing pathogens, to toxins produced by pathogens, and finally non-toxic behaviour-modifying chemicals. However, classical biological control is the term restricted to the use of parasites and predators for pest control.

Prior and Greathead (1989) reviewed the potential for classical biological control of locusts and grasshoppers. One of the earliest recorded biological control introductions against any pest was the successful establishment of the Indian mynah (*Acridotheres tristis*) in Mauritius in 1762 to control red locust (*Nomadacris septemfasciata*) out breaks. Insect parasitoids and predators of locusts inflict substantial mortality on solitary and gregarious populations. However, they do not prevent out breaks although they may hasten their decline. Unfortunately parasitoids and predators can not be used effectively in augmentive releases against swarms because of the logistical problems of mass breeding the natural enemies in their living hosts (see Prior and Greathead, 1989). There have been no long term successful biocontrol programmes for locust todate.

1.4. Microbial Control as an Alternative to Chemical Control of the Desert Locust, *Schistocerca gregaria.*

Steinhaus describes the term " microbial control" as that type of biological control involving the use of microorganisms for the control of pests (Steinhaus, 1949). Such agents can be used either as microbial insecticides, or as introductions leading to establishment and persistence of the pathogens in new environments (Fuxa, 1987). This distinction depends on the capabilities of the organisms i.e those for introduction must spread from relatively small inocula and persist, allowing nature to take its course. Those unable to spread and persist must be used as microbial insecticides and applied both regularly and in larger quantities.

Klassen (1981) and Hall (1982) suggested that microorganisms can be used as components of integrated pest management (IPM).

1.4.1. Main Advantages of Microbial Control in Relation to Other Control Methods.

Some of the major advantages of microbial control methods in relation to other methods can be listed as follows:

(a) they are 'safe' in comparison with some of the more toxic compounds (chemicals) used for pest control and

in particular the microbes are not accumulated in food chains. However, the safety testing of some insect pathogens is very expensive and safety considerations have until recently seriously limited the application of some control agents-viruses in particular. It is obvious that living organisms can replicate unlike chemicals and might prove impossible to contain if they are subsequently found to be hazardous.

(b) microbial control agents can be persistent, giving lasting control. This does not apply in all cases but certainly there are examples in which a microbial control agent, once introduced, has contained a pest problem at a sub-economic level.

(c) microbial control agents and biological control agents in general, have at most slight effects on the ecological balance. in particular they do not eradicate the natural enemy complex i.e. all those organisms that naturally help to keep a pest or pathogen in check.

(d) microbial control agents are often compatible with other control agents, including chemicals and can be used in conjunction with them. This is probably the most realistic approach to microbial control. Any way it is also unrealistic to think that microbial control agents will ever completely replace the existing control methods.

Despite the above summary of advantages, there are a number of disadvantages of microbial control which include the long term study that is necessary in relation to the scale on which it is subsequently going to be used and the

fact that micro-organisms cannot easily be patented; industry is therefore reluctant to invest in the development programmes. In addition over-specificity (when a pest control is involved), slow speed of action and expense can pre-clude the effective use of mycrobial pesticides.

However, there are certain factors that must be considered before a microorganism can be contemplated for use in biocontrol. Like conventional insecticides, it must be cheap to produce, be appropriately formulated so that it can be used in existing application equipment. Further more, the product must be host specific, suitable for the environment in which it is to be used and have a rapid effect on the population sprayed.

These guide lines rule out the majority of microbial biocontrol agents for control of locusts. Firstly, lack of specificity rules out a number of entomopathogens. Secondly, the harsh environmental conditions rule out the remaining agents. For instance, while *Nosema locustae* are effective control agents of the desert locust in captivity, the protozoan's requirement for moist conditions rule it out as an agent that can be used in desert conditions. Similarly, the known nematodes of the desert locust also require fresh water to infect their hosts (Prior and Greathead, 1989). A number of potential pathogens must also be ruled out due to cost of mass production eg. nuclear polyhedrosis virus (NPV) (Bensimon *et al* (1987).

The remaining microbial pathogens, fungi and bacteria, have the greatest potential, not least because these are representatives which are not obligate parasites and can be produced in *vitro*. However, a number of bacteria are not selective enough to be included in microbial control methods, since they are also pathogenic to man. The toxigenic bacterium *Baccillus thuringiensis* (Bt) is the insect pathogen most widely used for pest control. Unfortunately at present there are not isolates with toxins active against Acrididae. Therefore fungal pathogens are the lead candidates for microbiological control of locusts.

1.4.2. Entomopathogenic Fungi as Potential Control Agents of the Desert Locust.

There are approximately 100 genera of fungi that contain numerous species pathogenic to insects (Hall and Papierok, 1982; Zimmermann 1986). Of these, deuteromycete fungi have received most attention as they are among the easiest to produce in *vitro* and several have a broad host range. *Metarhizium anisopliae*, for example, has more than 200 known hosts among Coleoptera, Lepidoptera, Orthoptera and Hemiptera, whereas *Beauveria* spp. have been identified from about 500 host species, principally Lepidoptera, and Coleoptera (Hall and Papierok 1982). Individual strains of the same fungal species, however often have different host ranges and pathogenicities. Apart from deuteromycete fungi, the Entomophthorales (Zygomycetes) contain by far

the most insect-pathogenic isolates, but although the optimal growth temperatures for the deuteromycetes (20-30°C) fit them best for use against tropical and subtropical pests, members of the Entomophthorales appear more effective in temperate climates where they often produce extensive, if slow-acting, natural epizootics (Wilding, 1981).

The infective propagule in natural deuteromycete infections is the conidiospore. Although a few strains enter the host through the gut or through the respiratory tract, the majority invade insects through the cuticle. Fungi unlike other insect pathogens do not in general have to be ingested to be effective. They have potential for the control of sap-feeding Arthropods which do not ingest pathogens on plant surfaces (Payne, 1988).

Fungal disease development can be divided into a number of stages (Burges, 1981): (1) attachment of the infective unit (e.g. conidium or zoospore). (2) germination of the infective unit in the cuticle. (3) penetration of the cuticle, either directly by germ tubes or by infection pegs from appressoria. (4) multiplication in the yeast phase (hyphal bodies) in the haemocoel. (5) production of toxic metabolites. (6) death of the host. (7) growth in the mycelial phase with invasion of virtually all host organs. (8) penetration of hyphae from the interior through the cuticle to the exterior of the insect. (9) production of infective units on the exterior of the insect.

Epizootics caused by entomopathogenic fungi are relatively common, though this has only been realised comparatively recently (Ferron, 1978). However, the value of this control is still doubtful because it is unpredictable (dependent on many variables) and often too late to be of any economic significance. Attempts to improve the efficiency of endemic fungi by changing agronomic practices to optimize environmental conditions has received little attention (Ferron, 1978; Ignoffo, 1978).

However, the alternative to introduce inocula artificially, has been the subject of much study. Many attempts have been made to use artificial inocula to control insect pests on small scale, but there are relatively few cases where production has been on a commercial scale and application has been over a wide area. Most of these appear to have been done with Deuteromycete fungi (Deacon, 1983)

There are a number of reasons why entomopathogenic fungi have not become widely available as off-the-shelf pesticidal agents for use as an integral part of crop protectionists' armoury. The problem areas include: (i) a combination of suitable environmental conditions (particularly high humidity) is necessary for the development, growth and spread of disease; the difficulty of producing a virulent, stable production on a large scale. In addition susceptibility to fungicides complicates the process of fitting entomopathogenic fungi into

integrated control programme. However, there is general if not universal compatibility with insecticides.

Fungi are probably more dependent on appropriate microclimate conditions for their success than any other group of insect pathogens. In particular, the high relative humidity (RH) required for spore germination is often very restricting (Drummond *et al*, 1986). Among deuteromycetes infecting terrestrial insects, the lower limit for spore germination is probably about 92% RH and some may require a film of water to germinate (Payne, 1988).

Environmental susceptibility, poor shelf life and slow kill have all contributed to the limited use of mycopesticides. The details of current commercial scale use of fungal pathogens for insect control are given in table 1.1.

Table 1.1 Current commercial scale use of fungal pathogens for insect pest control

Product	Fungus	Pest	Company
Microgermin	*Verticillium lecanii*	Glasshouse whitefly & aphids	Christian Hansen Biosystems Denmark
Mycotal	*V. lecanii*	Glasshouse whitefly	Koppert Netherlands
Vertalec	*V. lecanii*	Glasshouse aphids	Koppert
?	*Metarhizium anisopliae*	Vine weevil	Christian Hansen
?	*Neozygites floridana*	Mites	Incitec Australia
Boverin	*Beauveria bassiana*	Colorado beetle	Co-op, Russia
Metaquino	*M. anisopliae*	spittle bugs	Co-op, Brazil
Bio-Path	*M. anisopliae*	cockroaches	Ecoscience USA
Bio-Blast	*M. anisopliae*	termites	Ecoscience (with Terminix) USA
?	*B. bassiana*	grasshoppers	Mycotech, USA
Naturalis-L	*B. bassiana*	cotton boll worms	Fermone, Corp USA
?	*B. brogniartii*	cockchafer	Andermatt Biocontrol Switzerland

1.4.3. Developing and Application of Mycopesticides

1.4.3.1: Mycopesticide formulations.

Pesticides are biologically active in extremely small quantities, so that the chemical has to be formulated in a form that it is convenient to use and and that can be spread evenly over a large area (Matthews, 1979). In the same way, the development of an insecticide based on a pathogenic fungus viz. a "mycoinsecticide" requires a suitable formulation.

In the past mycoinsecticide formulations were prepared in the form of suspension of spores in water. However, Prior *et al* (1988) showed for the first time that conidia of Deuteromycete fungi like *Beauveria* can be formulated in oil and give significantly better kill than in water. Their lipophilic cell walls enable conidia to suspend easily after vigorous agitation of suitable carrier oils poured over the sporulating culture. Oils are preferred in ultr-low volume (ULV) formulations since evaporation of the carrier liquid must be minimised when using small droplets. Oils also stick readily and spread on insect exo-cuticles, and may assist in transport of conidia into areas such as intersegmental membranes which are particularly susceptible to hyphal penetration (Prior *et al*, 1988). It is also important to retain viability and virulence of the infective units during storage and application. More recently Bateman *et al (1991)* showed that sprayed conidia of *Metarhizium flavoviride* in oil based formulation killed

locusts under the actual tropical environments in Africa.

1.4.3.2. Mycopesticide Spraying: Controlled Droplet Application.

For a spray application method to be effective, a good coverage of active ingredient must be achieved. Conventionally, this has been produced using large volumes of spray liquid as a wide range of droplet sizes. Although this may be effective, ultimately a large proportion of spray is wasted to run off, missing the target and overdosing. In addition, this method of spraying involves a large amount of time spent in mixing and refilling (Amsden, 1985). There are also increased risks from handling undiluted formulations and equipment costs are high.

Where water, money and time is scarce, and where a < 5 l/ha application rate is sought, ULV may prove to be an economically viable alternative. The use of very small quantities of formulation by controlled droplet application (CDA) is often possible by controlling the size and uniformity of droplets in a way that is most likely to achieve impaction on the biological target (Bals, 1975). The optimum droplet is based on the type of pesticide used, and the type of target it is being sprayed against Ford (1987).

Locust control often involves aerial and vehicle mounted spray equipment at about 2 l/ha application rate.

However, hand-held equipment is also used for small scale operations and field trials.

The use of small volumes (0.5-8 l/ha), and lack of mixing equipment, make it safer to use, the equipment is relatively cheap, and has already been adopted by farmers, extensively used on cotton (Matthews, 1977). The most commonly used equipment for CDA spraying employ hand-held, battery operated spinning disc or centrifugal energy nozzles. The hand-held spinning disc sprayer has a bottle (usually 0.5-1 litre) containing the spray liquid. this is fed under gravity through a restrictor on to a spinning disc connected to a battery powered D.C motor. The droplet size produced from such a sprayer is dependent upon the flow rate the spray liquid onto the disc (i.e the restrictor size and viscosity of the spray liquid) and the speed of the disc (the number of batteries charging the motor).

The present work is a continuation from that of Prior *et al* (1988) who showed that *Beauveria bassiana* conidia in oil based formulation were over 30 time more infective than spores formulated in water against *Pantorhytes plutus*. Bateman (1993) has also showed that target locusts will be more vulnerable over a time to increasing conidia, if these are applied in oil than in water.

The first objective of this project was to study the pathogenicity of *Metarhizium flavoviride* against the desert locust, *Schistocerca gregaria,* when the fungus was applied in an ultra low volume, control droplet, spray. A track

sprayer was used for this work, as a useful intermediate between laboratory bioassays and field application. Subsequently field tests were carried out in Benin, West Africa, to help establish protocols for the use of oil-based formulations of *M. flavoviride* against locusts in the field.

Most current work on mycopesticides is focussed on producing death of the target pest. However, sub-lethal or pre-lethal effects of microbial infections may contribute significantly to the crop protection value of the treatment. Therefore, finally in this project, the effects of mycosis on feeding, flight and energy metabolism in adult desert locusts were investigated.

Chapter II: Laboratory Experiments Using an Experimental Track Sprayer.

2.1: Introduction

Conventional pesticides are usually applied to desert locust in the form of oil-based ultra-low-volume (ULV) sprays and these techniques should be compatible with spores in oil rather than in water.

The conidia of fungi such as *Metarhizium* have lipophilic cell walls and it is relatively easy to prepare oil-based suspension formulations that are compatible with ULV application equipment. Unlike bacteria, viruses, nematodes and protozoans, infection occurs through the cuticle rather than in the digestive tract so these fungi can be applied as contact acting sprays and need not be formulated as baits (which are expensive to make and difficult to apply over large areas).

The objective of experiments using an experimental track sprayer was to check pathogenicity of *Metarhizium flavoviride* formulations applied as sprays to adult desert locusts, before carrying out field trials. This therefore constituted an intermediate stage between applying known amounts of fungal suspensions in laboratory bioassays and field testing.

2.2: Materials and Methods

2.2.1 Sources and maintenance of experimental insects

Adult locusts, were used in experiments 7-10 days after fledging. They had been reared and maintained in the locust rearing facility at the International Institute of Biological Control (IIBC), under a constant room temperature of 30°c with 12 hour light/dark periods. They were kept in metal cages and provided green-house grown

wheat seedlings and supplemented by wheat bran, hay and water.

2.2.2 Media preparation, formulations and viability checks.

All experiments were with *Metarihizium flavoviride* strain IMI 330189 cultured on Molisch's agar (Speare 1920). This agar consists of consists of 0.3% peptone, agar, sucrose, potassiumbiphosphate (K_2HPO_4) and magnesium sulphate mixed with one litre of distilled water. The ingredients were dissolved by placing the plugged flask in a hot water bath for 90 minutes. The solution was then decanted into small McCartney bottles for autoclaving under 15 p.s.i. for 15 mins.at 121°c. Autoclaved media was cooled at a slant and then inoculated by streaking with a sterile loop. They were stored at 25 °c for between 20-30 days depending on experiment, following which the conidia were made up in to a suspension with cotton seed or ground nut oil (Sigma) depending on availability, sonicated for two minutes and filtered through either a 90 μm mesh or a double layer of sterilised muslin (chapters 4 and 5).

The cultures were incubated at room temperature and sporulation occurred within a week. Spores were harvested by half filling bottles with cotton seed oil and agitated using an ultra-sonicator, when necessary. The resulting suspension was removed and conidia counted by a haemocytometer using a phase contrast microscope. The required dilution (4.5 $x10^7$ conidia/ml) was obtained by serial dilution of concentration with cotton seed oil. The final mixture contained 70 %Shellsol T which was added to decrease the viscosity of the formulations. The spray material was kept in the control room (30°c) at least 2hr. before use to warm to the ambient temperature.

Viability tests of the pathogen were carried out before each experiment by placing agar containing conidia into a sterile microscope slide similar to that used by

Seyoum (1990). The slides were observed down a microscope and the percentage germination of 500 conidia was recorded. Viability of was considered normal when conidial germination was not less than 90%.

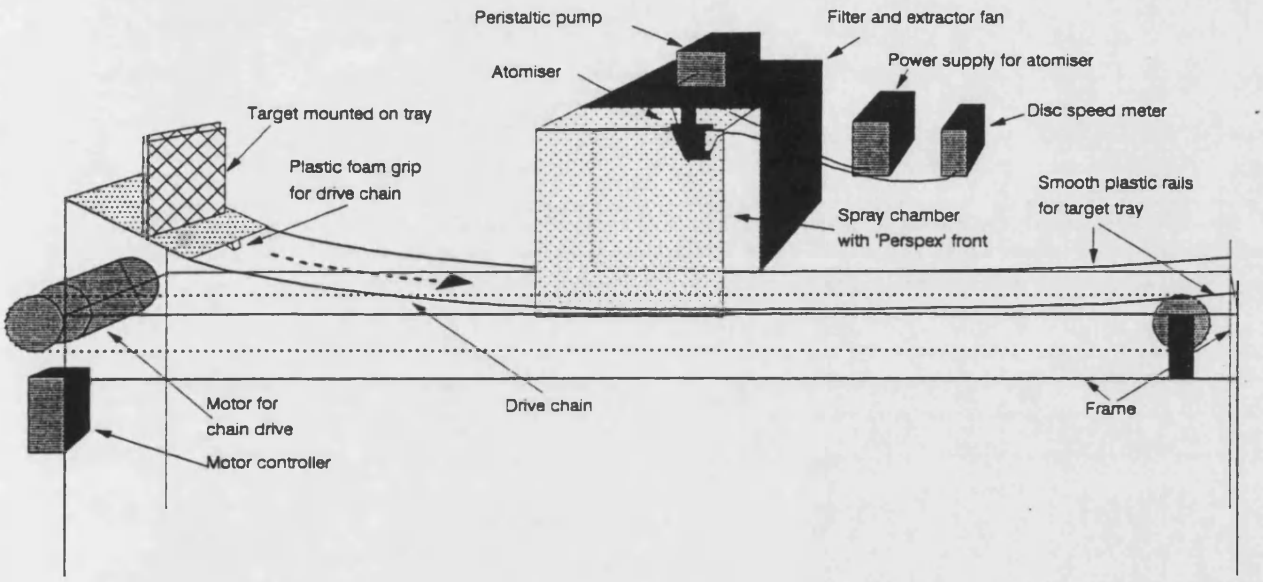
2.2.2.1: Application of fungal formulations.

The IIBC prototype biopesticide track sprayer (Fig.2.1) consists of a frame, a variable speed plastic chain drive, a target platform a spray chamber with an extractor fan, a sectorial spinning disc atomizer assembly and a liquid feed assembly (Bateman, 1994). This was developed in an attempt to produce reasonably reproducible deposits on target surfaces that are similar in magnitude to those encountered in the field.

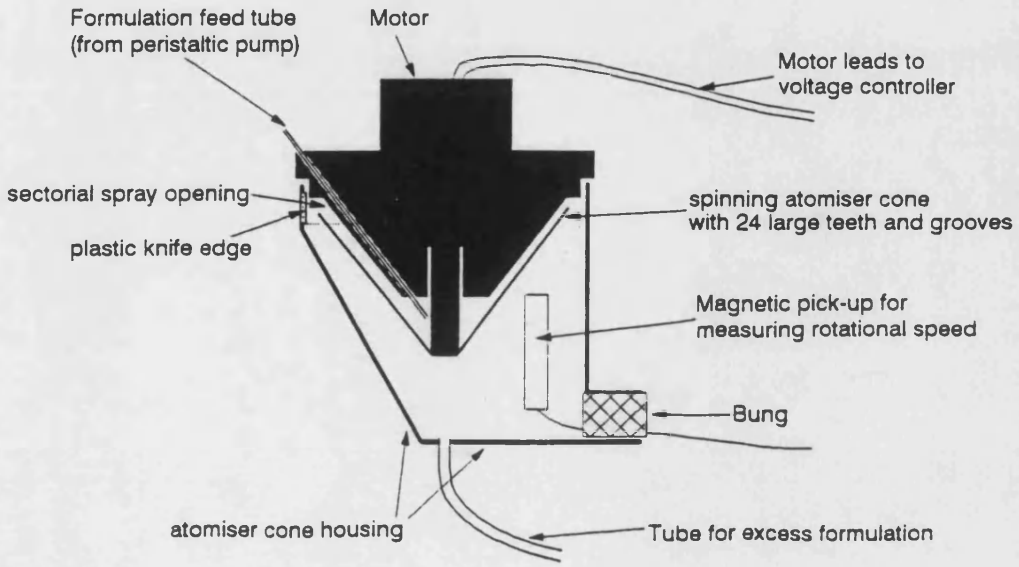
The atomiser was shrouded and mounted over the disc so that spray was emitted over a ~60° angle. Reproducible disc speeds were obtained by an adjustable voltage regulator connected to the atomiser. A fan was positioned behind the spray chamber to provide air current for impaction of small droplets and to remove excess spray. A digital magnetic pick up was fixed within the shroud and positioned approximately 1mm. away from the spinning disc onto which a strip of metal had been fixed; this was connected to a meter which gave a reading in revolutions per second.

Figure 2.1. An experimental track sprayer with rotary atomiser (Courtesy to R.P. Bateman)

General construction



Construction of Atomiser



An unmodified Micro Ulva (Micron CDA Ltd.) with a 52 mm. diameter disc and 360 teeth was used in the original (1991) experiments; this was connected to a reservoir with a constant liquid head to provide an even flow of formulation at 30 ml/min. This was found to apply excessive numbers of droplets so a modified atomiser with 24 toothed disc and a feed rate of 6.7ml/min was used for the 1993 series of experiments.

Targets were mounted on a plastic tray with a foam strip glued to its base so that it is engaged onto a plastic chain driven by a variable speed motor controller set at about 1.3 m/second. In 1991 locusts were suspended in a harness consisting of a rubber band which passed through a thin metal tube attached to the target carrier around the pronotum behind the fore legs. The use of rubber bands or clips was found to be better than passing a pin through the posterior edge of the pronotum (as used by MacCuaig, 1962). Before being passed through the spray chamber insects were blown-on lightly to initiate flying.

In 1993, the locusts were caged between two sheets of stainless steel gauze (2.5 mm mesh) separated by a 10 mm circular spacer. This reduced handling and enabled a greater number of insects to be tested. Impaction of droplets was assessed by mixing fluorescent tracer with the formulation and droplet size was determined by using magnesium oxide coated slides inserted into separate cages that were passed through the spray curtain. The locusts were sprayed in a group of five at a time (Table 2.7).

Sprayed insects were kept individually in labelled small plastic boxes lined with sterile tissue and were maintained in a CT room under conditions similar to the locusts rearing room. The locusts were not given food after treatment as adults under actual conditions could live on their body fat. Fresh food would have also raise the relative humidity. Mortality records were taken at 24

hour intervals. Dead locusts were removed and placed on fresh wet tissue in a similar box and were incubated at 30°C to assess that mortality was caused by mycosis.

2.2.2.2: Determination of droplet size and quantity of spray collected by locusts

Magnesium oxide slides were prepared as a sampling surface by burning two strips of magnesium ribbon underneath glass slides placed on metal strips so that the central area was coated uniformly. These MgO slides were passed through the spray chamber of the track sprayer belt, disc speed, and flow rate set according to the experiments carried out. The slides were positioned and sprayed with saturn yellow fluorescent tracer in a similar way to those treated with fungal spores. A Flemming particle size analyzer (Matthews, 1979) was used for droplet counting and measurement on six slides for each disc speed.

Insects were sprayed with "saturn yellow" fluorescent tracer in an oil mixture. Twenty flying and another 20 resting (1991) and 10 resting (1993) locusts were sprayed and droplets were counted per insect body parts (table 2.2). Sprayed locusts were killed by freezing and droplets were counted by observing with a microscope and an ultra violet light.

2.3 Results of the track sprayer experiments-pre-modification (1991).

Operating conditions and results for the 1991 experiments are summarised in tables 2.1-3 with analyses of results given in Appendix 1.

Table 2.1: Track sprayer experiment 1; 30-11-1991

Target: *Schistocerca gregaria* adults, simulated flying.

Formulation: *Metarhizium flavoviride* (IMI 330189), @ $4.5x10^7$ spores/ml. in 30% cotton seed oil, 70% Shellsol T.

Treatments: beside controls, sprays of two droplet sizes: "Medium" droplets produced at 120 rps (70μm VMD). "Small" droplets 180 rps. (55μm VMD).

		% cummulative mortality at days after treatment		
Treatment	N	5	6	7
Control	12	0	0	0
Small	12	30	85.9	90
Medium	12	40.2	90	90

Table 2.2:Track sprayer experiment 2 (1991)

Formulation: *Metarhizium flavoviride* (IMI 330189),@ 4.5x 10^7 spores/ml. in 30% groundnut oil, 70% Shellsol T.

Treatments: as in experiment 1.

% Cumulative mortality

at days after teatment

Treatment	**Total**	**No / rep.**	5	6	7	8
control	20	5	0	0	0	0
small	20	5	22.8	71.6	77	90
medium	20	5	30	77	90	90

Table 2.3: Track sprayer experiment 3, 21-02-91

The experiments were repeated under similar conditions, with 20 insects to confirm the findings.

Formulation: *Metarhizium flavoviride* (IMI 330189),@ 4.5x10^7spores/ml.in 30% groundnut oil, 70%Shellsol T.

Treatments: controls and pathogenic formulation of one droplet size, disc rotation approx. 180 rps. (55μm measured VMD).

% Cummulative mortality

at days after treatment:

Treatment	**n**	5	6	7	8	9
control	20	0	0	18.4	18.4	18.4
small	20	5	20.3	67.2	71.6	90
medium	20	33.2	71.6	90	90	90

N.B. Mortality in the controls was not due to *Metarhizium* infection, and probably was due to bacterial attack.

Percentage cummulative values for each experiment were transformed into arcsine.

The results of the three experiments (1991) showed no significant differences in mortality due to infection between replicates, but due to treatments (disc speed). Differences due to treatments at day 5 after spray were significant in all the 1991 experiments. Analysis for day six in the third experiment has showed significant difference at 95 % (2 df) between treatments. This did not however happen in the first two experiments.

2.3.1: Spray deposition on live locusts and size of droplets

Deposition of spray on both flying and resting locusts was estimated using fluorescent tracer to count droplets impinging on insect body parts. Mean spray deposition of droplets on flying insects counted (965) was about 4 times greater than deposition counted on insects sprayed while resting (293). Both the flying and resting insects were exposed to similar spray cloud. The results are summarized in table 2.4, and droplet size measurements for different disc speeds carried out at the same time are given in table 2.5.

Table 2.4: Average number of droplets on locust body parts (1991).

Conditions	No	H+A	Tx	Wg	Abdn	LG	Mean
Flying	16	82	139	213	378	144	956
Resting	16	31	34	152	37	39	293

H+A: head+antenna; TX:thorax; Wg:wing; Abdn:abdomen; Lg:leg

Table 2.5 Measured spray characteristics from atomiser assembly.

70 % raw cotton seed oil, 30% Shellsol T, flow rate 40 ml/min.

Disc speed	VMD	NMD	ADV	Span
80	81	22.00	82	0.78
100	73	11.56	45	0.78
120	69	11.48	39	0.86
140	63	11.62	32	0.91
160	60	11.80	28	0.93
180	58	10.56	24	0.92
200	60	10.48	16	1.16

Disc Speed in revolutions per second (r.p.s.)
VMD: ($Dv_{0.5}$) Volume Median Diameter in μm,
NMD: ($Dn_{0.5}$) Number Median Diameter in μm,
ADV: Average Droplet Volume - $D_{3,0}$ (pl),
Span: $\frac{Dv_{0.9}-Dv_{0.1}}{Dv0.5}$

Table 2.5 shows droplet size spectrum and flow rate of formulation used as control in the track sprayer experiments when analysed with the Malvern 2600 particle size analyser

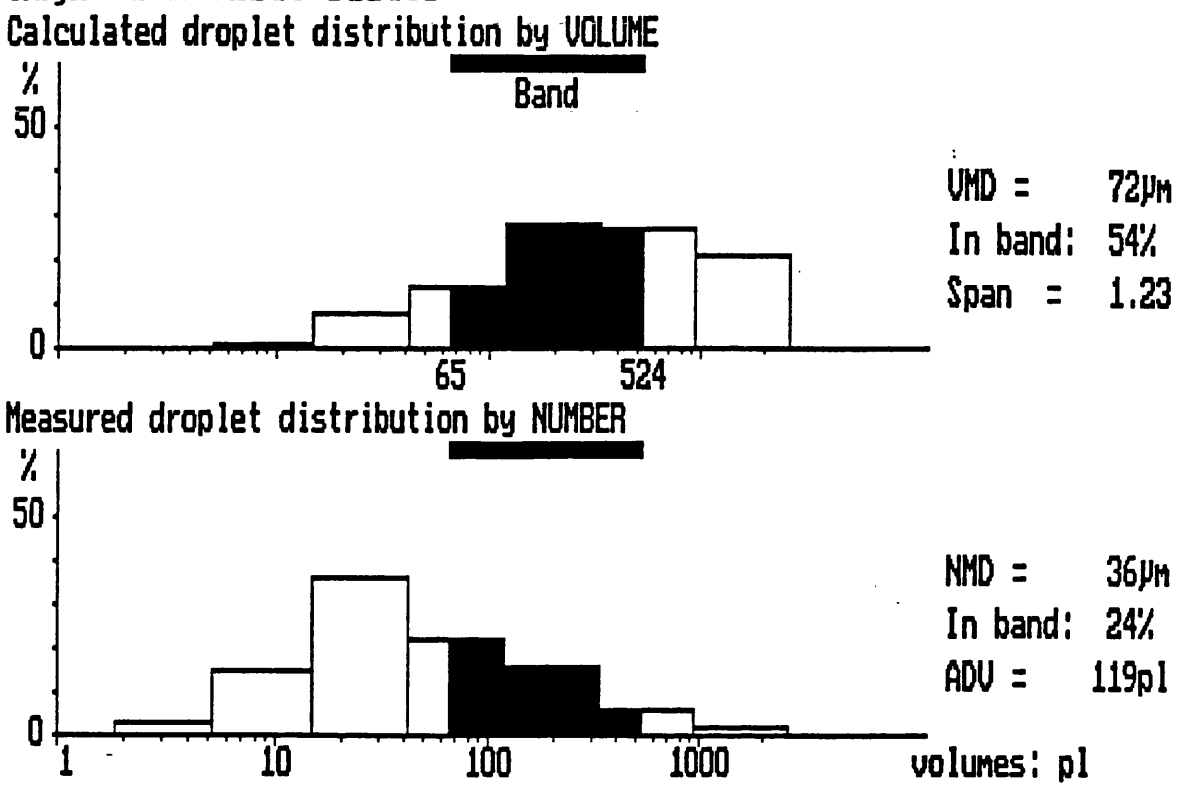
2.4: Results of the Track Sprayer Experiment (1993)

2.4.1: Determination of droplet size

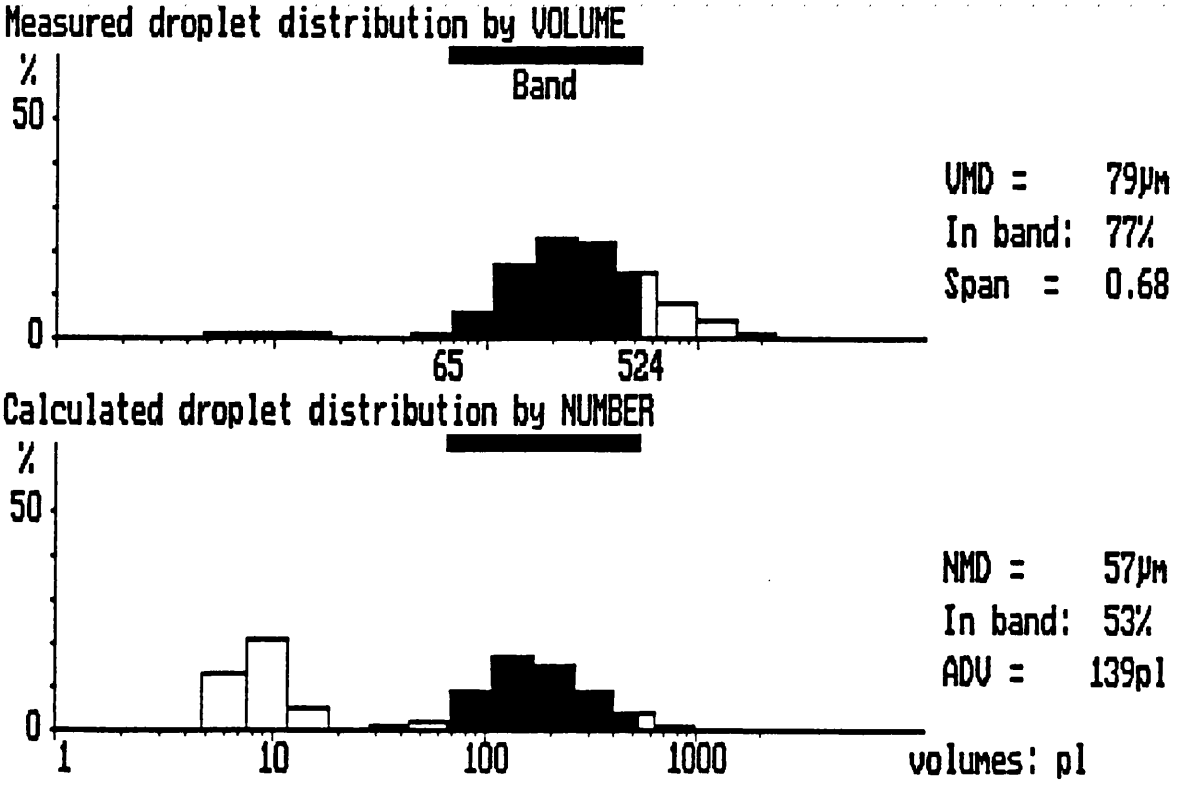
In this experiment, a 24 tooth track sprayer atomiser at 5500 R.P.M was used. Figure 2.4 illustrates calculated and measured droplet sizes by volume (VMD) and number (NMD), 72μm and 36μm, respectively, on magnesium oxide slides using Fleming particle size analyser. The droplet size spectrum of similar disc speed was also analysed using Malvern 2600 particle size analyser and the VMD and NMD values were 79μm and 57μm respectively. The spray uniformity in is indicated by the values for SPAN and VMD/NMD ratio, 1.23 and about 1.4, respectively (figure 2.2). These indicate that there was a reasonably uniform spray reaching the targets.

Fig. 2.2 Droplet data for 24 tooth atomiser at 5500 R.P.M. spraying a blank formulation of 50% Ondina EL oil with Shellsol 'T'

a) Magnesium oxide slides



b) Malvern 2600 particle size analyser



The main objective of the 1993 track sprayer experiment was to test a modified version of the equipment and compare insect mortality at different concentrations of formulation applied at volumes more commensurate with field practice. The results (appendix 4) were analysed using a computer program "Meltimore" which was written to store and calculate simple statistics for biopesticide assays (Bateman *et al.*, in press).

In order to incorporate the rest of data, the average survival time (AST) has been used where n^t is the number of individuals that die on day t ; out of a population N treated insects, between day 1 and the end point of the assay (u).

$$AST\ (\bar{t}) = \frac{\sum_{t=1}^{u} n^t.(\ t - 0.5\ )}{N}$$

2.4.2: Spray deposits on caged locusts.

Table 2.6: Average number of droplet recovered by locust body parts

N	H+A	Tx	Wg	Lg	Abdn	Mouth	Mean
10	7.8	11.9	31.4	4.0	9.4	2.3	66.8

H+a: Head+antenna; Tx:thorax; Wg:wing; Abdn:abdomen; Lg:leg.

Table 2.6 ilustrates that the average number of droplet recovered per insect was about 67± 3.24 (mean ± s.e) with an estimated 120-140 pl (about 8-10nl /insect). Droplets per insect indicate in the above table were lower

than in table 2.4. This was as a result of of modification of the the spray equipment (from 160 to 24 teeth).

Table 2.7: Average survival time (AST) of adult *Schistocerca* sprayed with an experimental track sprayer

(1993)

Estimated conidia per:

ml	**Insect**	**% kill at day 8**	**AST (Days)**
$4x10^7$	200	86.67	6.70
$1x10^8$	500	93.33	6.37
$2x10^8$	1000	73.33	7.03
$4x10^8$	2000	80.00	7.30
$1x10^9$	5000	93.33	6.10

No mortality was observed in the control insects.

There was no significant difference in mortality due to the different conidial concentrations applied (over all $x^2 = 3.622$, on 4 df ($P>0.05$).

Table 2.7 shows the results of the (1993) experiment and it further illustrates the lack of difference in average survival time between estimated conidial concentrations applied.

2.5 Discussion.

These results indicate that sprays containing conidia of *M. flavoviride* kill desert locusts under conditions of low relative humidity. This has been achieved by formulating the fungus in oil instead of water (Prior *et al.*, 1988 ; Bateman *et al*, 1993). Such formulations therefore show potential for use in arid field conditions

as well as controlled environments (e.g. Bateman *et al.*, 1993 ; Bateman, 1994). Further studies on the spray characteristics of *M.flavoviride* conidia formulated in oil were carried out in the field as described in the next chapter.

Chapter III: Spraying of *Metarhizium flavoviride* conidial formulations in the field: spray deposition and distribution on target surfaces.

3.1 Introduction

For the purposes of this study microbial insecticides are treated like conventional chemical pesticides which must be formulated in an appropriate carrier and reach the target in sufficient quantity to be effective. The "active ingredient" consists of whole numbers of infective conidia rather than continuously variable quantities as with dissolved chemical insecticides. From a practical point of view, it would be useful to correlate droplet size and number of conidia transferred to the target which in turn affects the speed of kill.

The objectives of field tests were to:

(a) Study the downwind distribution and deposition of the oil-based mycoinsecticide formulation when applied as a drift spray.

(b) compare the droplet impaction on artificial targets and live locusts and estimate the numbers of conidia reaching target surfaces.

(c) attempt to relate spray deposition with locust mortality.

3.2: Materials and methods

3.2.1: General Design

An area of 90 m x 70 m land was cleared at the IITA research site and the central 82.5 m x 60 m was laid out and used to carry out trials (Fig 3.1). 24 arenas (3m x 3m each) were sited in four parallel sampling lines 20 m apart at six sampling points: 5, 10, 15, 20, 25, 30 m downwind line. The sampling lines were marked out with a spacing of 20 m between centres. 5 m zones were sprayed leaving the 15 m between lines unsprayed. Each sampling position consisted of square section poles 50 cm high and branches of cassava (of similar height) for locusts to perch on. Artificial targets were attached vertically to the sampling posts facing upwind and were sprayed concurrently with the locusts.

The desert locusts had been obtained from the IITA-BCP locust rearing facility at a temperature of 30-32°C and a 12 hour day/light periods. 15 newly fledged (10-15 days) adult locusts were placed in each arena; their fore-wings were restrained with paper clips in order to minimise the number of escapees during and after spray. Treated insects were kept in cages and assessed daily.

3.2.2: Spraying and spray-materials

Formulations consisted of:

(1) An oil based suspension of *M. flavoviride* conidia containing: $1x10^9$ for trial 1 and $4x10^9$ conidia/ml was

used in trials 2, 3, 4 and 5. The formulating medium contained 50% Shellsol K and 50 % groundnut oil. 'Lumogen' fluorescent tracer was added to estimate droplet numbers under ultra-violet light.

(2) Controls were sprayed with a blank oil mixture (as above) containing oil soluble waxolene red dye for estimating droplet numbers on Kromekote cards.

A hand-held 'Micro Ulva' sprayer was powered by 5 batteries and fitted with a 1.56 mm.(red) restrictor; flow rate 60 ml/min disc speed (approx.) 165 r.p.s for all trials. Each test consisted of 4 passes along the spray line (Fig. 3.1). The nozzle was held at a height of 1.3 m and the average walking speed was 1.2 m/s. The amount of spray for each trial was checked by measuring spray-material before and after each trial. The sprayer was switched on at marked points of 2.5 m distance before and switched of at 2.5 m away after each row of targets.

Spraying was carried out during the afternoon (approximately 4:30-5.30) over a five day period. Measured environmental factors included humidity, wind speed and temperature. Dry and wet bulb readings were recorded at the IITA weather station.

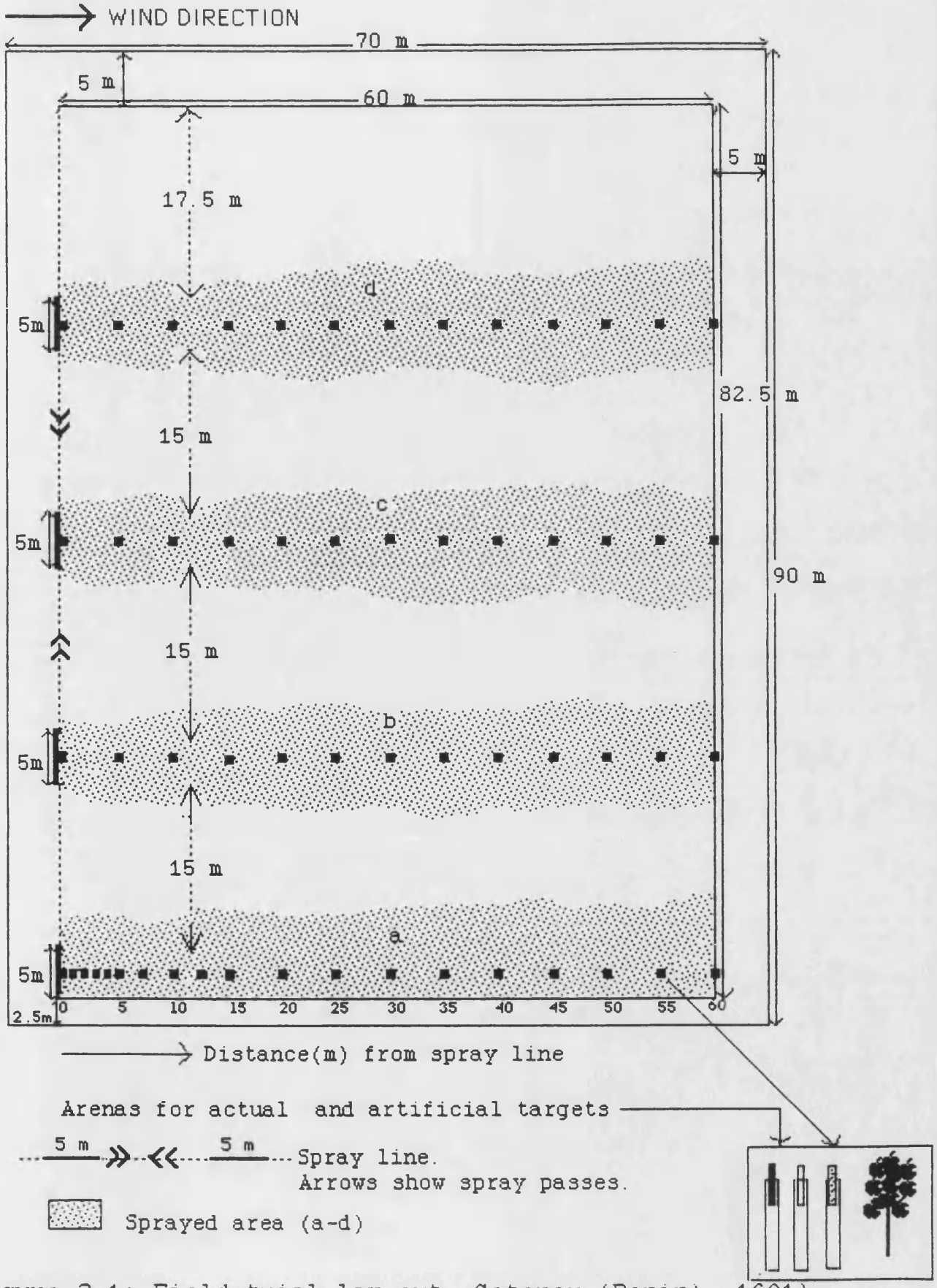


Figure 3.1: Field trial lay out, Cotonou (Benin), 1991).

3.2.3. Methods of estimation of spray deposition.

Artificial targets consisted of:

(1) 'Kromekote' and Black cards of approximately 2 x 6.5cm

(2) Microscope slides (7.5 x 6 cm) with and without a coating of nutrient agar. This agar used was similar to that used in laboratory work with antibiotic chlorophenicol added at a rate of 0.3 g per 200 ml.

'Kromekote' and Black card targets were positioned at 0, 1, 2, 3, 4, 5, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 m from passage of nozzle in order to obtain extra detail of the downwind spray pattern. Droplet numbers were estimated using an index (Bateman, 1989, 1993). Droplet marks on artificial targets or plant material were compared with a sheet containing dotted areas which increase in a doubling progression with index number. Each index (I) represents a range rather than a specific droplet density and is related by the formula:

Equivalent mean droplet per $cm^2 = 2^I-1$

The actual ranges described are as follows:

Index(I)	Range	Index	Range
0	Absent over a large area	5	21-40
1	< 2	6	41-80
2	2-5	7	81-160
3	6-10	8	161-320
4	11-20	9	> 320

Slides sprayed with fungal formulations were examined with a compound microscope for droplets counts/cm^2 and numbers

of conidia from at least 10 drops/cm² Estimation of drops/cm². was possible by producing a similar sized paper with a measured and cut out 1 cm² centre and fixed to the under side of each slide.

3.2.4: Assessment of conidial viability

Compatibility tests of the pathogen with all formulating materials were carried out before and after field testing. Samples were inoculated onto nutrient agar (containing Bacteriocide) in petri dishes. Live formulations were compared with standards containig similar concentrations of the fungus in pure groundnut oil.

Similar tests were also carried out on the agar coated microscope slides and petri dishes which had been sprayed in the field tests. Viability tests consisted of 500 spores counted after 24 and 48 hours of germination. In all cases, at least 90% of the spores counted were viable.

3.3: Results

3.3.1: Distribution and deposition of spray droplets on actual and artificial targets.

The raw data from Kromekote and Black cards were analysed by the computer program "swath" which converts indices back into mean number of droplets per square centimetre. The software produces graphs, an example of which is illustrated in Figs 3.2a and 3.2b. The area under

curve (A.U.C.) gives an estimate of total number of spray droplets along a downwind transect, 1 cm wide. When these swath patterns are super-imposed on one another in positions equivalent to the track spacing, it is then possible to estimate the mean numbers of droplets/cm^2 in a large field; the variance/mean indicates of evenness of coverage. Table 3.1 presents data from all tests in this way with blank formulations assessed with 'Kromekote' cards and the "live" suspension using black cards. In the illustration a track spacing of 5 m has been selected and the "contamination beyond field" helps to plan appropriate plot spacing in field trials.

Table 3.1 Summary of spraying conditions and assessment of total droplet deposits (see text)

		Conditions			Droplet deposit assessment		
Date	**formulation**	**wind**	**dry**	**wet**	**A.U.C.**	**Mean**	**Var/mean.**
27/5/91	blank	4.0	31	26	51360	102	0.03
	"live"	3.5			41490	83	0.03
28/5/91	blank	3.5	33	25	26490	53	0.71
	"live"	3.0			16540	33	1.16
29/5/91	blank	4.5	33	25	30550	61	0.84
	"live"	3.5			18820	38	1.73
30/5/91	blank	4.5	31	25	41650	83	0.20
	"live"	3.5			22570	45	0.68
31/5/91	blank	4.5	34	26	50500	101	1.06
	"live"	5.0			101360	202	0.15

Data includes: Approximate wind speed (m/s); dry and wet bulb readings in °C; A.U.C: Area under curve; droplets in downwind 10 m of field; Var. variance;

Trial 1 (27/5/91) was carried out by spraying *Metarhizium flavoviride* suspension ($1x10^9$/ml) on strips of Black cards, microscope slides and on experimental adult locusts at the same time, and therefore regression (linear) analysis was performed for the dependent variable (mean number of either droplets or spores /cm^2) on the associated predictor variable (downwind distance).

Table 3.2: Summarized results on mean droplet by target type against downwind distance of trial 1(27/5/91)

Distance (m)	**mean No.of droplets/cm^2 or /insect /spray pass. Microscope slides**	**Black cards(V)**	**Locusts (I)**	**Kromekote cards(V)**
5	31.3	31.75	35.225	52.03
10	22.3	32.24	22.525	18.72
15	17.6	9.41	16.25	9.46
20	13.7	6.97	6.975	7.10
25	10.2	4.86	4.35	5.60
30	9.4	4.85	3.25	3.68

I: Data taken from appendix 2(I); V: from appendix 2(V).

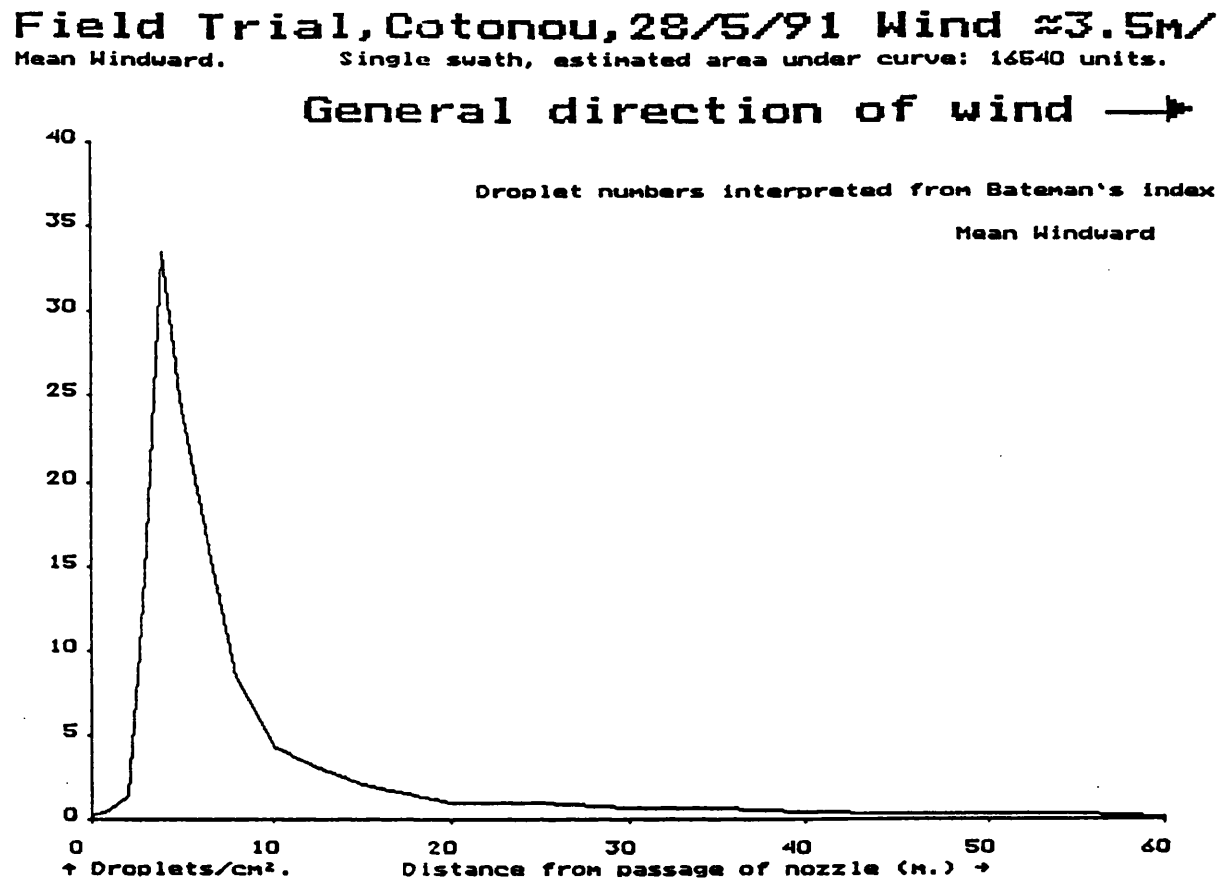
Data were used for the analysis in table 3.1.

The following equation was developed and used for the statistical (regression) analysis of the above results:

$Y = C \times (I)\ (cf)$

where, Y is dependent variable; C: constant which represents the y-axis intercept of the regression line.(cf): coefficients (slopes) and I: independent variable. All negative correlation coefficients (cf) showed that an increase in the independent variable (downwind distance) was associated with a decreasing trend to the respective dependent variable This trend is illustrated on table 3.2 and figs. 3.4a and 3.4b in which mean droplet density on different targets has seen similar relationship (trend) so that variation in droplet density was not due to variations in target type but mainly due to variation in downwind distance.

Figure 3.2a. Spray deposit down wind/spray pass.



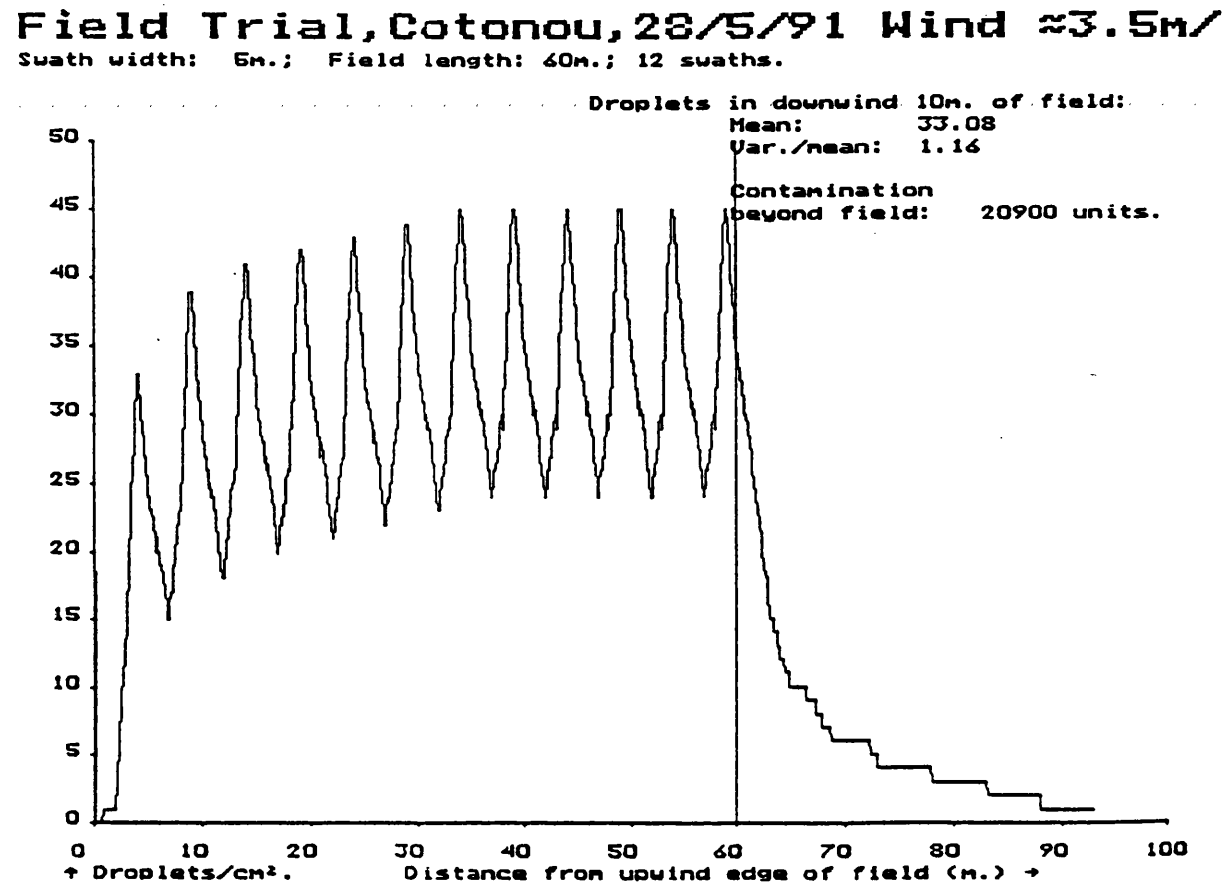
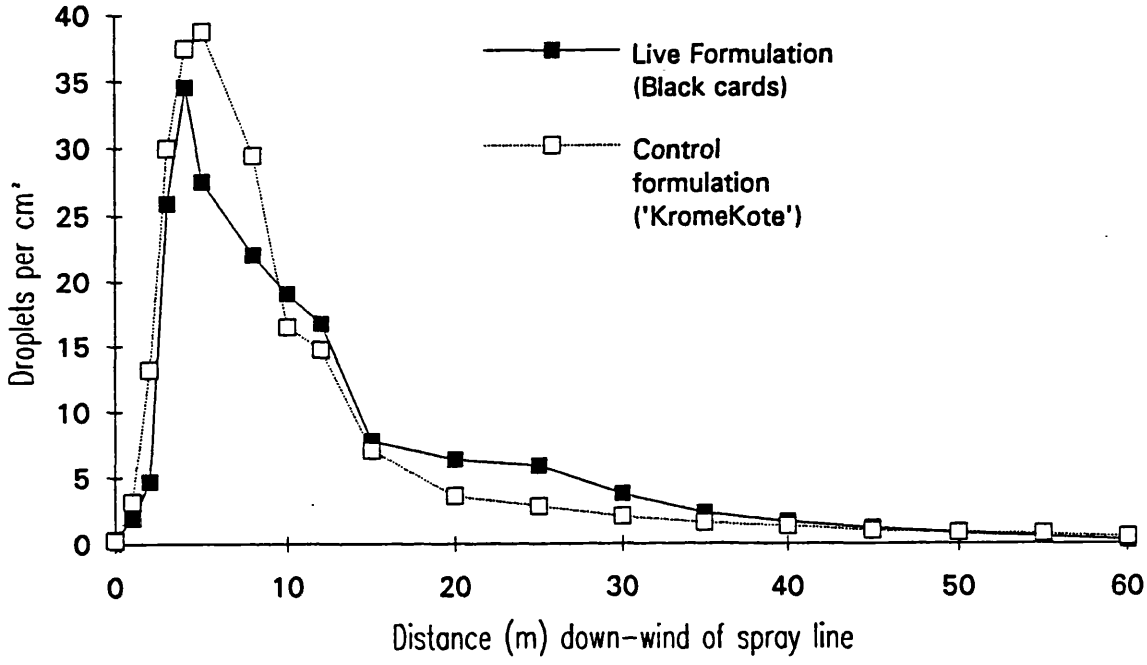


Figure 3.2b. Incremental spray deposition.

Therefore, depicted in figure 3.4a is mean droplet density/cm^2 of live conidial formulation (black) of two tests and whereas figure 3.4b shows deposition on different artificial targets and on live locusts sprayed at the same time and conditions. Despite the differences in target surface type, the trends of spray deposition were similar and they were all positively correlated against downwind distance (m). Further details of outputs from the linear regression against downwind distance of spray line are shown in table 3.3

Figure 3.4

a. Down-wind deposition of droplets on card targets (means from 5 tests)



b. Comparison of droplet deposition on Locusts and artificial targets (means from tests 1 and 4)

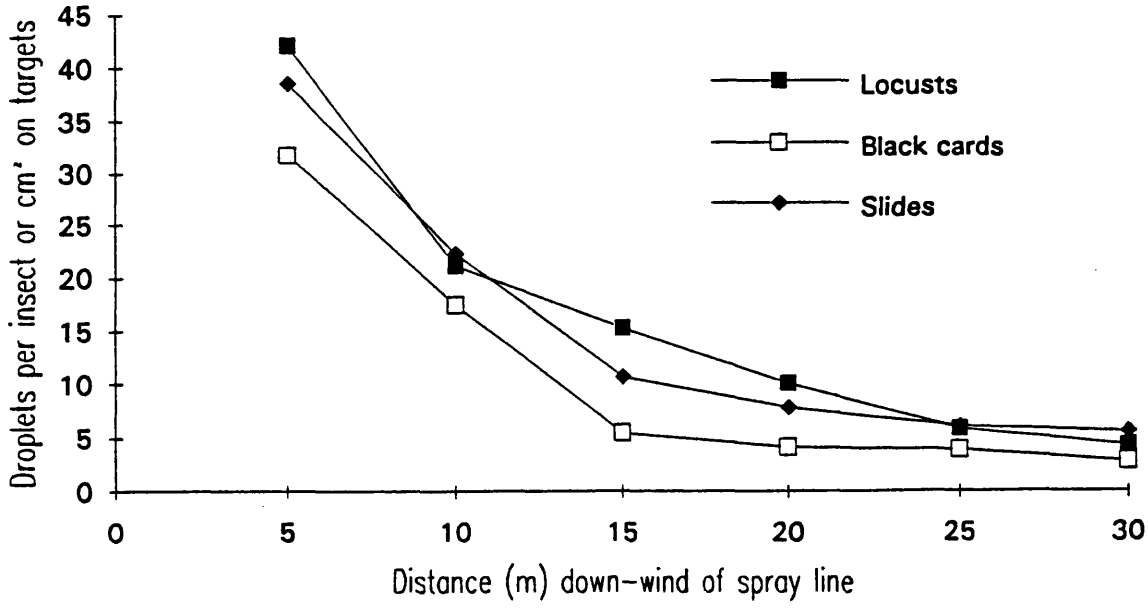


Table 3.3. Summary of regression analysis on spray deposition on target surfaces against down wind distance.

Explanatory variable	dependent variable	r^2	df	(cf)
Distance	Drops on slide	0.97	4	-0.69
Distance	Mean No drop on BC	0.75	4	-0.97
Distance	Mean No.drop on KC	0.99	4	-1.47
Distance	Mean No.drop on Loc	0.91	4	-1.38
Distance	Mean No.spores/cm²	0.97	4	-1.28

BC and KC: Black and Kromekote cards; Loc: locusts.

Details on estimated number of fungal spores/cm^2/spray pass of this trial are indicated on appendix 2 (III), table 3.4 which shows that an increase in calculated mean estimated fungal spore was associated with similar trend of mean droplet deposit/cm^2 but both declined as downwind distance increased.

Table 3.4: Average droplet and estimated conidial deposition /cm^2 against down wind distance (m) for trial 4.

Downwind distance(M)	Estimated mean No/cm^2/spray pass of: drops	conidia
5	31	3650
10	22	1951
15	18	1274
20	14	815
25	11	477
30	9	374

Results for trial 4 (30/5/91) were obtained from a formulation consisting 4 x 10^9 conidia/ml of *M. flavoviride* as described in the materials and methods. These results were summarized by target type against downwind distance from passage of nozzle as follows (table 3.5).

Table 3.5: Average droplet number per square centimetre collected and recovered on artificial targets.

Distance (m)	Mean number of droplets by target type			
	Black cards(V)	M.slides	Locust(II)	KC(V)
5	21.75	52.75	47.1	41.80
10	2.75	34.875	19.75	22.80
15	1.5	22.375	14.31	3.80
20	1.25	18.563	13.2	2.00
25	0.88	13.313	7.32	2.00
30	0.75	10.938	5.30	1.80

KC: 'KromeKote' cards; (V): Raw data taken from appendix 2 (V); (II) data from appendix 2 (II).

Similar statistical methods of analysis and equation to that of trial 1 were used. Details on estimation of mean fungal spores/cm^2 are indicated on appendix 2(IV).

Details of outputs of the linear regression analysis are indicated bellow (table 3.6)

Appendix 2 (IV) and table 3.7 show details of results of the mean fungal spores and mean droplet density containing them down-wind distance (m).

Table 3.6. Summary of mean conidial and droplet deposition/cm^2 against down wind distance (m).

Predictor variable	dependent variable	Coefficient(s) r^2	df	(cf)
Distance(m)	Mean drop/cm^2 on Bc	0.94	4	-1.815
Drops/cm^2	Mean spores/cm^2/pass	0.91	4	1.838
Distance(m)	Mean drop/insect	0.96	4	-1.135
Distance(m)	Mean drop/cm^2 on slide	0.98	4	0.883
Distance (m)	Mean drop/cm^2 on Kc	0.92	4	- 1.442

BC,KC: Mean number of drops on Black and Kromekote cards.

Table 3.7: Average droplet and estimated conidial deposition /cm^2 against down wind distance (m) for trial 1.

Downwind dist(m)	Mean No/cm^2/pass of: drops	conidia
5	53	8266
10	35	5032
15	23	2871
20	19	2093
25	14	978
30	11	367

Results of mean spray deposits/cm^2 for trials 2, 3 and 5 (dates, 28/5, 29/5 and 31/5/91, respectively) were collected from spraying as described in the materials and methods on two different artificial targets but sampling posts were placed at equal sampling points in number and of

downwind distance from passage of nozzle. Therefore, the results on these targets were analysed for effects due to differences both in time and target type using one way analysis of variance.

Table 3.8: Comparison (anova) of spray deposition by trial against both time and down wind distance (m).

Trial	variable	source	df	ss	ms	f	p
2	Distance	between	18	3.6	200	32.66	0.0001**
	Time	between	1	52.9	53	0.52	0.4753ns
	T.type	between	1	52.9	53	0.52	0.4753ns
3	Distance	between	18	4.9	275	18.03	0.0001**
	Time	between	1	72.0	72	0.50	0.4841ns
	T. type	between	1	71.6	72	0.50	0.4841ns
5	Distance	between	18	1.1	595	6.91	0.0001**
	Time	between	1	498.0	498	1.51	0.2266ns
	T.type	between	1	498.0	498	1.51	0.2266ns

T.type: target type;

NB: Total degrees of freedom for comparison of f-value of each variable was the same (37). The F test assumes that the within a variable variances were the same for all the groups.

The analysis shows that neither difference in time nor in target type have showed significant effects as F-values for mean droplet density/cm^2 on time, target type and on distance(m) downwind of spray line by trial.

3.3.2: Compatibility and viability of *M. flavoviride* conidia in formulations

The compatibility and viability tests for the pathogen were carried out as described in the materials and methods for the field trials and germination percentage results of counted 500 spores from sprayed agar coated microscope slides and petri dishes with similar media in placed at similar downwind distances of spray line. The following summary (table 3.9) is from the results of these tests from those slides and petri dishes 48 hr. after spray.

Table 3.9: Percentage germination of *M. flavoviride*.

			Downwind distance (m)					
Trial		Date	5	10	15	20	25	30
Slides	1	27/5 /91	95	98	98	92	98	96
	4	30/5 /91	96	90	94	98	94	95
Petri	1	27/5/91	91	92	94	94	96	98
dishes	4	27/5/91	98	90	90	94	98	92

3.3.3: Locust mortality

Daily observations were taken for all locusts retrieved from arenas. Unfortunately, at the time of these tests the locust stock was heavily infected with *Malamoeba*. Most of the insects died within 3 days, and no differences could be discerned between fungus treated and control groups.

3.4: Discussion.

With a consistent cross wind, the pattern of droplet deposition shown in Figures 3.2 and 3.3. are typical for emissions of ULV spraying equipment (e.g. Heinrichs *et.al.* 1981). The arena design was developed to carry out preliminary field tests with limited resources of live fungal inoculum, manpower and available land. It produces a number of small plots (arenas) receiving a range of mean spray deposits. Had the locust stocks been more dependable, it should have then been possible to correlate speed of kill with droplet impactions.

The position and magnitude of the initial "peak" and diminution of droplet numbers further down-wind is dependent on droplet size, emission height wind speed, air turbulence and the characteristics of the formulation (Johnstone, 1991). The ULV mixture used was based on well established formulating principles where a non-evaporative viscous oil (in this case groundnut oil) is mixed with a solvent (Coutts and Parish, 1967). With chemical pesticides the solvent is used to dissolve the active ingredient, but in this case the Shellsol was added to reduce viscosity and produce a consistent flow with gravity fed atomisers. These results show that this mixture was not harmful to the conidia of *M. flavoviride*. When sprayed in the field, the formulation produces deposits that contain live conidia, and are very similar in magnitude to the "blank" mixture - which is equivalent to a conventional ULV solution (Bateman,1993).

Shellsol K is a paraffinic oil that is produced in the distillation range of 190-225°C. In hot climates it may evaporate from small droplets, however the diameters of droplets containing 50% non evaporative oil cannot reduce to less than 79% of their original size. Assuming that conidia are distributed in spray droplets according to their original volume, an increase in conidial concentration of up to 2 times might be expected in droplets collected far from spray track. The further down-wind droplets had been recovered, the smaller the numbers of conidia counted, and there were no significant differences between the regression analysis for the various droplet assessments (table 3.6). This implies that larger droplets containing greater numbers of conidia settled close to the spray path, and the effects of evaporation are minimal. However, assumptions on droplet size and conidial distribution have been made which require further investigation.

Chapter IV:The effect of infection by *Metarhizium flavoviride* on the behaviour of the desert locust, *Schistocerca gregaria*.

4.1: Introduction

Different species of locusts and grasshoppers behave in different ways. The behaviour of a single species also changes with age, and the size and density of the population. Behaviour is affected by a wide range of external factors which characterise the habitat and among which the weather is dominant as far as locusts are concerned. The knowledge of locust behaviour (with emphasis on flight and feeding in the present work) and of the factors which determine it are essential for an efficient locust control programme (Steedman, 1990)

The purpose of insect control is to reduce crop losses caused by insect damage to plant tissue. Most control measures are designed to kill sufficient numbers of insects so that the total amount of feeding is reduced to an acceptable level. Consequently, routine tests of the efficacy of insecticides both chemical and microbial tend to be based on assessment of mortality. This approach is so common that in the field of crop protection, efficacy is treated as a synonym of mortality. However, insecticides can be effective in protecting crops for a number of other reasons, including reduction of pest food consumption, development, reproduction, and activities such as flight. This is particularly true of microbial agents applied to

debilitate pest insects while remaining endemic (Johnson and Pavlikova, 1986).

Therefore the object of the work described in this chapter was to investigate possible pre-lethal effects of fungal infection on flight and food intake in adult desert locusts.

4.2: Materials and Methods.

The effects of infection on:

4.2.1. Short term flight:

The experimental adults (20 days after fledgling) of which 50 % were male were inoculated topically under the pronotum with an oil-based fungal spore suspension of $4x10^7$/ml using a micro-applicator. Doses included 0.5 and 2μl/insect (low and high, respectively) of the inoculum and 2 μl cotton seed oil / insect for the controls. Nine (3 insect per treatment) mature adults were suspended by a harness around the neck in a wind tunnel of 1.05, 0.40 and 4.55 m in height, width and length, respectively with head-on into a 4m/ sec wind (plate 4.1).

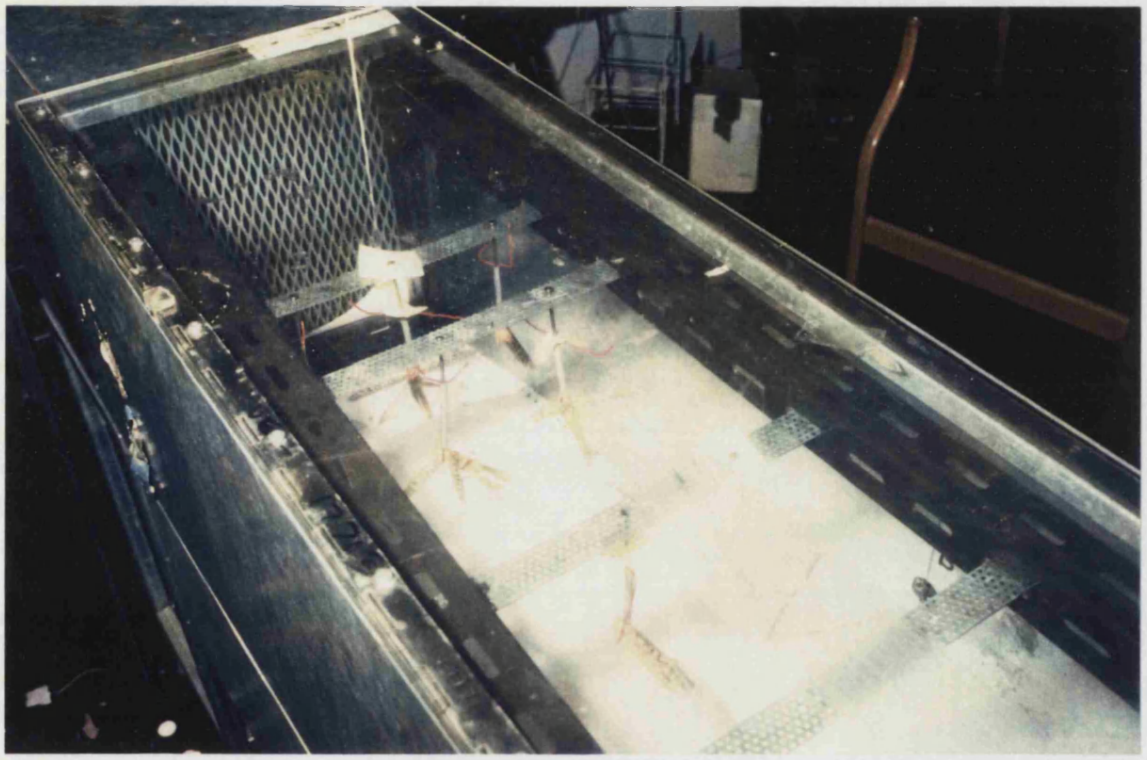


Plate 4.1. Flying locusts in wind tunnel.

See text for explanation.

Flight was initiated by the incoming air with no possibility for the locusts to change direction. The wind tunnel was switched on five minutes after the insects were placed in position. The locusts were forced to fly in two rounds (r1 and r2) each round consisting of four sub-rounds of 10 minutes flight time during which each insect was checked and recorded as flying or nonflying. The insects were rested for 20 minutes after every 10 mins. flight. There was a 40 minutes gap between r1 and r2 during which insects were at rest.Thus the maximal time one experimental adult could fly in the two rounds was 80 mins. The flight time was as % of the total possible (80 min). Temperature in the room and inside the wind tunnel during the day were maintained at 26-28 and 29-30 °c, respectively. The locusts were kept in small plastic boxes individually, provided with fresh wheat seedlings and were maintained at constant temperature (30°C). This experiment was repeated and results were pooled (see section 4.3.1).

4.2.2. Long term flight: This experiment was similar to the short term flight experiment described above, in terms of age, sex ratio, maintenance and type of formulations, suspension of insects in the tunnel, temperature and wind speed (4 m/sec.). However, treatments were 2 μl/insect of ($4x10^7$/ml) fungal spore suspension for those mycosed and 2μl/insect of pure cotton seed oil for the controls. The experimental locusts were flown for 5 h continuously. Each insect was checked every 30 min at minute intervals for five minutes (5 observations every 30 min), and recorded as

flying and nonflying on each occasion. Thus the maximal flight score for a given insect was 50. The experiment was conducted for 5 days after which mycosed insects were all dead. The position of the insects within the wind tunnel was randomised daily. This was possible as each insect was identified with a mark on the head. The experimental adults were kept individually in small labelled plastic boxes placed in a room at constant 30°c temperature and fed at night with fresh wheat seedlings.

4.2.3.Feeding: This experiment was carried out concurrently with the above flight experiment (4.2.2) using the same insects. Each locust was provided with a known fresh weight of wheat seedling during the night. The remaining uneaten food of each day per insect was kept in paper bags, dried (in the oven at 90°c for 24 hr.) and re weighed. The amount of dry matter [(food(mg/insect)] consumed was determined in relation to the dry weight of aliquots of undamaged wheat seedling.

4.2.4: Wing beat frequency: the effect of mycosis on the wing beat performance of twelve adults (6 inoculated and 6 controls) was investigated. The study was carried out using a stroboscope (DAW 201 D). Age, sex ratio, treatments and maintenance of insects were similar to that described in the short time flight (4.2.1) above. The insects were suspended in the wind tunnel (see plate 4.1 and methods for the short time flight for description). However, contrary to the other flight experiments, locusts were flown singly in a dark room. The tunnel was also covered from outside

with black Crèpe-paper to make sure that light was reaching the respective experimental insect in place only from the same source (stroboscope). The frequency of the stroboscope flash was adjusted until the wings appeared to be stationary. The readings (flash per second) from the stroboscope were taken as equivalent to wing beat / second of each insect.

4.3: Results

]4.3.1. Effect of fungal infection on flight capability of desert locusts.

4.3.1.1. Short term flight.

The results of two tethered short flight experiments were pooled, analysed and are presented in table 4.1.

Locusts infected with *Metarhizium flavoviride* had a significantly reduced short term flight performance by comparison with controls from day three post application onwards. This was true for insects that received high (about 90,000) and low (about 22,500) conidia.

Flight performance of control insects was not significantly different between rounds (see table 4.2).

4.3.1.2. Long term flight

The decline in flight capability of mycosed insects noted in short flights was observed with long flights. Once again a significant difference between treatments was observed 3 days after inoculation (table 4.3).

Table 4.1. The effects of *M.flavoviride* infection on short term flight tethered flight performance in locusts. Two sample t-test was used.

Treatment means	**Days after treatment ± s.e**				
	1	2	3	4	5
Control	56.7	57.7	56.2	46.0	52.2
	±2.22	±1.76	±1.92	±3.71	±0.60
Low dose	58.0	53.0	51.8	35.4	40.5
	±3.32	±1.59	±2.46	±2.38	±2.70
High dose	56.8	52.7	44.6	26.30	24.50
	±3.63	±2.22	±2.48	±2.02	±1.71
Treatment comparisons					
Low v Con	0.33	1.96	1.19	2.40*	4.24*
Low v high	0.24	0.77	2.08	2.91*	4.39*
High v con	0.04	2.12	3.42*	4.61**	17.9***

Con, *, **, ***, = Significant at 5 %, 1% and 0.1%, respectively.

Low dose = 22,500, high = 90, 000 conidia/insect

control=2μl cotton seed oil/insect.

Table 4.2: Comparison of short flight performance of uninoculated insects by round (r1, r2).

Day after treatment	Mean flight score r1	r2	df	F	P
1	28.50	28.17	10	0.02	N.S
2	27.00	30.67	10	2.85	N.S
3	28.00	27.67	10	0.03	N.S
4	26.25	19.75	10	4.45	N.S
5	26.75	24.50	10	1.00	N.S

flight score = actual number of flying insects expressed as % of the total uninoculated locusts.

Table 4.3. The effects of *Metarhizium flavoviride* infection on long term tethered flight performance of locusts. Means ± se as percentage of total flight score possible.

Day after treatment	Control	Mycosed			
	Mean ±se	Mean ±se	Df	t	P
1	71.33±7.21	70.50±5.03	22	0.09	N.S
2	56.50±4.41	56.83±4.27	22	1.07	N.S
3	60.50±4.10	42.83±3.27	22	3.36	<0.05
4	58.33±3.58	28.91±7.99	21	3.46	<0.05
5	65.00±7.55	19.38±5.07	17	5.04	<0.05

Means ± se as percentage of total flight score possible.

4.3.2. The effect of fungal infection on feeding by locusts.

The food consumption of the locusts used in the long term flight experiment was also determined. Mycosed insects ate significantly less wheat seedling than controls from day 3 after inoculation (table 4.4, fig 4.2).

Table 4.4. The effect of *Metarhizium flavoviride* infection on feeding by locusts.

	Control		Mycosed			
	mg dry wt eaten					
Dat.	Mean±s.e	n	Mean±s.e	n	t	P
1	104.0±16.17	12	107.3±15.16	11	0.15	0.470ns
2	91.3±11.06	12	118.1±7.18	12	2.03	0.054ns
3	94.5±12.22	12	51.5±13.00	12	2.41	0.0250*
4	150.1±18.02	12	38.4±12.16	12	5.14	0.00001**
5	139.6±17.66	12	44.2±10.92	10	4.60	0.0002**
6	154.8±14.21	6	29.9±15.41	3	5.40	0.001**

Dat.; day after treatment.

n.s, * and **; non significant, significant and highly significant at the 5% level, respectively.

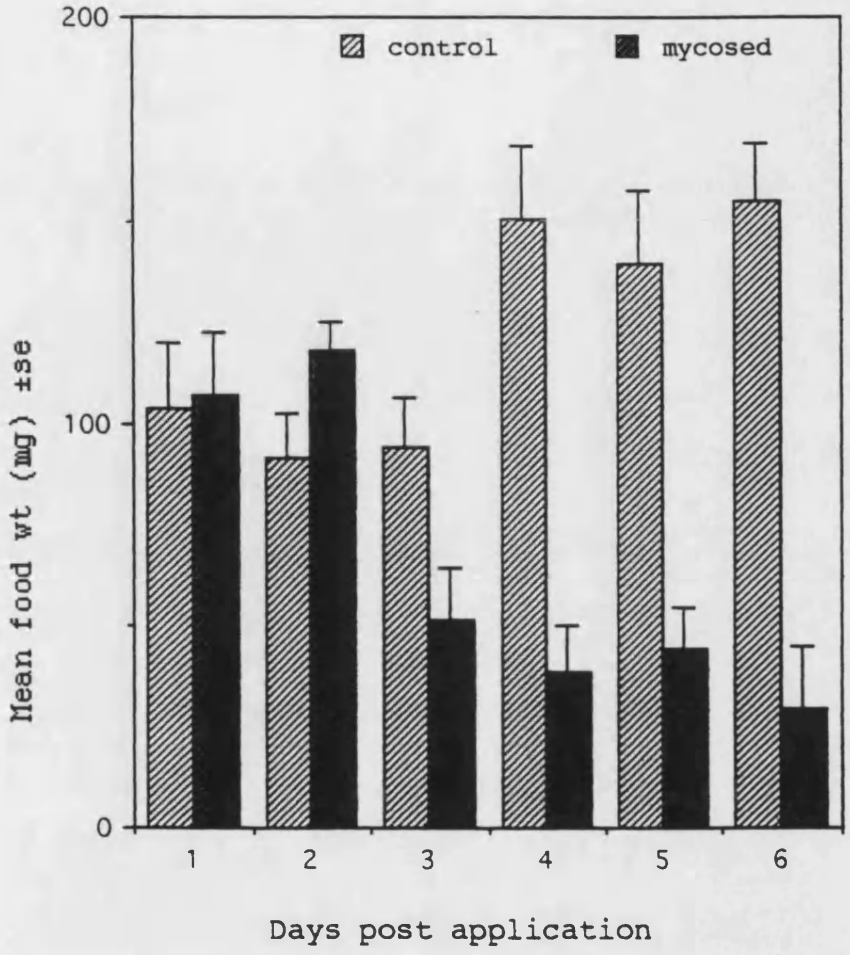


Figure 4.1: Mean food consumption (mg) per adult locust after treatment.

4.3.3. The effects of fungal infection on wing beat frequency of locusts.

The wing beat frequency of mycosed insects was determined using a stroboscope and the results are shown in table 4.5. Infected insects had a significantly lower wing beat frequency than that of controls on days 4 and 5 after inoculation.

Table 4.5.The effect of *Metarhizium flavoviride* infection on wing beat frequency of locusts.

D.a.t	**comparison(mean±se)**				
Dat.	**inoculated**	**control**			
	beats/sec (mean±se)		**DF**	**T**	**P**
1	33.07±2.061	34.93±4.74	10	0.36	N.S
2	28.98±2.18	35.90±3.093	10	1.83	N.S
3	20.12±3.07	27.07±1.621	10	2.03	N.S
4	22.5±2.693	30.26±1.111	10	2.671	0.0236*
5	16.2+2.732	28.67+1.231	7	4.91	0.0017**

N.S, *, ** : not significant, significant and highly significant at the 5% level respectively.

Dat: Day after treatment

4.4. Discussion

Infection with *Metarhizium flavoviride* causes a significant decline in flight capability and wing beat frequency of locusts. The difference between experimental and control insects becomes significant 3 day after inoculation which is around the time when the fungus invades the body cavity of the insect (Joshi, pers.com). It is not possible to make a direct comparison between the

present work and published data on locust flight because of the different methods used. However, the wing beat frequency of the uninoculated desert locusts was similar to that recorded by Goldsworthy and Coupland (1974) for adult male *Locusta migratoria*. They showed that the wing beat frequency after 10 min flight was about 20 Hz in their studies concerning flight performance of normal locusts and those in which the glandular lobes of the corpora cardiaca had been removed.

The cause of the poor flight performance could be related to the decline in food intake, in that a semi-starved insects may not have the metabolic reserves to support flight. This possibility was explored in the experiments described in the next chapter. Moore *et al*, (1992) have also shown that locusts infected with *M.flavoviride* eat significantly less food than the controls. They established a dose as low as $5.5x10^5$/ml conidia was sufficient to reduce food intake. This is not the only case where a pathogen has been shown to affect feeding behaviour of an insect. Johnson and Pavlikova (1986) indicated that infection with *Nosema locustae* Canning (Microsporidia:Nosematidae) causes significant reduction of consumption by Grasshoppers, *Melanoplus sanguinipes* species (Orthoptera:Acrididae).

Whatever the cause of the phenomena recorded here, it is clear the pre-lethal effects on flight performance and feeding behaviour are beneficial from a practical point of view, in that they might lead to reduced crop damage and

interference with swarming behaviour, which is an important aspect of locust pest biology.

Chapter V:Studies on the effects of infection by *Metarhizium flavoviride* on haemolymph lipid and carbohydrate levels of *Schistocerca gregaria*.

5.1 Introduction

5.1.1: Haemolymph lipid and carbohydrate levels.

The major activities of insects like other animals rely on the availability of nutrition in the right form as source of energy. Locusts in common with many other animals use fats as the major fuel during migration (Goldsworthy, 1990). Though carbohydrate is used to initiate flight it is readily available both in the form of muscle glycogen and as blood sugar (Goldsworthy, 1990). Previous work by Beenakkers (1983) indicates that the availability of blood substrates such as lipid and carbohydrate in locust varies considerably depending on developmental stages and is influenced by such internal and external factors such as: nutrition, temperature, sex, starvation, and diapause.

Jutsum and Goldsworthy (1976) found that in *Locusta migratoria*, carbohydrate is the predominant energy source for the first 20 to 30 min flight when is utilized at the rate of 120 μg/min. The switch to lipid utilization is brought about by the release of the adipokinetic hormone (AKH) from the glandular lobes of the corpora cardiaca (Jutsum and Goldsworthy (1975); Jutsum and Goldsworthy, 1976). AKH mobilizes lipid from the fat body and increases diacylglycerol levels in the haemolymph while increasing

lipid utilization by the flight muscles (Highnam and Hill, 1977).

Long term starvation of locusts also results in a gradual increase in haemolymph diacylglycerol which reaches levels similar to that in flown locusts or in locusts that have received injections of physiological amounts of AKH (Jutsum and Goldsworthy, (1975); Jutsum and Goldsworthy 1976); and Mwangi and Goldsworthy (1977a)). However, AKH is not responsible for starvation induced hyperlipaemia.

5.2: Materials and Methods

Experiment 5.2.1: **Quantification of total haemolymph lipid level of mycosed adult** *Schistocerca*.

Total lipid level of blood samples taken from 23 day old 6 locusts inoculated with *Metarhizium flavoviride* spores in oil and 6 treated with cotton seed oil 50% male adult *Schistocerca gregaria* were analysed using the vanillin reagent method (Goldsworthy *et al.*, 1972b) for the quantification of lipids. Insects were inoculated with 90, 000 spores as described in chapter 4. The animals were reared in the locust rearing facility of University of Bath . They were supplied with fresh wheat leaves, bran and maintained in a control room at 30°c with 12 h light/12 h photoperiod. Haemolymph was collected with a microcap from a puncture made in the arthrodial membrane at the base of the right hind leg of each animal 72 hr post inoculation. The blood sample was centrifuged using a micro-centrifuge (MSE) at 1300 rpm for 15 minutes to spin down cells and

fungal hyphae. 5 μl plasma was then transferred to the base of specimen tubes. A similar amount (5μl) of standard lipid (100μg cholestrol/10μl ethanol) was prepared in different tubes. 0.2ml of conc.H_2SO4 was added to all tubes. All tubes were placed in a dry block heater 100°c for exactly 10 minutes then placed on ice to cool.

3ml of Vanillin reagent (800ml ortho-phosphoric acid, 200ml dist. H_20, 2 g Vanillin) was added to all tubes and mixed thoroughly using a vortex mixer. The O.D of the solution was determined after 30 minutes using a double beam U.V Spectrophotometer at 620 nm Wave length against the blank (0.2ml of conc.H_2SO4 in 3ml of Vanillin reagent).

Experiment 5.2.2: **Effects of starvation on haemolymph total lipid level.**

Three week old adult locusts were placed in individual containers and fed on wheat seedling or starved without access to water for 72 h. 5 μl samples of blood were taken from fed and starved insects. The samples were analysed in a similar way to that described above (5.2.1)

Experiment 5.2. 3: **The effect of adipokinetic hormone on haemolymph lipid concentration of adult** *Schistocerca gregaria* **(SGR) infected with** *Metarhizium flavoviride*.

Twelve (6 controls and 6 experimentals) mixed sex adult (23 day old) *Schistocerca gregaria* treated and maintained as described above were injected with fresh corpus cardiacum (c.c) extracts prepared from the glandular

lobes of an adult male *Schistocerca*. The preparation of c.c extracts was carried out as follows. A mature male locust was decapitated using sharp scissors and the head was bissected with an asymmetric longitudinal cut. The portion containing the brain and foregut was placed in a dissecting dish and secured with two pins. The preparation was covered with ringer solution. The whole corpora cardiaca complex was removed from behind the brain using watch-maker forceps and fine scissors. The dissection was completed on a piece of glass embedded in the wax. Adhering trachea, fat body etc were teased away. Then corpora cardiaca was placed in an "Ependorf tube" containing 500 μl methanol. The tissue was disrupted by ultrasonicating for 2 min and the debris were removed by centrifugation at 12,000 g using the high speed microcentrifuge. The supernatant was then transferred to another "Ependorf tube". The methanol was evaporated from the resulting supernatant by freeze-drying. The residue was then dissolved in 200 μl of simple saline (7.5 g NaCl, 0.375 g KCl/litre) and kept on ice until used. Treatments (injections) were conducted in such a way that half of the control insects were injected with 20 μl saline each and the other half with same amount of saline containing a 0.05 pairs of c.c each. Half of those inoculated with fungal spores were also injected with 20μl saline each and the other halves with 20μl/insect of c.c extracts. Blood samples (from each group) for the analysis of haemolymph lipid concentration were taken before injection and an hour latter after injection.

5.2.3: Quantification of Haemolymph Carbohydrate Level of Adult *Schistocerca gregaria*.

Haemolymph carbohydrate levels of mycosed, starved and control adult desert locusts, reared and maintained as described in the total lipid experiments were investigated. Blood samples were taken from two groups of 23 day old mixed adults 3 day post inoculation with *Metarhizium flavoviride* spores and from another group of adults starved for 3 days (tables 5.4 and 5.5, respectively) as described in the methods for total haemolymph lipid experiments above.

In some experiments plasma was boiled for 15 min to precipitate protein and then centrifuged for 15 min at 1300 r.p.m and anthrone positive carbohydrate was determined in the supernatant.

The total haemolymph carbohydrate (Cho) levels were determined using the anthrone method developed by Roe (1955). The anthrone reagent was 0.05% anthrone, 1% thiourea in 66% v/v sulphuric acid, and was stored in a dark bottle. Thiourea was used as an anti-oxidant to prevent the oxidation of anthrone in sulphuric acid and so help storage. Anal a R glucose was dried in vacuum oven and dissolved in saturated benzoic acid, 100 mg /100ml, to give a standard glucose solution. Haemolymph was centrifuged for 15 min in a microfuge at 1300 rpm to remove haemocytes and fungal hyphae.

5 ml of anthrone was added to 3μl of plasma and to 3 μl of saline. This gave a final H_2SO_4 concentration of 60%. The tubes were placed in a boiling water bath (in a metal tray to cut down direct light) for 15 minutes, then they were stored in the dark for 30 minutes to minimise exposure to light as the blue colour formed by the reaction of the anthrone reagent with carbohydrate fades in light.

The absorbance of the solutions was measured against a blank. Carbohydrate was quantified with respect to a glucose standard at 620 nm using a Cecil CE 505 double beam U.V spectrophotometer. Locusts were flown for 10, 30 and 60 min in a wind tunnel as described in the previous chapter.

5.3: Results

Experiment 5.3.1:Quantification of total haemolymph lipid level of mycosed and starved *S.gregaria*

Table 5.1: The effect of fungal infection on total haemolymph lipid of mixed sex adult *Schistocerca*.

Control		Mycosed			
total lipid (μg/μl)					
Mean±s.e	n	Mean±s.e	n	T	P
6.74±0.90	6	3.6±0.80	6	2.6	0.026*

*: Significant at the 5% level (10 df) (two sample t-test).

The haemolymph lipid level of infected insects was significantly less than that in uninoculated insects. One possible reason for this is the utilization of the insect lipids by the fungus. Other possibilities are (1) that the reduced food intake by the mycosed insects was insufficient to maintain haemolymph lipid levels and (2) mycosed locusts were unable to mobilise lipid stored in the fat body to maintain the haemolymph concentration.

Table 5.2: The effect of starvation on haemolymph lipid level of adult *Schistocerca*.

Treatment	Fed	n	Starved	n	T	P
Mean(μg/μl)	8.485	6	6.213	6	1.27	0.234

Starvation had no significant effect on the haemolymph lipid level of the experimental adult locusts(table 5.2).

Experiment 5.3.3: **The effect of Adipokinetic Hormone on haemolymph lipid concentrations of mycosed locusts.**

In this experiment the mycosed insects did not have a significantly lower concentration of lipid than that in controls. However, Insects from both treatments had significantly elevated haemolymph levels following injection of c.c extract (table 5.3). In neither case did injection of saline have any effect.

Table 5.3: Haemolymph lipid concentration before and after injection of c.c extracts and saline.

	Total lipid ($\mu g/\mu l$)±se					
Treatment	before	n	after	n	T	P
Cont.+c.c	4.864±0.73	7	19.9±4.0	7	3.73	<0.05)*
Cont.+sa.	6.281±1.134	7	5.1±1.7	7	0.60	0.60ns
Myco.+c.c	3.213±0.614	7	16.0±2.9	7	4.7	<0.05)*
Myco.+sa.	4.53± 1.001	7	4.6±1.3	7	0.59	0.57ns

*, ns; significant and Not significant at the 5 % level, respectively.

c.c, sa: corpora cardiacum and saline, respectively

control = treated with saline

mycosed = inoculated with *Metarhizium flavoviride*

Thus even though infection 3 days after inoculation was well advanced (appearance of fungal hyphae in the haemolymph), the diseased locusts were able to mobilize the reserve lipid in response to exogenous AKH.

Table 5.4. The effect of starvation on carbohydrate - haemolymph plasma

	carbohydrate concentration (μg/μl)					
	fed		starved			
	mean±	n	mean±se	n	t	P
Native plasma	91.5±9.24	6	74.12±12.23	6	1.13	0.34
Deproteinised plasma	165±8.19	6	104±4.71	6	6.45	<0.001

Concentration of anthrone positive carbohydrate- the haemolymph plasma of starved insects was not significantly different from that of fed insects (table 5.4). When the plasma was deproteinised by boiling, however, the carbohydrate concentration increased substantially in insects of both treatments. Carbohydrate concentration-the haemolymph plasma of fed locusts was significantly greater than that of starved locusts (table 5.4).

Table 5.5. The effect of mycosis on haemolymph plasma carbohydrate level of adult *Schistocerca*.

	Carbohydrate concentration (μg/μl)					
	Control		mycosed			
	mean±se	n	mean±se	n	t	P
Normal plasma	73.5±6.74	6	32.05±4.26	6	5.21	0.0004
Deproteinised	165±11.1	6	107.7±6.72	6	4.42	0.0013

In contrast to 3 day starvation, infection with *Metarhizium flavoviride* resulted in a significantly lower

carbohydrate concentration (table 5.4) whether the plasma was used native or deproteinised. Statistical comparisons were made between the two experiments (starvation and fungal infection). These revealed that while there was significantly less carbohydrate in the native plasma of mycosed insects than in the plasma of starved insects ($P<0.001$), this was not so with deproteinised plasma ($P=0.649$).

Table 5.6. Haemolymph plasma total carbohydrate level of mycosed (3 day post inoculation) locusts after flight.

Treatment		flight time (min)	Carbohydrate concentration in plasma (μg/μl)	
			n	mean±se
f1	mycosed	10	9	50.53±11.44
f2	mycosed	30	9	39.43±10.10
f3	mycosed	60	9	38.97±12.29
f4	mycosed	0	9	59.53±4.31
c1	control	10	9	78.33±12.41
c2	control	30	9	72.27±6.37
c3	control	60	9	66.72±7.32
c4	control	0	9	108.6±4.88
c5	untreated	0	9	112.1+7.81

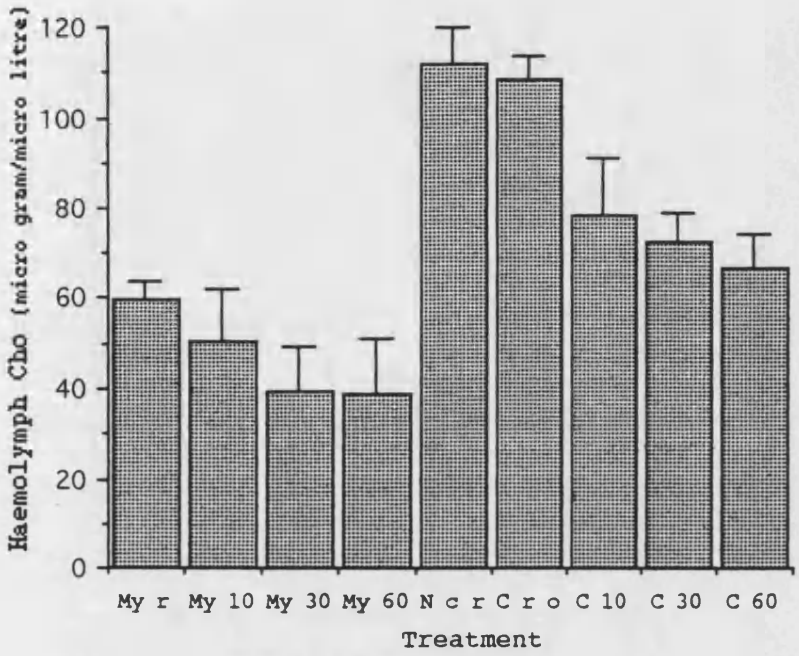


Figure 5.1. Effects of infection (72 hr) on haemolymph carbohydrate level of resting and flown locusts (Bars s.e. values).

N.B: My r, My 10, 30, 60; Mycosed resting, mycosed flown for 10, 30, and 60 min, respectively.
N c r, C r o, C 10, C 30, C 60; Untreated resting, oil treated resting controls, oil treated controls flown for 10, 30 and 60 min, respectively.

10 min of flight caused a significant drop in the haemolymph plasma carbohydrate of control locusts (Table 5.6 and Fig.5.1). Longer flight resulted in a further, but not significant reduction in carbohydrate levels. As expected plasma carbohydrate in mycosed insects was significantly less than their control counterparts. Flight also caused a reduction in plasma carbohydrate of mycosed insects, but the difference was only significantly differed from that at rest after 30 min of flight (between mucosed groups)

5.4. Discussion

Adult locusts infected with *M. flavoviride* had significantly less haemolymph plasma lipid than controls. This analysis was carried out only three days after inoculation. At this time the fungus has invaded the body cavity, but has not grown extensively in the blood. Although plasma lipid was determined in the present work, the values were similar to the total blood lipid concentration recorded for adult *Schistocerca gregaria* in the present work and by others (e.g. a mean of 8.1 ± 0.95 $\mu g/\mu l$: Charnley, pers comm.) suggesting, as one might expect that the haemocytes do not contain much of the detectable lipid in the haemolymph.

As suggested in the results, the effect of mycosis on blood lipid in the locust could be due either to a direct effect of the fungus or indirectly to a semi-starved condition brought on by the reduced food intake in infected

insects (see last chapter). The last named seems unlikely as 3d of total food deprivation had no significant effect on blood lipid. Indeed further starvation would probably have caused an increase in blood lipid, as seen in *Locusta migratoria* (Cheeseman and Goldsworthy, 1979), rather than the decline observed in infected insects. One alternative explanation is that mycosed insects are unable to mobilise lipid from their fat body or that there is so little lipid in their fat bodies that they are unable to maintain plasma levels. However, neither of these explanations appears to be correct since injection of an extract of the corpora cardiaca containing the adipokinetic hormone caused a large significant increase in plasma lipid in both control and mycosed insects. The extent of the hyperlipaemia was similar to that recorded by Goldsworthy and Mordue (1973) when they injected an extract equivalent to 0.05 of a gland pair into a desert locust.

Plasma carbohydrate levels were also adversely affected by mycosis. As was the case for lipid the result could not be explained on the basis of semi-starvation as even total food deprivation did not cause any effect on native plasma carbohydrate concentration. However, a different picture emerged when carbohydrate levels were determined in blood that had been deproteinised by boiling. Considerably more carbohydrate was present in boiled plasma from all treatments than was determined in native plasma. In addition starved and mycosed deproteinised plasma had significantly less carbohydrate than in the respective controls. These results are difficult to explain. If time

had permitted it would have been useful to compare the effect of an alternative deproteinising procedure, eg denaturation with trichloroacetic acid, on plasma carbohydrate. Without this information any suggestions are purely speculative. One possibility is that boiling solubilised some glycoprotein which increased the anthrone positive plasma carbohydrate. This component of the plasma carbohydrate was equally affected by starvation and mycosis. However, only further work will reveal the significance of this observation.

When locusts start to fly they use carbohydrate initially as a fuel. Subsequently they make use of their more substantial stores of lipid (Goldsworthy *et al* 1972a; Cheeseman *et al* 1976). Although during early flight carbohydrate, in the form of trehalose, is mobilised from the fat body the haemolymph carbohydrate concentration declines over ca the first 30 min (Goldsworthy *et al*, 1973). A decline in plasma carbohydrate was seen here with both control and infected locusts, though only with control locusts was the change significant after 30 min (between the control groups).

In the light of the above the hypothesis was made that one of the reasons for the decline in flight capability of mycosed locusts observed in the previous chapter was a failure of the insects to provide the necessary metabolic fuel. This possibility is investigated in the next chapter.

Chapter VI: The influence of exogenous trehalose on the flight performance of adult *Schistocerca* after infection by *M.flavoviride.*

6.1:Introduction

It was established earlier in this work that mycosed insects do not fly well and have reduced concentrations of haemolymph plasma lipid and carbohydrate. The latter can't be accounted for on the basis of reduced food intake. The hypothesis is made that the fungus either interferes with the mobilisation of energy reserves or competes with the insect for energy reserves which as a consequence are less available for flight. If the poor flight performance of mycosed locusts is due at least in part to reduced fuel then it follows that injected supplement should improve flight performance. This possibility was investigated in the experiments described in this chapter. Trehalose was chosen to be injected into the locust as an energy supplement because carbohydrate is the fuel used at the onset of flight in the locust (Goldsworthy and Coupland 1974) and trehalose makes up >90% of haemolymph soluble carbohydrate (Goldsworthy, 1990).

6.2. Materials and Methods

The influence of trehalose on tethered flight performance of mycosed and control adult *Schistocerca* in a wind-tunnel (plate 6.1) was investigated using two methods.

Method 1: Equal number of male and female 23 day old adult locusts were used in this experiment. Insects were tethered in the wind tunnel and the air turned on. They were then recorded as flying or non flying at: (a) 5, 10, 30 and 60 min or (b) 10, 40, 100 min. Insects were then injected either with 25 μl saline (7.5 g NaCl, 0.375 g KCl/litre) or 6 mg trehalose in 25 μl saline (Goldsworthy and Coupland, 1974) and then placed back in the wind tunnel. They were

recorded as flying or nonflying at intervals as before. The "flight score" was the number of observation when flying was noted.

Method 2: In this experiment either equal number of males and females were used or the sexes were treated separately as indicated in the results section. The flight performance of the locusts in the wind tunnel was assessed over a period of 1 h before and after injection with 25 μl of saline or 6 mg trehalose in 25 μl of saline. The locusts flight was recorded on video (camcorder) with a timing device attached. The video was played back through a monitor and the performance of each insect was assessed (time in seconds spent flying during the observation period).

The position of the experimental locusts in the wind tunnel was altered between each flight (before and after treating with trehalose).

6.3: Results

Method 1 was used to record the flight activity of mycosed and control locusts before and after injection with saline or trehalose. Equal number of male and female locusts were used in the experiment.

Table 6.1: Flight performance of tethered locusts infected with *Metarhizium flavoviride*.

(i) before injection with trehalose supplement.

	flight score					
Time	**Mean±se**		**Mean±se**			
(min.)	**Control**	**n**	**Mycosed**	**n**	**T**	**P**
5	9.48±1.26	12	7.175±0.1553	12	1.85	0.0815ns
10	11.04±1.2	30	6.483±1.031	30	2.89	0.0055*
30	9.72±2.7	12	4.167±2.28	12	1.59	0.1271ns
40	9.48±1.5	18	4.466±1.281	18	2.49	0.0177*
60	12.00±1.9	12	5.555±2.051	12	2.31	0.0306*
100	9.874±2.2	12	4.320±1.713	18	2.09	0.0444*

* significant at 5% level (two sample t-test).

(ii) after injection with trehalose supplement

	flight score							
Time	**Mean±se**				**Mean±se**			
Time	**Control**				**Mycosed**			
(min)	**sa**	**n**	**tr**	**n**	**sa**	**n**	**tr**	**n**
5	10.2[a]	6	10.2[t]	6	5.57[at]	6	8.4	6
10	9.20	6	10.22	6	5.56	6	8.1	6
30	9.59	6	9.17	6	5.84	6	7.51	6
40	7.22	6	9.45	6	4.08	6	5.19	6
60	9.72	6	9.72	6	5.84	6	11.5	6
100	8.04	6	11.5[xi]	6	5.92[x]	6	5.64[i]	6

Results with same letter are significant at 5 % level (two sample t-test)

Table 6.1 (i-ii) and figure 6.1 show the flight performance of tethered locusts before and after an injected supplement of trehalose. Mycosed insects flew consistently less well than control insects through out the test period confirming the experiment described in chapter 4 at all but two assessment times before injection with trehalose or saline. Mycosed insects had a significantly lower flight score than controls.

The total flight scores for each treatment, before and after injection with trehalose or saline are shown in tables 6.1(i-ii). There are no significant differences between treatments. However, consistent with the experiment described in chapter 4, the mean flight score of mycosed insects was less than that of the controls. There was an indication that a trehalose supplement may be boosting the flight performance when saline did not.

Experiments measuring flight performance of tethered locusts whether as here in the wind tunnel or on a flight mill (Goldsworthy *et al*, 1972a) show much variability with treatments. In the present case the method used to determine flight performance (method 1) was not very sensitive. In addition the experiments were performed on insects 3 day after inoculation. A time when flight performance and feeding were first affected. Using insects later in the infection process may have produced greater differences between treatments but other factors including death of infected insects may have clouded the shine. The experiment was repeated using method 2.

Figure 6.1. Flight performance in locusts

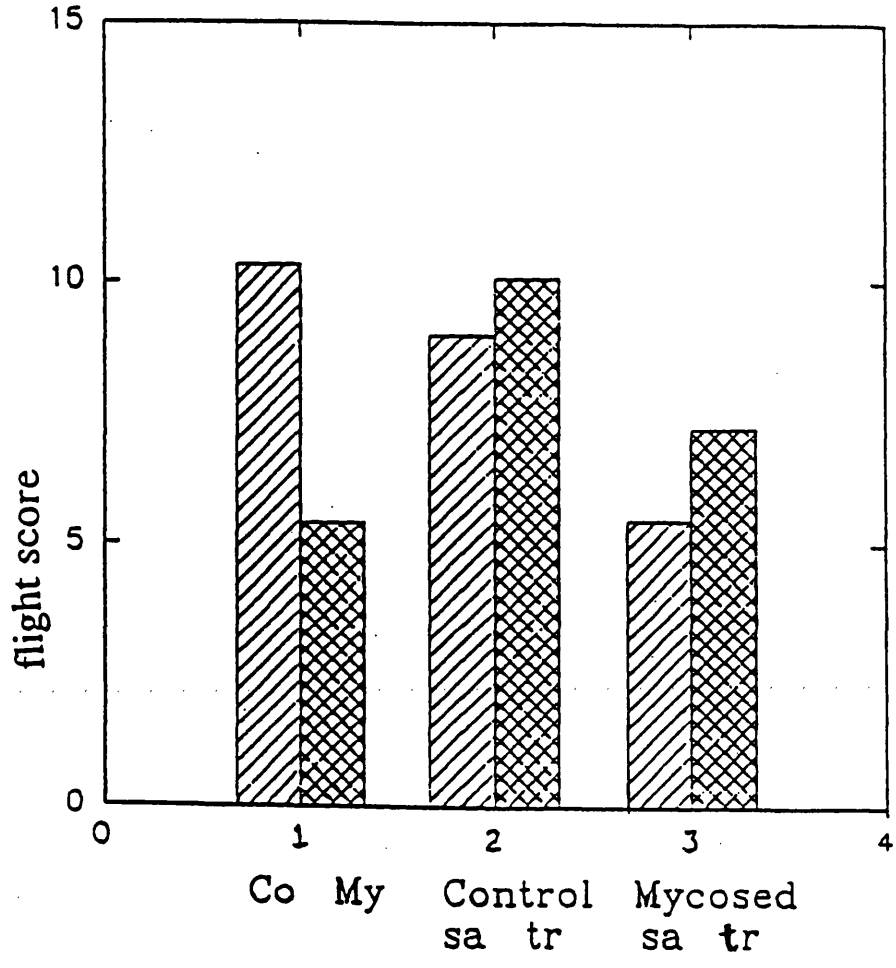


Table 6.2: The effect of mycosis on the tethered flight performance of adult *Schistocerca*.

(a): before injection		**(b):after injection:**			
Control	**Mycosed**	**Control**		**Mycosed**	
Mean±se	**Mean±se**	**Mean±se**		**Mean±se**	
		Sa	**Tr**	**Sa**	**Tr**
1026±305.1	445.2±212	647±416	1181±416	352±181	1170±554

before injection = 12 insects/treatment

after injection = 6 insects/treatment

Table 6.2 and figure 6.2 show the flight performance of locusts before and after injection with either saline or trehalose. A camcorder (Method 2) was used to video the flying locusts and the tape was played back to determine how long each insect flew during the 1 h experiment period. A similar pattern of results was achieved to the last experiment in that mycosed insects performed less well than controls and trehalose tended to improve the flight performance of mycosed insects. But again there were no significant differences between the treatments, due to large variability in the flight performance of the locusts.

Plate 6.1. Flying locusts in wind tunnel. Flight being recorded with a camcorder. Note image on TV monitor.

A third experiment was performed. Particular care was taken with this experiment to ensure consistency in the procedure. In addition the performance of male and female locusts was recorded separately. In the previous experiments mixed sex populations were used and no separate record of male and female flight performance was taken. The results of this experiment are shown in table 6.3 and figure 6.3 and 6.4 . The flight performance of female locusts did not differ significantly between any of the treatments and none of the trends observed in the previous experiment were seen (tables 6.1 (i-ii), 6. 2 (a-b) and figs. 6.1 and 6.2). However, with male locusts not only was the flight time of mycosed individuals significantly less than that of controls, but a trehalose supplement significantly boosted pre-injection flight performance ($t = 2.901$, $p<0.02$) whereas saline did not ($t= 1.541$, $p>0.1$) (tables 6.3-6.6, figs.6.4 and 6.4).

Table 6.3: Flight performance of female and male locusts before injecting trehalose (method 2).

flight performed (seconds) by:

	Mycosed		**Control**	
.	**Female**	**Male**	**Female**	**Male**
Mean	1322	562	1375	1426
S.e	305	129	365	372

Table 6.4: Comparison of flight performance of locusts by gender before injection with trehalose supplement(Method 2)

(a) male locusts:

Groups compared	Mean1	n	Mean2	n	T	P
con(m) vs my(m)	1426.3	12	562.1	12	2.194	0.039*

(b) female locusts:

Groups compared	Mean1	n	Mean2	n	T	P
con(f) vs my(f)	1375.4	12	1321.6	12	0.113	0.911

f, m, con, my; female, male, control and mycosed respectively. * significant at 5 % level. Flight score in table 6.4 were used for analysis.

Figure 6.2. FLIGHT PERFORMANCE IN LOCUSTS

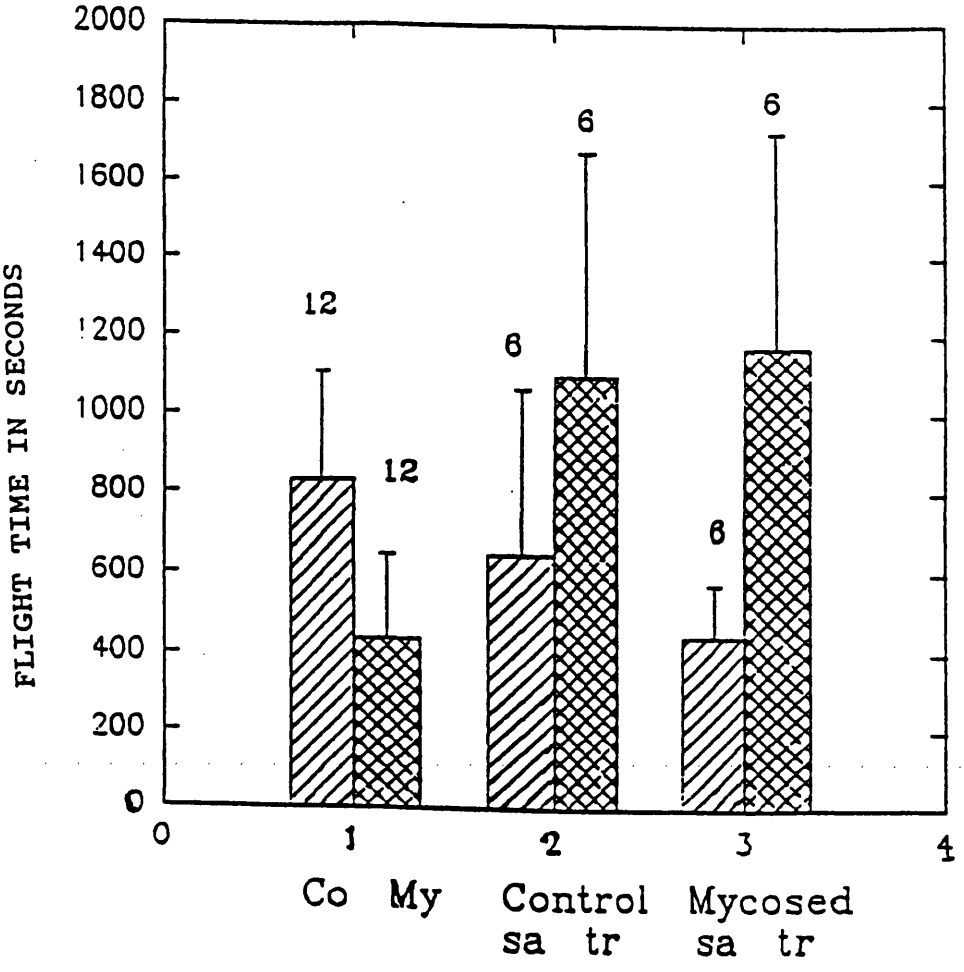


Table 6.5: The effect of trehalose on the flight performance of *Schistocerca*.

	Flight performed by:							
	Mycosed+:				Control+:			
	Saline		Trehalo.		Saline		Trehalo.	
No	F	M	F	M	F	M	F	M
1	639	1147	2556	769	2507	1878	673	863
2	571	602	591	994	1116	2235	3600	1235
3	1152	771	2400	2205	2470	2400	1842	2852
4	2540	873	2342	588	1106	856	1596	1458
5	2870	536	976	2731	1037	934	1159	1270
6	3124	430	1027	125	1225	725	1630	1173
Mean	1816	727	1649	1010	1577	1505	1627	1475
S.e	474	107	357	169	289	197	348	287

N.B: F, M, female , male respectively.

Table 6.6: The flight performance of male locusts after treating with trehalose.

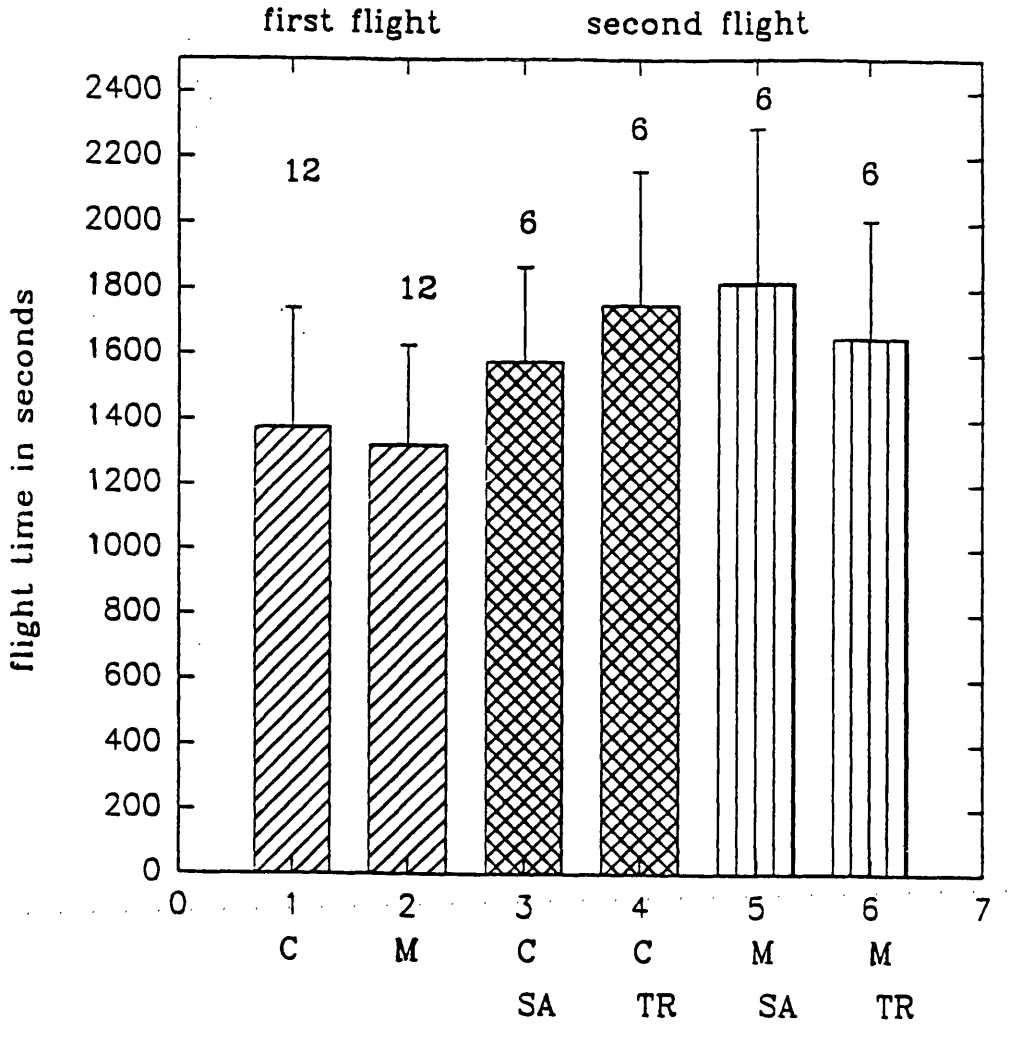
(i) Male *Schistocerca*.

Groups compared.	Mean1	n	Mean2	n	T	P
con(m+sa) vs con(m+tr)	1504.7	6	1475.2	6	0.10	0.94
con(m+sa) vs my(m+sa)	1505.0	6	727.0	6	2.39	.038*
con(m+sa) vs myc(m+tr)	1504.7	6	1235.3	6	0.53	0.61
myc(m+sa) vs myc(m+tr)	726.5	6	1235.3	6	1.20	0.26
con(m+tr) vs myc(m+sa)	1475.2	6	726.5	6	2.45	0.034*

myc(m+sa), my(m+tr): mycosed male treated with saline and trehalose, respectively.

Con(m+sa),con(m+tr): control male treated with saline and trehalose, respectively.

Figure 6.3. Flight performance of female locusts



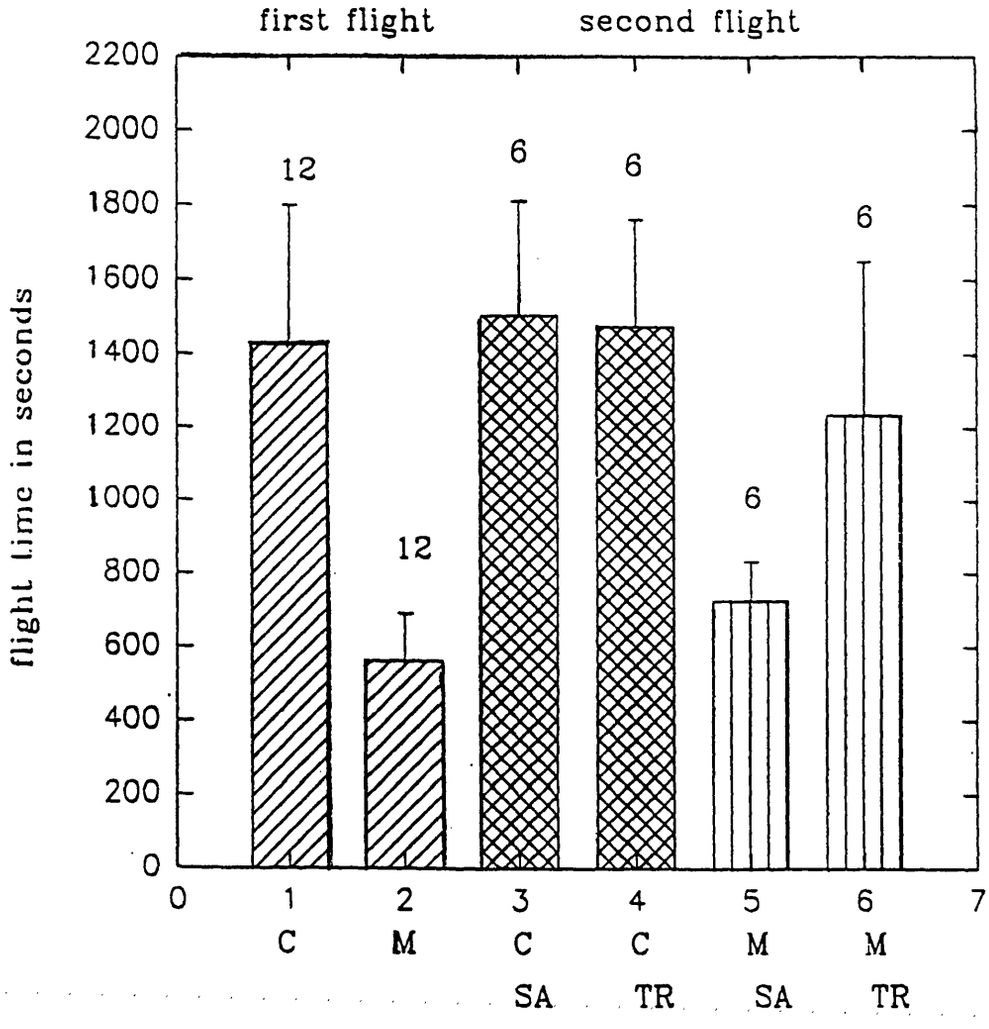
(ii) Female *Schistocerca*

Groups compared.	Mean1	n	Mean2	n	T	P
con(f+sa) vs con(f+tr)	1576.8	6	1750.0	6	0.35	0.74
con(f+sa) vs myc(f+sa)	1576.8	6	1816.0	6	0.43	0.68
con(f+sa) vs myc(f+tr)	1576.8	6	1648.7	6	0.16	0.88
con(f+tr) vs myc(f+sa)	1750.0	6	1816.0	6	0.11	0.92
con(f+tr) vs myc(f+tr)	1750.0	6	1648.7	6	0.19	0.86
myc(f+sa) vs myc(f+tr)	1816.0	6	1648.7	6	0.28	0.78

myc(f+sa), my(f+tr): mycosed female treated with saline and trehalose, respectively.

Con(f+sa), con(f+tr): control female treated with saline and trehalose, respectively.

Figure 6.4. Flight performance of male locusts



6.3: Discussion

This chapter describes attempts to improve the flight performance of mycosed insects by giving them an injected supplement of trehalose. Two experimental protocols were used. However, with both of them there was considerable intra treatment variation in flight performance. Other authors have noted variability in the flight of tethered locusts (Goldsworthy and Coupland, 1974). As a result though consistent trends were observed in the experiments, the differences between treatments were not always significant. Locusts were used also only 3 day after inoculation, the time when a significant reduction in flight performance first appeared (see chapter 4). It is likely that the differences between treatments would have been more marked if day 4 or day 5 locusts have been used. However, at this time other detrimental effects of infection would complicate the picture.

One of the controllable sources of variation in the initial experiments was the use of mixed sexed populations. When the performance of males and females was recorded separately, it was found that the flight performance of mycosed males was significantly reduced whereas the flight performance mycosed females was not. This may be due to the greater size and more extensive energy reserves of females though this needs further investigation.

Injection of a trehalose supplement significantly boosted flight performance of male insects whereas saline

did not. The results suggest that reduced flight performance of mycosed insects may be accounted for at least in part by the low titre of metabolic fuel in the blood. The latter would be due to its utilization by the fungus. This presumes that the fungus has also severely depleted the energy reserves in the fat body, which would normally replenish the blood. However, the effect of mycosis on fat body carbohydrate and lipid has not been determined nor is it known yet whether the fungal biomass in the insect at day 3 is sufficient to account for large scale use of carbohydrate. An alternative explanation is that either the fungus prevents mobilisation of energy reserves from the fat body or that the insect itself responds to the presence of the fungus by reducing blood sugar and lipid. The latter could be used as an attempt by the insect to reduce access of the pathogen to its energy reserves n.b. the fungus is restricted to the blood prior to death.

Metarhizium spp. can utilize trehalose as sole source of carbon liquid culture (Joshi, 1993). The fungus produces both extracellular and intracellular forms of trehalase (Joshi *et al*, 1994). Therefore it could either take up trehalose (a disaccharide) and hydrolyse it internally or break it down externally and absorb the glucose released.

Much interest has focussed on the role of enzymes in fungal penetration of insects and the impact of insecticidal fungal toxins. However, few studies have

addressed the effects of fungal pathogens on insect physiology and behaviour. Thus the possible link between the metabolic activities of the fungus and the flight behaviour of the locust is worthy of further investigation.

Chapter VII: General Discussion

Metarhizium flavoride caused high mortality at low RH among desert locusts in laboratory trials, when the fungus was applied in an oil-based formulation using a track sprayer. This confirms the observations of Bateman *et al* (1993), who first established the efficacy of oil-based formulations of *M.flavoviride* against desert locusts. Bateman *et al* (1993) applied the fungus in 2μl of oil beneath the pronotal shield. The LD50 was 8.9 x 10^3 conidia / insect at 5 day and 1.2 x 10^3 conidia at 8 day. The improved efficiency may be either because the spray delivered the spores to the most favourable sites for adhesion, germination and penetration, or infection from many sites simultaneously provides a greater threat to the immune system than mass invasion from a single site, or a high dose of conidia in close proximity mutually may improve germination.

The demonstration that oil-based formulation of spores can be used to initiate infection at low humidities may prove to be of great significance for the future of mycoinsecticides. It has been generally thought (eg.Drummond *et al*, 1986) that a requirement for a high environmental RH of spore germination was a major limitation on the use of entomopathogenic fungi for pest control. As a consequence research has focussed on pests in moist environments eg.rice pests, stem borers, soil-borne pests. For the future it may be possible to apply in oil other entomopathogenic fungi such as *Paecilomyces* spp,

Beauveria spp or *Metarhizium* spp which like *M.flavoviride* have lipophilic conidia for use against pests in drier environments.

In the present work droplet size was important with medium size (70 μm) droplets killing insects quicker than small droplets (55μm). This is probably because there is critical size of drops below which an increasing number of droplets do not contain conidia. In addition it is possible that pick up of small droplets is more variable than that of medium size droplets. Estimates of spray deposition showed that with flying locusts the greatest number of droplets collected on the wings. It is not known whether the fungus can invade the insect from the wing. The wing is a bilaminate living structure. The surfaces are held together by extensions of the epidermal cells, though there are many spaces (Uvarov, 1966). *In vitro* experiments (James, pers. com) have shown that *M.flavoviride* conidia can germinate on the surface of the wing. The fungus penetrates the cuticle and proliferates in the epidermal cells and lacunae between the upper and lower surfaces. Haemolymph flows in the lacunae of the wing and in the main veins (Uvarov, 1966). Thus elements of the fungus could be carried in the haemolymph from the wings to infect the rest of the body. However, significant droplets land on other parts of the locust , in addition to the wings, and these may be responsible for causing infection.

The track sprayer had the same spray head (disc) as that employed in field application and proved a useful

intermediate between laboratory bioassays and field tests. But field measurements of spray characteristics performed in Benin highlighted further the problems of predicting mycopesticide performance from laboratory assays. Spray deposition in field tests was up to 2 orders of magnitude lower than in the track sprayer experiments, despite the fact that 10x greater concentration of spores was used. The walking speed of a spray operator (1.2m/ sec) is not very different from the rate of progression of the track sprayer (1.3 m/sec). Thus the main factor reducing impact was wind drift. This was supported by field trials using Kromekote and Black cards to compare impaction in relation to wind speed.

The work described in the first chapters 2 and 3 supports the strategy of ULV application of conidia in oil-based formulations directly onto the insect. However, in practice unless a dense flying swarm is the target, it may be difficult to hit widely distributed insects with a sufficient number of droplets (spores). Under these circumstances it will be important for a mobile insect to pick up a lethal dose by accidental contact or through ingestion. However, for secondary pick up to be effective the spores must remain viable for an extended period after spraying. Unfortunately the desert locust lives in environments with high temperature, high UV and low RH, which are detrimental to spore survival (Moore pers.com). Indeed basking by locusts may lift body temperatures above ambient and interfere with the development of mycosis, such

behaviour helps North American grasshoppers avoid infection by *Entomophthora grylli* (Carruthers *et al*, 1992). Addition of uv screens to formulations serve a protective function in laboratory experiments (Moore unpublished data) but such adjuvants would probably be too expensive for field application

Emphasis of research on locust control by entomopathogenic fungi has been on mortality as the only criterion of success, because it is easy to measure. However, sub-lethal and pre-lethal effects of chemical insecticides can contribute to crop protection. Similarly with mycopesticides, behavioural and/or physiological defects brought about by mycosis may promote plant protection. Moorhouse (1990) found that low lethal doses of *M.anisopliae* dramatically reduced egg laying by the live vine weevil *Otiorhynchus sulcatus*. Fargues *et al* (1991) also found that fecundity and egg fertility was significantly less in Colorado beetles sub-lethally infected with *B.bassiana*. In the present work M.flavoviride significantly reduced feeding of the desert locust within 3 days of inoculation. Insect aggregation is central to the development of locust plagues and reduced flight capability in infected locusts should interfere in the wild with swarm development.

Reduction of blood carbohydrate concentration in mycosed locusts and significant decrease in flight performance of infected male locusts point to a competition

between insect fungus for metabolic reserves. However, further work is needed to shed light on this aspect of *M.flavoviride*-locust interaction.

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APPENDIX 1

1: TRACK SPRAYER: Experiment 1

Analysis of variance on % mortality results by day on which death was recorded has been carried out for each experiment

Analysis of variance table for day 5

source	df	ss	ms	F	p
TR(A)	2	2218.15	109.08	11.40	0.09*
REP(B)	3	63.149	21.050	2.20	0.89ns
A*B	6	57.408	9.5681		
Total	11	38.71			
Grand Average	1	281.30			

*:significant at 95%(on 2 df)

ns:not significant

Analysis of variance table for day 6

Source	df	ss	ms	F	p
TR(A)	2	562.60	281.30	21.00	0.0020*
REP(B)	3	22.963	7.6544	0.57	0.6542ns
A*B	6	80.372	13.395		
Total	11	665.94			
Grand Av.	1	1125.2			

*:significant at95%(on 2 df)

ns:not-significant at 95%(on 3 df)

Analysis of variance table for day 7

Source	df	ss	ms	f	p
TR(A)	2	103.33	51.667	2.45	0.1664ns
REP(B)	3	63.149	21.050	1.00	0.4547ns
A*B	6	126.30			
Total	11	292.78			
Grand Av.	1	51.667			

2: TRACK SPRAYER Experiment 2

Analysis of variance table for day 5

Source	df	ss	ms	f	p
TR(A)	2	154.17	77.083	5.84	0.039*
REP(B)	3	8.3333	2.7778	0.21	0.89ns
A*B	6	79.1167	13.194		
Total	11	241.67			
Grand Av.	1	208.33			

Analysis of variance table for day 6

Source	df	ss	ms	f	p
TRT(a)	2	787.50	393.75	51.55	0.0002*
REP(b)	3	22.917	7.6389	1.00	0.4547ns
A*B	6	45.833	7.6389		
Total	11	856.25			
Grand Av.	1	1518.7			

Further comparison of means (LSD) of day 6 (ex2) by treatment was carried out to see the factor which contributed more for the significance in treatment (disc

speed)(p 0.0002). Although the mean for variable 1 (18.75) was more than that of treatment 2 (15.00), they were not significantly different.

Analysis of variance table for day 7

source	df	ss	ms	f	p
R(A)	2	12.500	6.2500	1.00	0.422ns
REP(B)	3	6.2500	2.0833	0.33	0.802ns
A*B	6	37.500	6.2500		
Total	11	56.250			
Grand Av.	1	18.750			

Though mortality of locusts was extended up to day seven for treatments 2 and 3 (10 % and 5%, respectively.), variation due to disc speed was not significantly different from one another.

TRACK SPRAYER: Experiment 3

Analysis of variance table for day 5

Source	df	ss	ms	f	p
TR(A)	2	112.50	56.250	9.00	0.0156*
REP(B)	3	6.2500	2.0833	0.33	0.8022ns
A*B	6	37.500	6.2500		
Total	11	156.25			
Grand Av.	1	168.75			

*: Variation between treatments, in this case, disc speed was significant (p:0.0156) i.e. still the larger the droplet size, the more was the % mortality at day 5. No significant difference was seen between replicates.

Analysis of variance table for day 6

Source	df	ss	ms	f	p
Treat.	2	5.132	37.566	2.51	0.161ns
Rep.	3	99.602	33.201	2.22	0.186ns
Trea*Rep	6	89.676	14.946		
Total	11	264.41			
Grand AV.	1	423.64			

No significant difference due to treatments (disc speed) was seen by day 6. This probably could be due to extended mortality on experimentals (up to day 8 for variable 1 and day 7 for variable2).

Analysis of variance table for day 7

source	df	ss	ms	f	p
Treat.	2	4.1667	2.0833	1.00	0.4219ns
Rep.	3	25.3333	8.3333	4.00	0.0701 ns
Treat*Rep	6	12.500	2.0833		
Total	11	41.667			
Grand Av.	1	8.3333			

As for day 6, no significant difference due to disc speed variation was seen by day 7. Analysis of variance for death at day 8 has also showed no significantly difference results both between treatments and replicates.

APPENDIX 2

I: Summarized data on estimation of spray deposition and distribution per adult locusts body and body parts of trial I.

Date,27/5/91, Cotonou (Benin):

	Distance (m)					
	5	10	15	20	25	30
No.of inscts	10	10	10	10	10	10
I.B.P.						
Drops counted from						
Head	196	49	55	44	40	10
Thorax	295	195	150	37	16	33
Abdomen	249	105	40	26	18	9.0
Wing	489	403	316	146	92	58
Leg+ant.	180	149	92	26	16	21
Droplets/ins/p.	35	23	16	7.0	4.4	3.3

II: Results (suumarized) on spray collection and distribution on adult desert locusts.

Date, 30/5/91, Cotonou (Benin):

	Distance (m)					
	5	10	15	20	25	30
No.of insects	8	8	8	8	8	8
I.B.P.						
Drops counted from						
Head	117	54	37	31	18	9
Thorax	217	138	96	96	27	28
Abdomen	197	88	51	48	25	23
Wing	545	243	191	182	103	73
Leg+ant.	375	139	83	65	61	36
Mean No./ins/p.	47	19.8	14.3	13.2	7.32	5.3

I.B.P: Insect body part; No./ins/p.;Number per insect per spray pass.

III: Summary of results on some characters of *Metarhizium flavoride* spores (oil-based suspension) sprayed on microscope slides.

Date, 27/5/91, Cotonou (Benin).

	Downwind distance(m)					
	5	10	15	20	25	30
Mean No.of drops/cm^2	125	89	70	55	41	38
Mean No.of Conted. Drops/cm^2	10	10	10	10	10	10
spores counted from						
% Droplets/cm^2 Containing						
1	7.5	15.0	37.5	45.0	52.5	60
2	7.5	7.5	10.0	2.5	7.5	10
>2 spore	87.5	77.5	60.0	47.5	45.0	40
Mean No. Droplets /cm^2/pass.	31.3	22.3	17.6	13.7	10.2	9.4
Mean No of spore /cm^2/pass	3650	1951	1274	815	477	374

IV: Summarized results on some characters of *M.flavoride* spores(oil-based suspension) sprayed on microscope slides. Date, 30/5/91 Cotonou (Benin).

	Down wind distance(m)					
	5	10	15	20	25	30
Mean No.of spores/drop	152	128	84	61	42	19
Mean No.of Conted. Droplets/cm^2 spores counted from	10	10	10	10	10	10
% Droplets Containing						
1)	7.3	41.6	37.5	45	52.5	60
2)	7.3	7.3	7.31	25	32.1	50
>2spores/cm^2	85.4	85.4	75.6	60	50.1	15
Mean No. droplet /cm^2/pass	53	35	23	19	13	11
Av.spore/cm^2	8266	5032	2871	2093	978	367

V: Mean number of droplets /cm^2/pass/target against down wind distance from passage of nozzle of field tials in Cotonou (Benin),1991.

	Trial-1		Trial-2		Trial-3		Tria-4		Trial-5	
Downwind	27/5/91		28/5/91		29/5/91		30/5/91		31/5/91	
Dist(m)	KC	BC	KC	BC	KC	BC	KC	BC	KC	BC
0	0.1	0.2	0.1	0.20	0.7	0.68	0.06	0.5	0.1	0.13
1	3.5	0.8	1.6	0.60	4.16	2.60	4.38	2.5	2.05	2.88
2	15	1.5	6.75	1.43	18.1	5.60	13.8	5.2	12.1	9.6
3	25	3.8	21.8	14.5	38.4	28.5	29.8	48.8	34.8	33.8
4	34	8.8	37.2	33.4	33.4	40.3	32.8	48.3	49.8	41.8
5	52	21.8	28.6	24.8	29.4	25.2	41.8	21.8	41.8	43.8
8	33	36.8	16.6	8.4	19.9	7.0	34.8	10.3	42.8	47.8
10	19	32.2	9.0	4.3	12.0	3.9	22.3	3.0	19.8	51.8
12	16	19.0	8.2	3.2	7.2	3.2	21.2	2.2	20.8	55.8
15	10	9.4	3.9	2.0	4.8	2.3	3.8	1.5	12.8	23.8
20	7	1.0	7.0	2.4	1.0	2.7	1.6	2.0	1.3	23.8
25	6	6.9	1.5	1.0	1.4	0.8	2.0	0.9	3.3	19.8
30	4	4.9	1.2	0.7	1.0	0.6	1.8	0.8	2.3	11.8
35	3	2.4	0.8	0.6	0.8	0.4	1.3	0.8	1.8	7.3
40	3	1.7	0.7	0.4	0.8	0.3	0.8	0.8	1.3	5.1
45	2	0.3	0.7	0.4	0.7	0.2	0.9	0.9	0.6	4.1
50	2	0.2	0.6	0.3	0.5	0.2	0.5	0.8	0.5	2.3
55	2	0.2	0.5	0.3	0.4	0.2	0.5	0.2	0.3	1.8
60	1	0.1	0.3	0.1	0.4	0.2	0.4	0.1	0.3	0.6

KC:Kromekote card; BC: Black card

APPENDIX 3

3.1: Effects of corpora cardiaca (c.c) extracts on hemolymph lipid mobilization of adult *Schistocerca gregaria*

Analysed hemolymph lipid level (μg/μl) before and after injection of c.c extracts and saline (20μl/insect each) of two experiments.

3.1a: **before injection (N ≥13/treatment):**

Haemolymph lipid (μg/μl)/insect

Insects->		1	2	3	4	5	6	7	8
Treatment:									
Control:	(exp1)	2.05	3.91	4.53	8.87	5.77	0.81	----------	
	(exp2)	6.44	8.09	4.24	4.79	9.75	5.34	5.34	8.09
Mycosed	(exp1)	-0.87	0.81	2.67	5.15	3.91	----------		
	(exp2)	0.94	4.24	3.14	3.7	6.7	9.75	4.24	3.14

3.1b: **after injection (N ≥ 6/treatment):**

Haemolymph lipid level (μg/μl)/insect

Treatment		1	2	3	4
Control+saline	(exp1)	3.19	6.39	0.08	--
	(exp2)	4.47	2.64	4.47	14.23
Control+c.c	(exp1)	4.79	28.75	25.56	---
	(exp2)	11.18	33.74	13.00	22.15
Mycosed+saline	(exp1)	4.79	2.40	---	----
	(exp2)	3.25	5.69	1.42	9.96
Mycosed+c.c	(exp1)	9.58	23.96	11.82	---
	(exp2)	19.71	17.88	4.47	24.59

N.B: exp1, exp2; experiments 1 and 2, respectively. Data in 3.1a and 3.1b used for the statistical analysis in table 5.2 (Chapter 5)

3.1c: **Summarized comparison of results on the effects of c.c extracts on haemolymph lipid mobilization of adult *S.gregaria* before and after injection of** (3.1b)

Treatments (compared)	Mean before	after	%increase	%decrease
Control vs Cont.+sa	43.97	35.47	----	19.33
Control vs Cont+c.c	34.05	139.17	408.72	----
Mycosed vs Myco+sa	33.18	27.51	----	17.1
Mycosed vs Myco+c.c	15.49	112.01	723	----

3.4: Total and mean hemolymph carbohydrate (μg/μl) level of mycosed and control resting and flown adult *Schistocerca gregaria* by replicate.

Treatment	rep1	rep2	rep3	Mean
Cont.(res+c)	353.6	386.3	268.5	112.
Cont.(res+o)	371.4	305.9	300.2	108
Cont.(10)	110.3	208.9	307.4	78.3
Cont.(30)	135.6	203.6	239.0	72.0
Cont.(60)	130.0	166.1	237.7	66.7
Fungus(10)	33.59	148.2	222.5	50.5
Fungus (30)	23.01	105.4	186.8	39.4
Fungus (60)	13.01	101.8	197.0	39.0
fungus(rest)	191.7	153.7	192.7	59.8

N.B: Cont., Rest+c, rest+o; control, control resting insects from cage and control resting locusts treated with oil, respectively.

At least 3 haemolymph samples from 3 mature locusts per replicate were analysed.

Appendix 4:

4.1: Summary on mortality of sprayed adult *Schistocerca* (track experiment 1(1993) by day post application.

	% cummulative mortality at day:						
Conidia/ml	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Untreated	-	-	-	-	-	-	-
Ondina oil	-	-	-	-	-	-	-
$4x10^7$	-	26.7	73.3	86.7	93.3	100	100
$1x10^8$	6.7	6.7	6.7	53.3	73.3	93.3	100
$2x10^8$	6.7	53.3	73.3	73.3	73.3	93.3	100
$4x10^8$	-	13.3	40.0	80.0	86.7	100	100
$1x10^9$	26.7	66.7	80.0	100	100	100	100

15 insects/treatment of 3 replicates each and 5 locusts/replicate

-: denotes no mortality