Control of the Pasture Scarab *Adoryphorus couloni* with *Metarhizium anisopliae*

by

Andrew Charles Rath

B.Sc.(Hons.) (Univ. of Sydney)

Faculty of Agricultural Science

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This thesis contains no material which has been accepted for the award of any other degree or graduate diploma in any tertiary institution and that, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Andrew Rath

ABSTRACT

The redheaded cockchafer, *Adoryphorus couloni,* is a serious pasture pest of south-eastern Australia. No chemical insecticides are registered for use against *A. couloni.* Investigations into the biological control of this pest using the fungus *Metarhizium anisopliae*, covered three main areas. The first of these were laboratory studies on the affect of both spore concentration and temperature on the virulence of the fungus. Secondly, field experiments were established to determine the short-term and long-term biocontrol. Finally, an environmental study examined the influence of abiotic factors on the distribution and abundance of the natural *M. anisopliae* flora in pasture soils.

1. Laboratory experiments

Screening experiments using *M. anisopliae, M. flavoviride and Beauveria bassiana,* isolated from *A. couloni* or similar pests, showed that one isolate of *M. anisopliae*, DAT F-001, was highly virulent to L3 larvae of *A. couloni.* This isolate was used in all subsequent laboratory and field experiments.

M. anisopliae DAT F-001 was pathogenic to L3 larvae at all concentrations tested (10^1 - 10^7 spores/g sand-peat). These experiments, undertaken at 20°C, showed that the virulence of the fungus declined as the concentration declined. The LT_{50}'s ranged from 83 days at 10^1 spores/g to 19 days at 10^7 spores/g sand-peat. Eggs, L1 larvae and adults were also susceptible to the fungus but only at concentrations of 10^5 spores/g or higher.

DAT F-001 germinated at all temperatures tested (2° - 25°C) though the germination rate was slowed as temperatures declined. Reducing the temperature also resulted in a decline in virulence. The LT_{50} of L3 larvae, exposed to a concentration of 2×10^6 spores/g sand-peat, was 36 days at 15°C and 190 days at 5°C.

Temperature had a greater impact on the virulence of *M. anisopliae* for L3 larvae than spore concentration. The interaction between temperature and spore concentration was modelled to enable examination of concentration x temperature combinations which had not been tested in the laboratory. Two such mortality models were then used to investigate the timing of mortality events in the field. By incorporating the mean monthly temperatures at field sites, the efficacy of various concentrations as well as application timing, were examined on the computer before field experiments were established.

2. Field experiments

Field experiments were established in 1989 and 1991. Experiments established in 1989 showed that the efficacy of the fungus closely matched the model predictions. These experiments, conducted at three separate sites, resulted in up to 83% mortality of L3 larvae in the year of application. The adults, which are non-feeding and are found at a depth of 20 - 30 cm, were not killed prior to mating flights.

The concentration of DAT F-001 in the soil remained close to the applied concentration for the three years of these experiments. At one site, the

concentration increased five to ten-fold while at a fourth site where no host was present, the fungal concentration declined over the three years. The retention of the fungus in the soil resulted in 60-70% mortality in both the overlapping *A. couloni* populations and the next generation of the primary population, in the second and third years after application. Reduction in the larval numbers in the third year resulted in reduced visible pasture damage.

Experiments conducted in 1991 tested two hypotheses. The first hypothesis was that *M. anisopliae* applied in spring (November), while adults were laying eggs, would reduce the numbers of larvae the following autumn (April - May) and minimize pasture damage. This strategy was ineffective in five separate paddocks.

The second experiment tested the model prediction that application rates as low as 1 kg/ha (~1 x 10^3 spores/g soil) would reduce the L3 population by 50% by early summer if applied in autumn. This experiment tested 1, 10 and 25 kg/ha (~1, 10 and 25 x 10^3 spores/g soil, respectively) and by December the reductions in larval numbers were 0, 17 and 35% respectively. Both this experiment and the November application experiments had reduced efficacy probably because of the prevailing dry weather conditions, which limited larval movement, development and the contact between fungal spores and the larvae.

The effect of *M. anisopliae* on non-target invertebrates was examined at several field sites. After application of *M. anisopliae* there were no differences in the numbers of other invertebrates during the first year, however, after three years there was a rise in the numbers of another pest, the blackheaded cockchafer (*Aphodius tasmaniae*), in treated plots.

3. Environment

The influence of abiotic factors on the distribution and abundance of *M. anisopliae* in Tasmanian pasture soils was examined. Four hundred and nineteen soil samples yielded 132 *M. anisopliae* var. *anisopliae* isolates which were classified into 14 strains. The distribution of the strains varied with soil-type and average annual rainfall. Soil pH, conductivity, temperature and altitude had minor or no effect on distribution. The abundance of the 14 strains varied from common to rare. The densities of the strains were not different. Modal density of 1 x 10^3 c.f.u./g soil. The density was not correlated with any of the environmental variables examined. The significance of this data to a biological control programme is discussed.

4. Commercialisation

M. anisopliae DAT F-001 is a promising long-term control agent for *A. couloni*. Commercialisation of the fungus is proceeding with much of the data presented in this thesis being used to register DAT F-001 as a pesticide. An agreement has now been signed with the commercializing partner (Bio-Care Technology Pty Ltd) and full registration and marketing is expected in 1993.

ACKNOWLEDGEMENTS

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Contents

Chapter 1

1.0 GENERAL INTRODUCTION

1.1 INTRODUCTION

This thesis describes the research and development programme initiated in an attempt to control the redheaded cockchafer, *Adoryphorus couloni* (Coleoptera: Scarabaeidae), with entomogenous fungi. The research has resulted in the development of a commercial formulation of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes), an industrial partner and a preliminary submission to the Agricultural Chemicals Advisory Committee (ACAC) for clearance of the product prior to registration.

In order to facilitate an understanding of this research a brief background is provided in this chapter. This includes the insect life cycle, pest status and current control measures as well as a description of *M. anisopliae* with regard to pest control.

1.2 *ADORYPHORUS COULONI*

1.2.1 Life cycle

The two-year life-cycle of *Adoryphorus couloni* (Burmeister) (Fig. 1.1.) has been described by McQuillan and Ireson (1987) and McQuillan (1990) and the salient points are described here. Eggs are laid during spring in the top 80 mm of the soil. Over summer the L1 and L2 larvae are generally found below the root zone which is dry and hard. After autumn rain, final instar (L3) larvae (Fig. 1.2) are found in the root zone and down to 150 mm deep but individuals migrate up and down the soil profile on a daily basis (McQuillan, pers. comm.). Early in the second summer, the larvae migrate down the soil profile to a depth of 150 - 300 mm, where they pupate. The adults (Fig. 1.2) remain in the soil at that depth until early spring when they move close to the soil surface prior to leaving the soil to mate.

1.2.2 Distribution

A. couloni is indigenous to pastures in the lower south-east of South Australia (Allen, 1986), much of Victoria and the southern tablelands of New South Wales (Blackburn, 1983) as well as King and Flinders Islands and across northern and

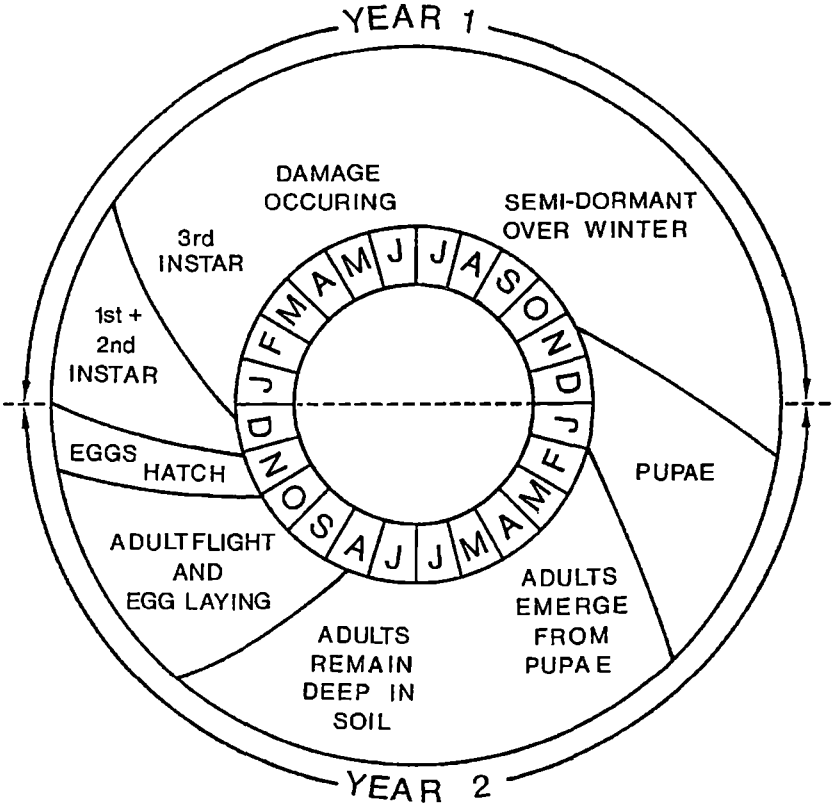

Figure 1.1 **The two-year life-cycle of *Adoryphorus couloni* (from McQuillan and Ireson, 1987).**

midland Tasmania (McQuillan and Ireson, 1987) (Fig. 1.3.). The insect has been introduced into the Canterbury region of New Zealand (Stufkens and Farrell, 1980). In Tasmania, at least, the distribution of the insect is expanding southward with new infestations being recorded biennially.

Two non-interbreeding populations are present in Tasmania (P.B. McQuillan, pers. comm.). In general, the populations are separated by time rather than geography. It is usual in the southern midlands, to find large numbers of L3 larvae in odd-numbered years (the primary population) followed by the subsequent adult population in even-numbered years. In the second population, defined here as the overlapping population, L3 larvae are present in even-numbered years. The overlapping population is usually small, $^{1}/_{10}$ to $^{1}/_{30}$ of the size of the primary population. In some areas of the state, notably the northwest and the northeast, the overlapping population is larger than the primary population. Few areas of the state have both populations in large numbers though this is more common in Victoria (Blackburn, 1983).

Figure 1.2 **L3 larva (top) and adult (bottom) of *Adoryphorus couloni* (from Watt 1984). Scale = 3 mm (larva); 1 mm (adult)**

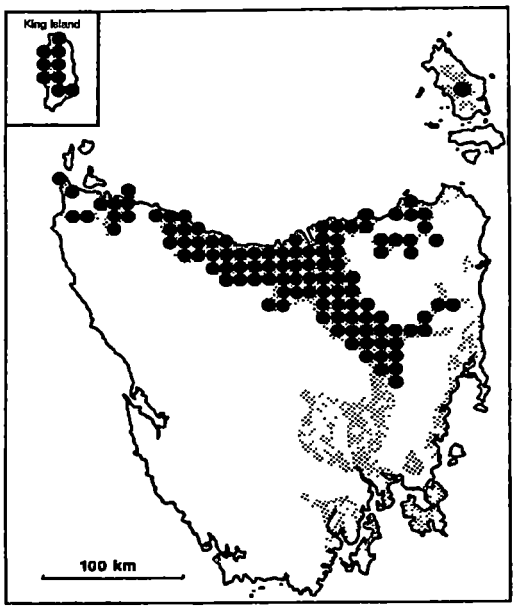

Figure 1.3 **Distribution of *Adoryphorus couloni* (●) in Tasmanian pastures (shaded) (modified from McQuillan and Ireson, 1987).**

1.2.3 Pasture Damage

Until 1967, *A. couloni* was only considered a minor pest in the north and north-west of Tasmania but in 1967, 1969, 1970 and 1971, the pest caused major damage to pastures in the northern midlands (Hardy and Tandy, 1971). The emergence of *A. couloni* as a pest has been generally associated with pasture improvement. Allan (1986) believed it was due to increased organic matter in improved pastures, while Blackburn (1983) believed that the shallow-rooted plants, such as perennial ryegrass (*Lolium perenne*), used to improved pastures, are more susceptible to attack. Probably both are correct, as *A. couloni* larvae feed on both roots and humus in the root zone (McQuillan and Ireson, 1987).

Damage to pastures is caused by L3 larvae severing roots during feeding, and the physical disruption of the roots during underground movement (McQuillan and Ireson, 1987). Undercutting the pasture plants promotes uprooting by stock and birds (Figs. 1.4 to 1.7) which during a dry spell, leads to plant death from moisture stress since the plants cannot tap soil moisture held at depth (McQuillan and Ireson, 1987). Severely damaged pastures can be rolled back in sheets (Hardy and Tandy, 1971)(Fig. 1.7).

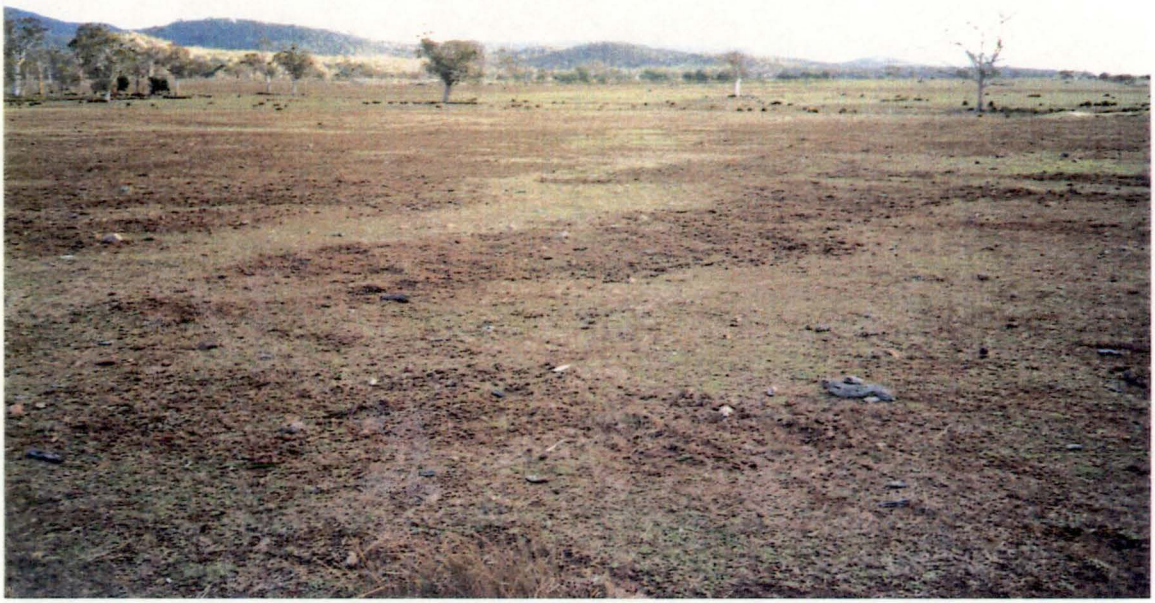

Figure 1.4 Pasture damage in the Royal George valley (148°00'E, 41°45'S), July 1991.

Figure 1.5 Pasture damage exacerbated by forest ravens (*Corvus* spp.) digging for *A. couloni* larvae at Woodbury (147°30'E, 42°15'S), May 1989)

Figure 1.6 Pasture damage in the Royal George valley, July 1991

Figure 1.7 Rolling back the pasture after extensive pasture damage (Royal George, July 1991)

Pasture plants can withstand remarkably high populations of root pruning insects before showing any detrimental effects (McQuillan, 1990). No damage to *Lolium* pastures occurs when numbers of L3 larvae are lower than 100/m^2 (pers. obs.) while in New Zealand no damage was detectable in an *Agrotis* - *Festuca* - *Cynosurus* - *Dactylis* pasture with 250 larvae/m^2 (Stufkens and Farrell, 1980). Severe damage occurs when numbers exceed 300 larvae/m^2 (Allen, 1986).

1.2.4 Economics

Pauley and Miller (1992) critically assessed the short-term economic loss due to *A. couloni* in Tasmania. They determined that in an average year, 16,000ha of improved pasture suffered medium-severe damage at a cost of A$1.1 million. Their calculation of economic loss was based on a reduction in livestock production. In their economic model, feed losses during winter have a greater impact on animal production than equivalent absolute losses in spring because, in winter, pasture growth rates are slower and the feed requirements of breeding livestock are increasing. Consequently, losses due to *A. couloni* which occur during the autumn/winter period result in large losses in animal production in economic terms.

Berg (1987) estimated that in Victoria, losses of up to A$100/ha occured in areas used for fat lambs production and up to A$500/ha in dairying areas. *A. couloni* is a sporadic pest of turf (Australia Turfgrass Research Institute, 1989) but during a pest outbreak, losses have been estimated at A$9,000/ha (Berg, 1989).

The short-term loss of pasture production is only part of the overall economic loss from *A. couloni* (McQuillan and Ireson 1987; Pauley and Miller, 1992), as damage by these pests leads to a loss of sown perennial pasture grasses due to selective larval feeding followed by weed invasion of open spaces. Ryegrass (*L. perenne*) is particularly susceptible to attack by larvae, while other pasture species such as phalaris (*Phalaris aquatica*) and cocksfoot (*Dactylis glomerata*) are more tolerant of cockchafer damage (McQuillan and Ireson, 1987). The presence of these latter grasses increases the threshold number of larvae which the pasture can accommodate before production losses occur. The selective loss of perennial grass species due to larval activity allows the invasion by annual grasses and weeds and leads to a decline in pasture quality which can

only be overcome by resowing the damaged pasture. Long-term loss and cost of recovery led Rath *et al.* (1990, appendix 7) to estimate that the total cost of cockchafer damage to pastures was 3 to 5 times higher than the short-term losses.

1.2.5 Control Measures

There are no chemical insecticides currently registered for the control of *A. couloni* in Australia (Blackburn, 1983; Ireson, 1990) and few prospects of future chemical control. McQuillan (1990) believed that insecticidal soil treatment against scarabs, even as a stop-gap method, was of dubious value in pastoral situations. Rath and Fulkerson (unpub. data) drilled two granulated organophosphates (25 mm deep) into dairy farms infested with L3 larvae of *A. couloni.* After twelve weeks, larval survival was still 100% (Appendix 3).

Biological control of *A. couloni* with nematodes has been studied in Victoria (Berg *et al.*, 1984; Berg, 1987, 1989) but the reduction in larval numbers was low at 25% (Berg *et al.*, 1984) to 40% (Anon, 1989). This form of control has not been successful because of the high cost of the nematodes (R.A. Bedding, pers. comm.) and the requirement for patent nematode application equipment (Berg *et al.*, 1987). This latter problem could be overcome by the use of New Zealand equipment used for the introduction of the bacterium *Serratia entomophila* into pastures for the control of the scarab *Costelytra zealandica* (Jackson *et al.*, 1989).

Crawford *et al.* (1985) investigated the susceptibility of L3 larvae of *A. couloni* to *Oryctes* baculovirus. They found that 96% of larvae died of the disease when the *Oryctes* baculovirus was injected into the haemocoel but that no larvae died when the baculovirus was ingested.

Cultural control methods which rely on pasture management are currently advised by Australian Departments of Agriculture (Blackburn, 1983; Allen 1986; Berg, 1987; McQuillan and Ireson, 1987). Grazing management strategies (Douglas, 1972; Roberts, 1984), cultivation in readiness for sowing forage crops, cutting spring pastures for hay, agistment and several other methods (Hardy and Tandy, 1971) have been suggested as possible control measures. Physical control, such as the use of heavy rollers (van Toor and Stewart, 1987) has not been evaluated.

1.3 *METARHIZIUM ANISOPLIAE*

1.3.1 Description

Metarhizium anisopliae (Deuteromycotina : Hyphomycetes) is a common entomopathogenic fungus isolated from insect cadavers and soil from around the world. Tulloch (1976) recognized two varieties based on conidial morphology; variety *anisopliae* (Metschn.) Sorokin with conidia measuring (3.5-) 5.0 - 8.0 (-9.0) x 2.5 - 3.5 (-4.5) µm and variety *major* (Johnson) Tulloch with conidia measuring 10.0 - 14.0 (-18.0) µm long.

Considerable genetic diversity can be found among various isolates of single species of entomopathogenic fungi from different hosts and localities (Roberts and St. Leger, 1990) and *M. anisopliae* is no exception. Guy and Rath (1990) were able to separate isolates of var. *anisopliae* using Enzyme - Linked Immunosorbent Assays (ELISA). Riba *et al.* (1990) were able to separate 11 isolates of var. *anisopliae* using isozyme analysis but found that ribosomal RNA was insufficient for strain separation. Duriez-Vaucelle *et al.* (1981) were unable to separate var. *anisopliae* isolates using zymograms of 23 enzyme activities. Riba *et al.* (1986) compared the conidial size, isoenzyme analysis and virulence towards the European corn borer of 96 *M. anisopliae* cultures originating from various hosts and countries. They found var. *anisopliae* to be very heterogeneous though some isolates could be separated into strains.

Yip, Rath and Koen (1992) characterized 204 cultures of var *anisopliae* isolated from Tasmanian pasture soils. They distinguished sixteen strains based on temperature requirements for germination and growth, conidial dimensions and sporulation colour. They also provided a key to these strains.

While *M. anisopliae* var. *anisopliae* has a wide host range, attacking species of insects within the orders Orthoptera, Coleoptera, Lepidoptera, Hemiptera, Hymenoptera and Arachnida (McCoy *et al.*, 1988), as well as Dermaptera, Isoptera, Diptera and Siphonaptera (Goettel *et al.*, 1990), individual strains usually show some degree of host preference (Roberts and St. Leger, 1990). Ferron *et al.* (1972) generally found that each species of scarab was only susceptible to fungus strains which had been originally isolated from that scarab species. Yip *et al.* (1992) tested 184 var. *anisopliae* strains for pathogenicity to the redheaded cockchafer (*A. couloni*). They found that 50% of isolates (15/31)

from strains 1 and 3 were pathogenic for L3 larvae, while in all other strains only 4% (6/153) were pathogenic. These authors showed, using hierarchical cluster analysis, that strains 1 and 3 were greater than 95% similar while all other strain groupings were separated at the 80% similarity level.

1.3.2 *M. anisopliae* as a biocontrol agent.

M. anisopliae was first used in 1879 by Metchnikoff in experiments on control of wheat cockchafer, *Anisoplia austriaca* Herbst and the sugarbeet curculio, *Cleonus punctiventris* Germ. Since then, Roberts and Yendol (1971) described it as the second most widely studied entomopathogenic fungus after *Beauveria bassiana*, though, as explained later, this situation may be quickly changing. The use of *M. anisopliae* to control pests has been reviewed by Ferron (1978, 1981), while more recent reviews of entomogenous fungi have included sections on *M. anisopliae* (Lisansky and Hall, 1983; Gillespie, 1988; McCoy *et al.*, 1988).

In spite of the many promising studies on insect control using *M. anisopliae*, the fungus is only registered for use against spittle bugs in both pastures and sugar cane in Brazil (Moscardi, 1988; Goettel *et al.*, 1990). This situation, and the status of *M. anisopliae* as a control agent, is likely to change shortly with the compilation of data for the registration of BIO 1020® by the multinational company Bayer Ag. This product is intended for use in controlling *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) a world-wide pest in glasshouses. Research has been conducted in Europe (Andersch *et al.*, 1990; Reinecke *et al.*, 1990), the United States of America (Storey *et al.*, 1990) and Australasia (Bayer Australia, pers. comm.), and consequently registration can be expected world-wide.

1.3.3 *M. anisopliae* control of subterranean soil pests

The soil is an important reservoir for *M. anisopliae* conidia (Milner, 1989). Milner (1989) considered that the fungus could be isolated from the majority of soils, however, Hokkanen and Zimmermann (1986) only isolated *M. anisopliae* from 5 out of 30 soil samples taken from a range of agro-forestry soils in Finland. The density of colony-forming units (CFU's) present in soil is usually low at 10^3 - 10^4 CFU/g soil (Milner, 1989).

Klein (1988) concluded that soil may be a suitable habitat for the development of fungal pathogens because natural fungal infections have been noted in larvae of Scarabaeidae, Curculionidae and Elateridae. He further stated that although more than 90% of insects have a stage in the soil, there are very few microorganisms now being used to manage soil insects. If *M. anisopliae* is to control soil pests, in general, and *A. couloni* in particular, the environmental factors which affect the survival and efficacy of the fungus, require study. McCoy *et al.*, (1988) have reviewed the environmental factors which affect entomogenous fungi in general, while Milner (1989) has reviewed these factors specifically in relation to *Metarhizium* use in the control of soil dwelling pests.

While *M. anisopliae* is not registered for use against soil-dwelling insect pests there have been many studies aimed at such control. Some of these studies have targeted the scarabs *Melolontha melolontha* (Ferron *et al.*, 1972; Ferron, 1978), *Oryctes rhinoceros* (Latch, 1976; Latch and Falloon, 1976), *Scapanes australis* (Prior and Arura, 1985), *Sericesthis* spp. (Milner and Lutton, 1976), *Costelytra zealandica* (Latch, 1965; Goh *et al.*, 1991), a diverse range of sugar-cane scarabs (Samuels *et al.*, 1990; Beckmann, 1991; Milner, 1992) as well as the curculionids *Otiorhynchus sulcatus* (Soares *et al.*, 1983; Gillespie *et al.*, 1989; Reinecke *et al.*, 1990), *Sitona* sp. (Muller-Kogler and Stein, 1976) and *Curculio caryae* (Gottwald and Tedders, 1983). Additionally, surface feeding insects pests which form vertical tunnels in the soil have been investigated (Coles and Pinnock, 1982, 1984; Latch and Kain, 1983). Some of the most recent programmes include scarab control in India (Yadava, 1992), the USA (Krueger *et al.*, 1990), Australia (Milner, 1990, 1992), Taro beetle control in Solomon Islands (I. Aloalii, personal communication 1991) and *Phyllophaga* spp. control in Costa Rica (P. Shannon, personal communication 1992).

1.4. Aim

The aim of the study reported in this thesis was to examine the prospects for biological control of *A. couloni* with entomogenous fungi. Early studies quickly established *M. anisopliae* var. *anisopliae* isolate DAT F-001 as a promising candidate control agent.

The aims of the research were then extended to cover specific studies of the laboratory and field efficacy of DAT F-001 against *A. couloni.*

The thesis is divided into separate studies, each of which reviews the literature pertinent to the study in either the introduction or the discussion section of each chapter. A flow chart of the experimentation pathway and the structure of the chapters in this thesis is given in Figure 1.8.

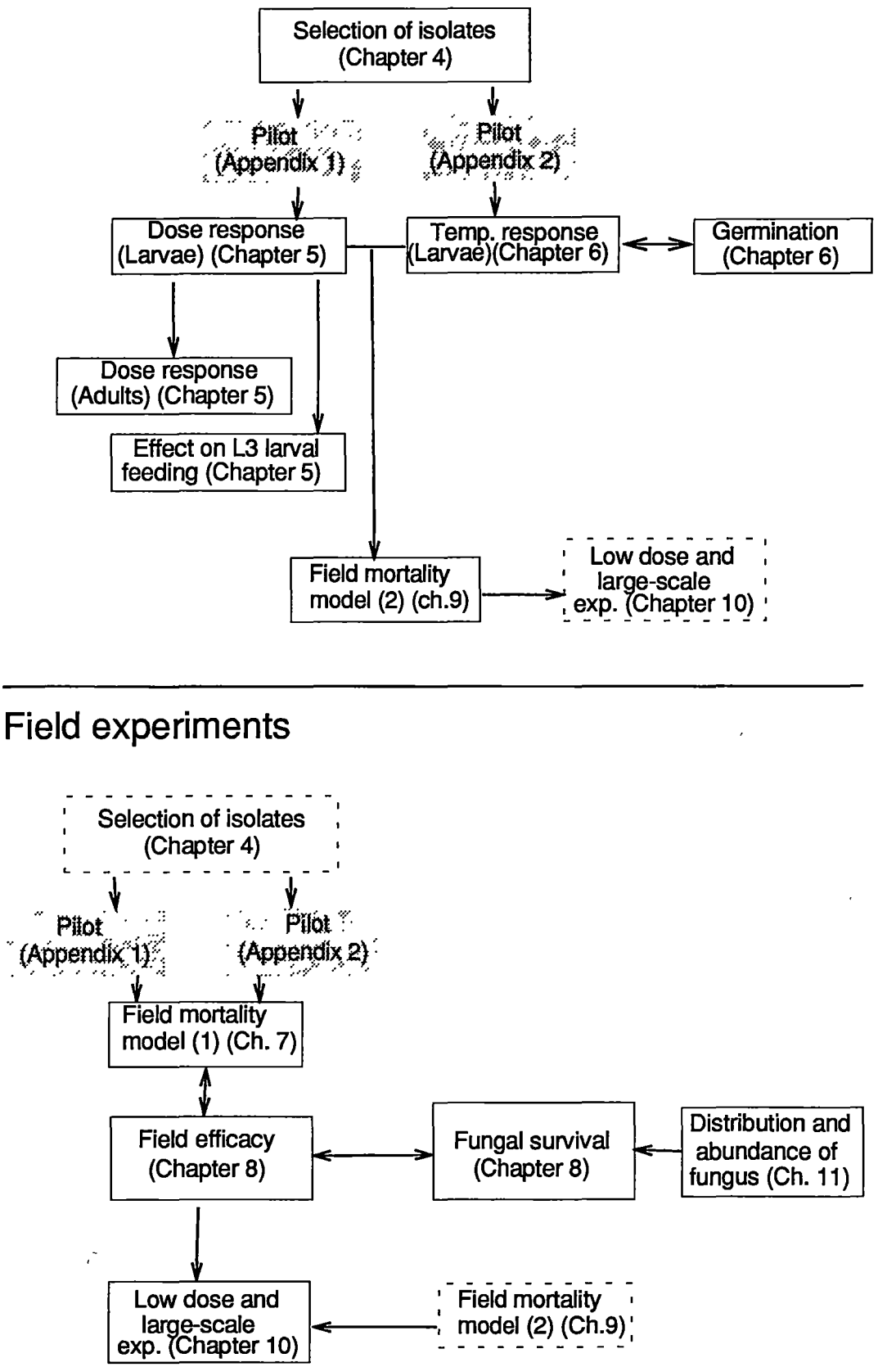

Figure 1.8 Flow chart of thesis experimentation

Chapter 2

2.0 PROGRAM SUMMARY

2.1 INTRODUCTION

Adoryphorus couloni (Burmeister), the redheaded cockchafer, is a subterranean pasture pest of the Australian states of Victoria, Tasmania, southern New South Wales and south-east South Australia but there are no chemical insecticides registered for their control.

The absence of control measures led to pressure from the farming community, followed by pressure from the government, to start a research program to investigate the microbiological control of *A. couloni*. In 1986 I commenced a research program to develop *Metarhizium anisopliae* as a biological control agent for the pest. The research progressed rapidly and I established several concurrent projects. These projects included studies on the distribution and abundance of *Metarhizium anisopliae* in Tasmanian soils and the mass production of the fungus. I have not used these studies in this thesis as I employed research assistants to undertake much of the work (their work was in their day-to-day control though the direction was set by me). This concurrent research cannot be ignored as much of it is central to the development of my thinking in the experiments presented in this thesis.

The ultimate aim of this research (if successful) was to initiate the development of a commercial product. Some aspects of this aim, such as farming methods, probable costings, etc, were also integral to my thinking and planning of this experimentation.

This chapter highlights the research and commercial settings in which the research was undertaken.

2.2 INTEGRATION WITH THE FARMING SYSTEM

Pasture: A low value crop

A microbial control system for *A. couloni* must take into account the low value of the pasture "crop". This means that the cost of the control product and its

application costs must be as low as possible because the gross margin for pasture in Tasmania is only A$350/ha annually (DPI, 1989).

Application

Surface application of *M. anisopliae* is not feasible (Appendix 1) but direct drill application at a depth of 20 - 25 mm (the standard depth at which pasture seed is sown) places the fungus at the appropriate depth for control of eggs (80 mm) and larvae (0-150 mm). However, once larvae move down the soil profile to pupate (150-300 mm), the effectiveness of the fungus would be lessened. Adults would only be exposed to the fungus immediately prior to mating or when the females returned to the soil to lay eggs.

Broad acre subterranean pest

A. couloni can be distributed over several hundred continuous hectares on each farm. This means that a *M. anisopliae* control agent must be used over large areas of each farm.

The subterranean nature of *A. couloni* also means that surface application of *M. anisopliae* will not control the pest (Appendix 1), and as early damage to pastures may not be discernible, the fungus must be drilled into the soil over large areas which may or may not contain damaging levels of larvae.

Long-term control

As a result of both the low value of pasture and the broad-acre nature of the pest, the fungus must have a long-term residual action to be economic. This would reduce the costs associated with the introduction of the fungus, and would enable the inclusion of *M. anisopliae* in pasture renovation program, thus reducing application costs. To be successful, the fungus would need to effectively reduce the pest populations for periods of 5-10 years (the life of the pasture).

Existing Management

If pathogen control of scarab pests is to become a reality then the use of a pathogen product must not greatly modify existing farm management

procedures. As an example of this, application of *M. anisopliae* to control *A. couloni* uses existing machinery (direct-drills for pasture renovation or crop sowing) and this implies that the product must be formulated for easy flow from the drills. More importantly it means that application timing must be compatible with the machinery. For example, application of *M. anisopliae* via a seed drill in mid January would not be practical as the ground is too hard for drill penetration. Any drill cuts that do not close because of the hardened soil, leave the fungus exposed to U.V. light and desiccation. If the fungus is being included in a pasture renovation programme, mid-winter is unsuitable for pasture establishment. These constraints may leave only a short period of time when the fungus could be introduced into a pasture and kill sufficient larvae to limit damage.

2.3 RELATIONSHIP BETWEEN DAT F-001 AND OTHER TASMANIAN *METARHIZIUM* ISOLATES

Studies commenced in 1987 to determine the distribution and abundance of *Metarhizium* strains in Tasmanian pasture soils and to assess the consequences of these for the biocontrol program (WRDC grant DAT 3 awarded to A.C. Rath). The incidence of natural *Metarhizium* disease in *A. couloni* populations was low with only rare findings of the fungus in larvae, although epizootics in large beetle populations after mating flights was more common. However, only isolates other than DAT F-001 have been recovered from adults (*M. anisopliae* DAT F-054; *M. flavoviride* DAT F-113) (Chapter 4).

Determination of the concentration of *M. anisopliae* in pasture soils relied on two selective agars. For the distribution and abundance studies where a diverse range of isolates was expected, the agar described by Milner and Lutton (1976) was found most suitable. Field studies on the survival of DAT F-001 used the agar of Doberski and Tribe (1980) as the crystal violet in this agar was found to hinder the germination of nearly all of the non-DAT F-001 types of *M. anisopliae*, while allowing >95% germination of DAT F-001.

Metarhizium was isolated from 32% of the 650 soil samples examined (Yip, 1990). These isolates have now been characterized into 16 strains (biotypes) of *M. anisopliae* based on temperature related growth rate, spore size and colour, as well as cultural characteristics (Yip, Rath and Koen, 1992). This classification enabled the rapid separation of isolates into cold-tolerant, meso-

Chapter 2

2.0 PROGRAM SUMMARY

2.1 INTRODUCTION

Adoryphorus couloni (Burmeister), the redheaded cockchafer, is a subterranean pasture pest of the Australian states of Victoria, Tasmania, southern New South Wales and south-east South Australia but there are no chemical insecticides registered for their control.

The absence of control measures led to pressure from the farming community, followed by pressure from the government, to start a research program to investigate the microbiological control of *A. couloni.* In 1986 I commenced a research program to develop *Metarhizium anisopliae* as a biological control agent for the pest. The research progressed rapidly and I established several concurrent projects. These projects included studies on the distribution and abundance of *Metarhizium anisopliae* in Tasmanian soils and the mass production of the fungus. I have not used these studies in this thesis as I employed research assistants to undertake much of the work (their work was in their day-to-day control though the direction was set by me). This concurrent research cannot be ignored as much of it is central to the development of my thinking in the experiments presented in this thesis.

The ultimate aim of this research (if successful) was to initiate the development of a commercial product. Some aspects of this aim, such as farming methods, probable costings, etc, were also integral to my thinking and planning of this experimentation.

This chapter highlights the research and commercial settings in which the research was undertaken.

2.2 INTEGRATION WITH THE FARMING SYSTEM

Pasture: A low value crop

A microbial control system for *A. couloni* must take into account the low value of the pasture "crop". This means that the cost of the control product and its

application costs must be as low as possible because the gross margin for pasture in Tasmania is only A$350/ha annually (DPI, 1989).

Application

Surface application of *M. anisopliae* is not feasible (Appendix 1) but direct drill application at a depth of 20 - 25 mm (the standard depth at which pasture seed is sown) places the fungus at the appropriate depth for control of eggs (80 mm) and larvae (0-150 mm). However, once larvae move down the soil profile to pupate (150-300 mm), the effectiveness of the fungus would be lessened. Adults would only be exposed to the fungus immediately prior to mating or when the females returned to the soil to lay eggs.

Broad acre subterranean pest

A. couloni can be distributed over several hundred continuous hectares on each farm. This means that a *M. anisopliae* control agent must be used over large areas of each farm.

The subterranean nature of *A. couloni* also means that surface application of *M. anisopliae* will not control the pest (Appendix 1), and as early damage to pastures may not be discernible, the fungus must be drilled into the soil over large areas which may or may not contain damaging levels of larvae.

Long-term control

As a result of both the low value of pasture and the broad-acre nature of the pest, the fungus must have a long-term residual action to be economic. This would reduce the costs associated with the introduction of the fungus, and would enable the inclusion of *M. anisopliae* in pasture renovation program, thus reducing application costs. To be successful, the fungus would need to effectively reduce the pest populations for periods of 5-10 years (the life of the pasture).

Existing Management

If pathogen control of scarab pests is to become a reality then the use of a pathogen product must not greatly modify existing farm management

procedures. As an example of this, application of *M. anisopliae* to control *A. couloni* uses existing machinery (direct-drills for pasture renovation or crop sowing) and this implies that the product must be formulated for easy flow from the drills. More importantly it means that application timing must be compatible with the machinery. For example, application of *M. anisopliae* via a seed drill in mid January would not be practical as the ground is too hard for drill penetration. Any drill cuts that do not close because of the hardened soil, leave the fungus exposed to U.V. light and desiccation. If the fungus is being included in a pasture renovation programme, mid-winter is unsuitable for pasture establishment. These constraints may leave only a short period of time when the fungus could be introduced into a pasture and kill sufficient larvae to limit damage.

2.3 RELATIONSHIP BETWEEN DAT F-001 AND OTHER TASMANIAN *METARHIZIUM* ISOLATES

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Determination of the concentration of *M. anisopliae* in pasture soils relied on two selective agars. For the distribution and abundance studies where a diverse range of isolates was expected, the agar described by Milner and Lutton (1976) was found most suitable. Field studies on the survival of DAT F-001 used the agar of Doberski and Tribe (1980) as the crystal violet in this agar was found to hinder the germination of nearly all of the non-DAT F-001 types of *M. anisopliae*, while allowing >95% germination of DAT F-001.

Metarhizium was isolated from 32% of the 650 soil samples examined (Yip, 1990). These isolates have now been characterized into 16 strains (biotypes) of *M. anisopliae* based on temperature related growth rate, spore size and colour, as well as cultural characteristics (Yip, Rath and Koen, 1992). This classification enabled the rapid separation of isolates into cold-tolerant, meso-

tolerant and heat-tolerant biotypes on the basis of spore colour. Yip *et al.* (1992) also found that within the DAT F-001 related biotypes (strains 1 to 3; DAT F-001 is the isolate on which field experiments were undertaken, Chapters 8 and 10), 50% (15/33) were pathogenic to L3 larvae of *A. couloni* while in the other biotypes only 4% (6/155) were pathogenic.

Analysis of 419 pasture soils yielded 132 *M. anisopliae* var. *anisopliae* isolates, which were classified into 14 strains. The distribution and abundance of the strains varied with soil-type and average annual rainfall (and this is fully studies in Chapter 11). Soil pH, conductivity, temperature and altitude had minor or no effect on distribution. The abundance of the 14 strains varied from common to rare. The densities of strains (CFU/g soil) were not significantly different ($P>0.05$) and the modal density was 1×10^3 CFU/g soil. The density of strains was not correlated with any of the environmental variables examined (Chapter 11).

The distribution and abundance of the pathogenic biotypes was found to be sporadic (Yip *et al.*, 1992) indicating that no effective natural control of *A. couloni* would be expected. I conclude that the use of DAT F-001 in most pastures can be regarded as a new introduction. While in those pastures with some level of DAT F-001 related strains present, supplementary application will still be required to evenly distribute the pathogen in the paddock.

2.5 MASS PRODUCTION

Commercial scale production of *M. anisopliae* is a major problem that must be solved before there will be widespread use of the fungus (Bartlett and Jaronski, 1988; McCoy *et al.* 1988). As an integral part of the study of *M. anisopliae* DAT F-001 as a control agent for *A. couloni*, Rath *et al.* (1990) (RCDF grant TDA 88/49 awarded to A.C. Rath 1988 - 1990) have been developing a fungal mass production system which requires very low technological input. The system utilizes local cereal grains and differs in a number of ways from that used in Brazil (Moscardi, 1988). Spore production is typically 1×10^9 spores/g grain. Using this production system, 500 kg of DAT F-001 was produced in 1989 and 1500 kg in 1990 for experimental field work.

The shelf-life of this product formulation is at least 12 weeks at temperatures of 10°C or less, but spore viability falls off rapidly at higher temperatures (Table

Table 2.1 **Effect of storage temperature on *M. anisopliae* DAT F-001 spore viability and predicted LT_{50}**

storage temp. (°C)	% viability after 12 weeks	predicted LT_{50}* (days)	
		10°C	16°C
5	100	137	65
10	100	137	65
15	50	147	70
20	13.5	168	79

* using the later model (section 2.4) and an application of 2.5×10^{13} spores/ha.

2.1). However, when LT_{50}'s are examined (Table 2.1) the loss of spore viability 'on-the-shelf' is not a major concern. The data suggest that while every effort must be made to maximize shelf life prior to sale, 'on-farm' storage for two to three weeks at 15°-20°C will have little detrimental effect.

The major disadvantage of this formulation is the considerable storage, handling, and transportation costs associated with a 10% a.i. product. There is scope to reduce this by coating pure *M. anisopliae* spores onto pasture seeds in a manner identical to the inoculation of clover (*Trifolium* spp.) with nitrogen fixing bacteria (*Rhizobium* spp.). Recent experiments (A.C. Rath and C.J. Carr, unpublished data) show that up to 5 kg of *M. anisopliae* can be stuck with methyl cellulose to 15 kg of ryegrass (*Lolium perenne*). There was no reduction in seed germination when *M. anisopliae* was coated on a range of commonly used pasture seeds.

Descriptions of the spore production experiments are not discussed in this thesis. The methods used to produce *M. anisopliae* spores for field experiments are outlined in the Materials and Methods sections of Chapter 8 and 10.

Chapter 3

3.0 GENERAL MATERIALS AND METHODS

3.1 PROCEDURES FOR FUNGI

Maintenance of cultures

Primary cultures were maintained on slopes of Sabouraud Dextrose Agar (SDA, Oxoid CM 41) at -20°C. A thin slice of agar, and mycelial and spore material was aseptically removed from the frozen culture annually. The slice was placed in the centre of a SDA plate. After 14 days incubation at 25°C, the resultant colony was subcultured onto SDA slopes. These secondary slope cultures were stored at 5°C for a maximum of one year. The secondary cultures were used to inoculate SDA plate cultures which were used as the inoculant for all experiments.

Inoculant preparation

Grain of Japanese millet (*Echinochloa utilis*) (150 g) was combined with distilled water (100 ml) in cottonwool stoppered 500 ml Erlenmeyer flasks and autoclaved at 121°C for 15 minutes. After cooling, the millet was aseptically inoculated with two squares (1 cm each) of the sporulating *M. anisopliae* (DAT F-001) culture cut directly from SDA plates. The grain/fungus mixtures were shaken every two to three days to avoid clumping of the grain during the four week incubation period at 20° ± 2°C. (The modification of this method for the production of large quantities required for field experiments is described in Chapters 8 and 10).

To determine the number of fungal spores per gram of the grain fungus (inoculant) mix, 20 g of the inoculant was suspended in 200 ml of sterile distilled water containing 0.01% Triton X-100. After orbital shaking for 10 minutes, a sterile disposable pipette was used to inoculate a single well of a Kova glasstic slide. The slide well was examined under the microscope (100 x) and the numbers of spores in five fields of view were recorded. These figures were then converted to spores/g grain and gave an estimate of the required weight of inoculant to be used in the bioassays.

3.2 PROCEDURES FOR INSECTS

Collection and pretreatment of insects

For all laboratory experiments, except the early screening tests (section 4.2, 4.3), third instar larvae and adults of *A. couloni* were hand-collected from infested paddocks at 'Inverell' York Plains, in the Midlands region of Tasmania during the autumn and winter of 1988 and 1989. For the experiments in section 4.2 and 4.3, the larvae were collected from 'Glenmorey' Woodbury (12 km north of York Plains). Two hundred to three hundred larvae or adults were placed into galvanized-iron trays with lids (400 x 300 x 80 mm) filled with a sand-peat mix (1:1) and maintained in a laboratory at 10°C for two weeks prior to experimentation. *A. couloni* could be maintained in the laboratory exclusively on sand-peat mix for their entire life-cycle. The larvae feed on the organic matter while the adults were non-feeding.

3.3 BIOASSAY METHODOLOGY

A bioassay was developed whereby *A. couloni* larvae or adults were exposed to concentrations of fungal conidia in mixes of sand-peat. To obtain each concentration a calculated amount of fungal grain was combined with 22.5 kg of non-sterile sand-peat (1:1) and sufficient tap water to give a moisture content of about 20% (w/w) when mixed in a concrete mixer for 10 minutes. At the completion of mixing, 7.5 kg of the inoculated (treated) or uninoculated (control) sand-peat was poured into galvanized-iron trays with lids (400 x 300 x 80 mm; L x W x H). Larvae or adults were placed onto the surface of the sand-peat and allowed to burrow into the mix. Equal numbers of treatment and control replicates were used. The number of larvae/replicate, and the number of replicate trays used, is reported separately for each experiment.

The effective concentration to which the larvae were exposed was estimated by taking two 40 g sand-peat samples at the completion of mixing. Each sample was then suspended in 200 ml of sterile Ringers solution and standard serial dilutions were plated in duplicate onto the medium of Doberski and Tribe (1980) (D&T). After 14 days incubation at 25°C the resulting plate counts were used to calculate the concentration in spores per gram of sand-peat. By comparison with the inoculant concentration determined by Kova counting, the plate counts

of the sand-peat mixes were approximately half the theoretical concentration. Spore germination averaged 95%.

At each sampling occasion (usually 7 or 14 days), each tray was emptied onto the working surface of a Class I biological safety cabinet and the sand-peat mix sifted by hand. The number of alive, mummified or dead (other than by fungus) insects were recorded. The sand-peat was then placed into the tray and the remaining alive larvae were placed on the surface of the sand-peat and allowed to burrow into the mix. Mummified or dead larvae were not reintroduced into the experiment to avoid an increase in exposure of surviving larvae to the fungus (or other diseases).

The gross weight of each tray of sand-peat was checked against the weight recorded initially. The weight was adjusted to the correct gross weight by spraying the surface of the sand-peat with tap water after the larvae had burrowed beneath the surface. Each tray was reincubated immediately after recording.

3.4 STATISTICAL PROCEDURES

Simple experiments used *t*-test, Analysis of Variance or Chi-square procedures (Minitab Reference Manual, 1989). The field experiments used a paired *t*-test. The methodology and statistical procedures for the field experiments was reported by Koen and Rath (1990, appendix 4). Canonical variate analysis was used in Chapter 11 and is described there.

Bioassays and germination experiments

Standard procedures of Least Squares analysis of variance and regression analysis, assuming Normal distribution of residuals, were inappropriate methods to analyze these data (Hall, 1982). Rather, the method of Generalised Linear Models (GLM) (McCullagh and Nelder, 1983) was used to analyze both experiments. An intuitive explanation of GLM procedures and their application to agricultural research has been given by Crosbie and Hinch (1985).

For these experiments, spore germination and larval survival follow a sigmoidal response curve when monitored across time. This S-shaped curve was adequately modelled by the logistic function:

but graphing the data may show that the regressions appear very different (and hence suggest that there are intrinsic differences between the doses). GLIM modelling of the 'fit' of the data to specific models can add support to this interpretation.

The GLIM 3.77 manual describes the applications of logistic models to grouped binomial data. An example of the fitting of the differing models used in my analyses and the order in which they were fitted, is given (The GLIM System, 1987; p.127-129) and an account of the measure of the 'Goodness of Fit' of a model is discussed in Appendix 5 and 6 of the GLIM System User's Guide (The GLIM System, 1987). While the GLIM system allows for detailed study of the parallelism or otherwise of fitted curves, my intention was to determine whether dose, temperature or germination curves were different. To this end the results are reported in terms of whether, for example, different dose response curves are significantly different. Whether these curves differed with respect to slope, intercept or both was determined (as per the example in the GLIM manual; The GLIM System, 1987) but was considered of minor importance compared to the initial question of significance. Information on this aspect can be ascertained by examination of the statistical table given with each analysis.

Chapter 4

4.0 FUNGUS SELECTION EXPERIMENTS

4.1 GENERAL INTRODUCTION

Entomogenous fungi are the pathogens of choice for coleopteran control (Roberts and Humber, 1984), and soil is an ideal reservoir for these fungi in control of subterranean pests (Klein, 1988; Milner, 1989). Roberts and Humber (1984) placed the fungi at the head of their list because with the exception of the pathogen control of *Oryctes rhinoceros* with *Oryctes* baculovirus and *Popilliae japonica* with *Bacillus popilliae*, effective viral, bacterial and protozoan diseases are unknown.* More recently, the bacterial disease of *Costelytra zealandica*, *Serratia entomophila*, has been added to this list (see below). However, the decision to use entomogenous fungi in the development of a programme for *Adoryphorus couloni* is not as obvious as Roberts and Humber (1984) suggest. Currently, there is only one registered fungus for the control of coleopterans (*Beauveria bassiana*, Boverin®) whilst there are two bacteria (*Bacillus popilliae* and *Serratia entomophila*) and one virus disease (*Oryctes* baculovirus).

Bacteria

The classic example of microbiological control of a subterranean pest is the control of Japanese beetle (*P. japonica*) with the bacterium *B. popilliae* (Dutky, 1963; Falcon, 1971; Klein, 1981, 1988). This control is cited as a 'classic' example because the pest was introduced into the U.S.A. and the disease may also have been introduced (Obenchain and Ellis, 1990a). The control of *P. japonica* utilizes mass-produced *B. popilliae* spores which are incorporated into the soil (2 g is applied to the soil surface in a grid at 1 m intervals) after which the disease becomes established in the pest population. *P. japonica* can be controlled for many years by the one application (Klein, 1988). This bacterium was commercialized in the U.S.A. in the 1940's (Daoust, 1990), and *B. popilliae* products are still marketed, including Grub Attack® marketed by the Ringer Corporation.

B. popilliae is mass-produced *in vivo* because it has been impossible to develop *in vitro* methods (Klein, 1988). This makes *B. popilliae* an expensive

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* A book reviewing this area of research will be published in May 1992: "The Use of Pathogens in Scarab Pest Management" (ed. T.A. Jackson and T.R. Glare). Intercept Press: Andover, U.K.

'insecticide' costing US$330 -880 /ha (Klein, 1988). Recent claims by Obenchain and Ellis (1990b) of an *in vitro* method for the production of *B. popilliae* have been retracted (T.R. Glare, personal communication, 1991) and consequently, a major disadvantage of the bacterium (*in vivo* production) remains. Klein (1988) believes that until the production problems are solved, *B. popilliae* will not be an important component in the suppression of soil-inhabiting insects.

B. popilliae has been isolated from many species of scarabs from many countries (Klein, 1981, 1988) including Australia (Milner, 1974, 1977, 1979). Milner (1981) was unable to infect *A. couloni* with *B. popilliae* var. *rhopaea* either by injection of 10^7 spores/larva or 10^9 spores/larva *per os.*

Serratia entomophila is an endemic disease of the New Zealand scarab, *Costelytra zealandica.* This bacterium is developed as an inundative control agent (Jackson and Pearson, 1986; Jackson, 1989). The vegetative cells of the bacterium are diluted with water and incorporated into the pasture soil with a triple disc drill (Jackson *et al.*, 1989). This bacterium was registered in New Zealand in 1990 under the tradename Invade® and is marketed by Monsanto N.Z. Ltd (Jackson, 1990). The development of the product is described by Jackson (1992). The disease is of low virulence for *A. couloni* (T.R. Glare, personal communication, 1990).

Viruses

Control of the palm rhinoceros beetle, *Oryctes rhinoceros*, with the *Oryctes* baculovirus has been described by Bedford (1981). Like the introduction of *B. popilliae*, *Oryctes* baculovirus was absent from the south Pacific nations where *O. rhinoceros* was a pest of coconut and oil palms (Bedford, 1981). Zelazny *et al.* (1992) believe that the reduction in the average life span of adult *O. rhinoceros* as a result of baculovirus infection, and the persistence of the disease at low host densities are the main reasons for the success of *Oryctes* baculovirus. The *Oryctes* baculovirus is unable to infect *A. couloni per os.* (Crawford *et al.*, 1985).

Other viral diseases (especially entomopoxviruses) have been found in pasture inhabiting scarabs in Europe (Hurpin and Robert, 1977) and Australia (Roberts,

1967; Goodwin and Roberts, 1975; Moore and Milner, 1973; Milner and Lutton, 1975), but not from *A. couloni*.

Fungi

The fungus *Beauveria bassiana* has been used successfully in field control of the Colorado beetle (Col.: Chrysomelidae: *Leptinotarsa. decimlineata*) in the Commonwealth of Independent States (C.I.S.) (Ferron, 1981) and the U.S.A. (Anderson *et al.*, 1988). The control of the scarab, *Melolontha melolontha*, with *Beauveria brongniartii* has been extensively studied (including Ferron, 1981, 1983; Keller, 1986, 1989: Keller *et al.*, 1986; Zimmermann, 1992).

In Australia, Milner and Lutton (1976) examined *Metarhizium anisopliae* pathogens of the scarab *Rhopaea verreauxi*, and Coles and Pinnock (1982, 1984) have a patent on the use of *Metarhizium anisopliae* for another scarab, the blackheaded cockchafer (*Aphodius tasmaniae*). This patent (AU-C-14863/83) includes in its claims that the selected strain of the fungus also controls *A. couloni* and *Sericesthis* spp. Throughout the 1980's, research by the Bureau of Sugar Experimental Stations, the Waite Institute of the University of Adelaide and the CSIRO (Milner, 1992) was directed towards scarab control in sugar cane. An isolate of *M. anisopliae* was isolated from *A. couloni* by C. Reinganum (R.J Milner, personal communication, 1985).

This chapter reports the initial studies undertaken to find isolates of entomogenous fungi virulent to *A. couloni* which could be of further experimental use.

4.2 INITIAL PATHOGENICITY STUDIES

4.2.1 Introduction

The relative pathogenicity of isolates of *Metarhizium anisopliae* obtained from CSIRO, the Waite Institute and some Tasmanian isolates for L3 larvae of *Adoryphorus couloni* are reported here.

4.2.2 Materials and Methods

The original host and the supplier of each of the nine isolates of *M. anisopliae* are listed in Table 4.1. After 1986, when the isolates were transferred to the Department of Agriculture, Tasmania (DAT) culture collection, the isolates were cultured and maintained as described in Chapter 3. In this experiment each isolate was point inoculated onto each of five petri dishes containing 15 ml of Sabouraud dextrose agar (SDA, Oxoid CM 41) and grown for 14 days at 25° ± 1°C.

The pathogenicity of the nine isolates was evaluated by comparing the survival and mummification of third instar (L3) larvae. The larvae were collected (from pastures in the Midlands region of Tasmania) and maintained in a sand-peat mix (1:1, 20% water) at room temperature for 14 days prior to inoculation. Ten L3 larvae were rolled in each of the 14-day old sporulating cultures (50 larvae/isolate). The rolled larvae were divided into duplicate trays (25 larvae/tray) containing non-sterile 1:1 sand-peat mix (20% water). Two replicates (25 larvae/tray) were used as an untreated control. The number of surviving and mummified larvae were recorded after 30 days incubation at 25° ± 1°C. Differences between the survival of untreated and treated larvae were assessed using the *t*-test procedure.

4.2.3 Results and Discussion

The virulence of isolates of *M. anisopliae* for *A. couloni* varied markedly (Table 4.1). Only isolates DAT F-001, F-002 F-025 and F-026 were significantly different ($P<0.05$) from the untreated controls. DAT F-001 and F-020 produced mummified cadavers but the mummification produced by DAT F-001 occurred in only one replicate.

Table 4.1 **The origin and virulence of 9 isolates of *M. anisopliae* to L3 larvae of *A. couloni* inoculated by rolling the larvae in fungal spores followed by incubation in sand-peat for 30 days at 25°C.**

DAT Accession number	strain origin	Original host (insect larvae)	% Survival*	% Mummified cadavers*
DAT F-001	CSIRO†	*Adoryphorus couloni*	0.0 ± 0.0[a]	28.0 ± 28.0
DAT F-017	Author‡	*Adoryphorus couloni*	74.0 ± 2.0	0
DAT F-020	Author	*Adoryphorus couloni*	16.0 ± 16.0	6.0 ± 2.0
DAT F-026	Author	*Adoryphorus couloni*	0.0 ± 0.0[a]	0
DAT F-002	CSIRO	*Aphodius tasmaniae*	32.0 ± 4.0[a]	0
DAT F-010	Waitpinga§	*Aphodius tasmaniae*	58.0 ± 2.0	0
DAT F-011	Eyre Peninsular§	*Aphodius tasmaniae*	56.0 ± 4.0	0
DAT F-012	Aldegate§	*Aphodius tasmaniae*	40.0 ± 8.0	0
DAT F-025	Author	*Aphodius tasmaniae*	4.0 ± 0.0[a]	0

* mean ± SE., n=2. Annotated values are significantly different ($P<0.05$) from control survival (72.7 ± 5.7%)

† Dr R Milner, CSIRO Division of Entomology, GPO Box 1700, Canberra Australia 2601

‡ Tasmanian isolates

§ Professor D Pinnock, Entomology Department, Waite Institute, University of Adelaide, South Australia

Rolling insects in fungus can be regarded as giving the insects the maximum possible dose, even though most of the spores on the cuticle would be dislodged by the larval movement through the sand-peat. In the study reported here, it is unlikely that any fungus found to have low virulence to *A. couloni*, would have high virulence when exposed to repeatable dose (or concentration) bioassays. This method is likely to generate more false positives (pathogens) than bioassays.

This method was also used by Milner (1990) and Yip *et al.* (1992) to screen *M. anisopliae* isolates for scarab control. Yip *et al.* (1992) screened 184 *M. anisopliae* cultures for pathogenicity to *A. couloni*. They found that only 10% of isolates were pathogenic whereas in this study 4/9 were pathogenic. This difference may be related to the source of the isolates. The *M. anisopliae* cultures of Yip *et al.* (1992) were isolated from pasture soils and would have a wide range of hosts, potentially covering many insect orders. By comparison, in this study the fungi were isolated from two host insects, one of which was *A. couloni*. However, only 2/4 of these *A. couloni* isolates (DAT F-001 and F-026) were pathogenic.

Two *A. couloni* isolates (DAT F-001 and DAT F-020) were the only isolates to produce mummified cadavers. These cadavers were proof that the fungus had at least invaded the larvae. The virulence of the pathogenic isolates is further examined in the following section (section 4.3).

4.3 COMPARATIVE VIRULENCE OF *M. ANISOPLIAE* DAT F-001, F-020, F-025 AND F-026.

4.3.1 Introduction

The previous study (section 4.2) showed that four isolates of *M. anisopliae* significantly reduced the survival of L3 larvae, however only one of these isolates produced mummified larvae (DAT F-001). A fifth isolate did not significantly reduce larval survival but did produce mummified cadavers (DAT F-020).

The pathogenicity of fungi to insect hosts can be quickly determined by rolling the insects in spores of the fungus (Milner, 1990, 1992; Yip *et al.*, 1992) but this method inoculates the insect with an extremely high dose which does not assess the virulence of an isolate. The virulence of isolates selected in this manner can only be evaluated by more rigorous bioassays.

If *A. couloni* are going to be infected by *M. anisopliae*, the larvae must contact an 'appropriate' dose in the soil. A bioassay based on dosing sand-peat mixes with *M. anisopliae* was developed (Chapter 3) and is used here to determine whether there are any differences between the virulence of select isolates examined earlier (section 4.2).

4.3.2 Materials and Methods

Concentrations from 1 x 10^2 - 5 x 10^5 spores/g sand-peat (20% moisture w/w) of each of four fungal isolates (DAT F-001, F-020, F-025 and F-026) were prepared as described in Chapter 3. 7.5 kg of each treatment (fungal isolate x dosage concentration) was placed in each of two galvanized iron trays with lids (100x 300x80 mm). For each fungal isolate, two trays, each with 7.5 kg of untreated sand-peat (20% moisture), were used as controls. L3 larvae (75) were placed onto the top of the sand-peat for each treated and untreated tray and allowed to burrow into the mix. The trays were incubated at 20° ± 2°C for 51 days. The effect of fungal concentration on virulence was evaluated by comparing the survival and mummification of the L3 larvae exposed to dosed sand-peat mixes. Neither, the response of larvae to fungal concentration nor mummification rates of the four fungi were statistically compared as graphical analysis (Fig. 4.1) was quite clear.

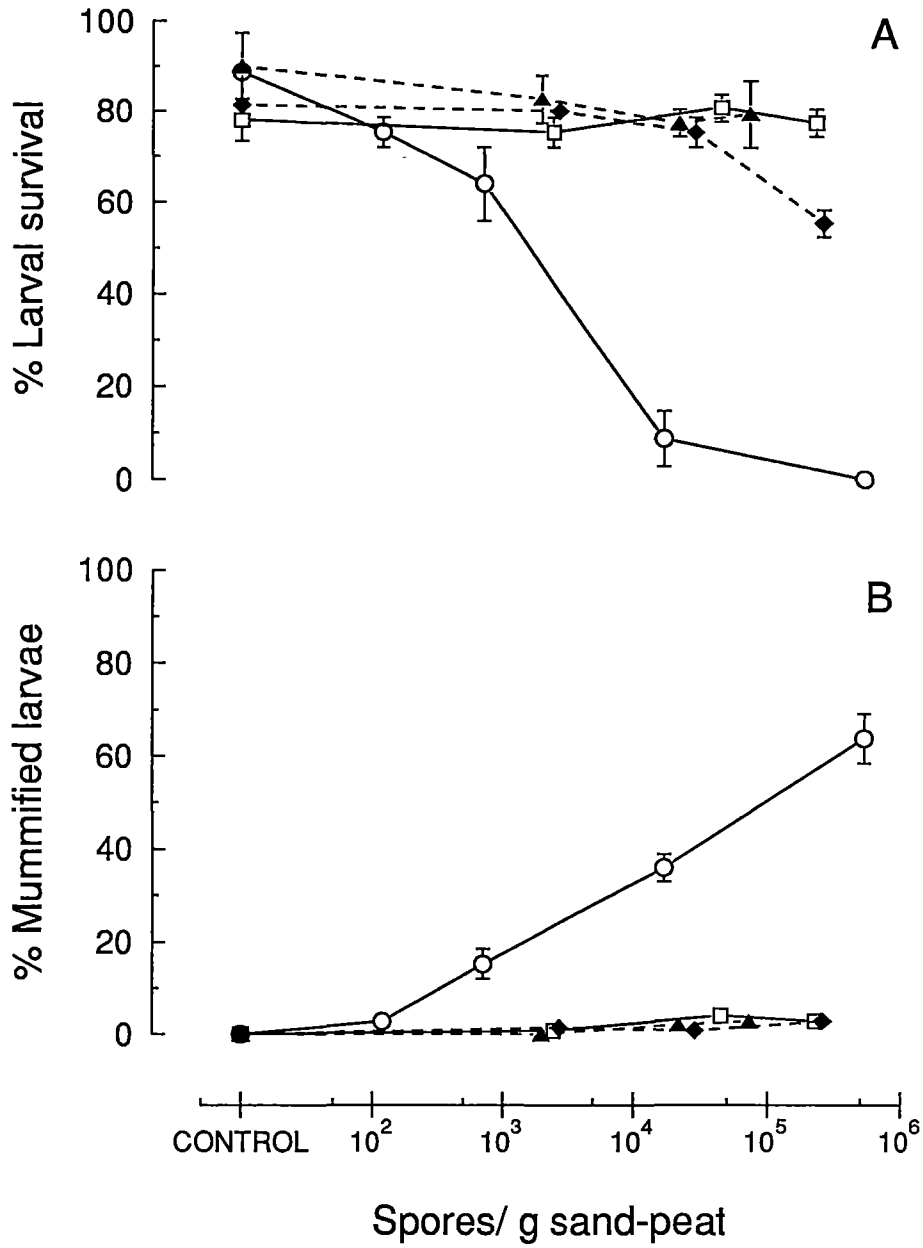

Figure 4.1 **The effect of spore concentration in sand-peat of four isolates of *M. anisopliae* (○ DAT F-001; ▲ DAT F-020; ❑ DAT F-025; ♦ DAT F-026) on (A) the survival and (B) the percentage of mummified cadavers of L3 larvae of *A. couloni* after 51 days at 20°C (mean ± SE.).**

4.3.3 Results

DAT F-001 reduced larval survival to less than 10% when concentrations of 1.7 x 10^4 spores/g sand-peat or higher were used (Fig. 4.1A) and the percentage of mummified larvae (Fig. 4.1B) increased with increasing spore concentration,

reaching 63% mummification at the highest concentration used. DAT F-026 reduced larval survival to 55% (*cf* untreated = 81% survival) at the highest concentration tested (2.6 x 10^5 spores/g sand-peat), however, mummification was less than 3%. Isolates DAT F-020 and F-025 did not reduce larval survival at the concentrations tested and mummification was less than 4%.

4.3.4 Discussion

M. anisopliae isolate DAT F-001 was the most virulent isolate tested, producing 90% larval mortality at a concentration of 1.7 x 10^4 spores/g sand-peat and 100% at a concentration of 5 x 10^5 spores/g sand-peat (Fig. 4.1). Isolate DAT F-026 produced 32% mortality at a concentration of 2.6 x 10^5 spores/g sand-peat (Fig. 4.1A), however this isolate was considerably less virulent than DAT F-001 which had produced 100% mortality at a similar concentration. Other isolates (DAT F-020 and F-025) which gave high larval mortalities in the earlier pathogenicity screening studies (section 4.2) were non-pathogenic at the concentrations tested (up to about 1 x 10^5 spores/g sand-peat; Fig. 4.1A), but may be pathogenic at higher concentrations. *M. anisopliae* concentrations of between 10^5 and 10^8 spores/g soil are commonly required to induce disease development in soil pests in the laboratory Ferron (1981).

The effect of spore concentration on the virulence of DAT F-001 is clearly seen in this experiment and may be shown for the other isolates by comparison with the earlier experiment (section 4.2). In that experiment DAT F-025 and F-026 significantly reduced survival of L3 larvae when given an extremely high dose, whereas in this experiment lower doses (concentrations) were unable to induce mortality (with the exception of DAT F-026 at 2.6 10^5 spores/g sand-peat).

Isolates DAT F-020, F-025 and F-026 do not warrant further investigation as the highly virulent nature of *M. anisopliae* isolate DAT F-001 is shown by the reduced survival and some mummification at concentrations of between 10^2 and 10^3 spore/g sand peat. The virulence of this isolate is further studied.

4.4 EFFECT OF *METARHIZIUM ANISOPLIAE, M. FLAVOVIRIDE* AND *BEAUVERIA BASSIANA* ON THE SURVIVAL OF L3 LARVAE OF *A. COULONI.*

4.4.1 Introduction

During the assessment of preliminary field experiments using *Metarhizium anisopliae* DAT F-001 (Rath, 1989; appendix 1), three adults from the overlapping population were found mummified with a different isolate of *M. anisopliae* (DAT F-054). Additionally, two naturally infected populations of adults were found after mating flights, one infected with *Beauveria bassiana* (DAT F-140) and the other with *Metarhizium flavoviride* (DAT F-133). Further, a *M. anisopliae* isolate (DAT F-141) was found infecting a larval population in Northern Tasmania in 1989 (Table 4).

The virulence of these isolates for L3 larvae of *A. couloni* in comparison with *M. anisopliae* DAT F-001 is reported here.

4.4.2 Materials and Methods

Experiment 1: Comparison of *M. anisopliae* DAT F-001, F-141, *M. flavoviride* DAT F-133 and *B. bassiana* DAT F-140

A concentration of 2 x 10^6 spores/g sand-peat mix was prepared for each fungal isolate (except *M. anisopliae* DAT F-054) as described in Chapter 3. L3 larvae (75) were placed onto the top of the sand-peat for each of the three replicate trays used for each fungal isolate as well as the untreated control, and allowed to burrow into the mix. The trays were incubated at 20° ± 2°C for up to 112 days. The effect of the fungal isolates was determined by recording the survival and mummification of larvae every 14 days. Mummified larvae were removed at the time of counting. 'Abbott's formula' (Finney, 1971) was used to correct treatment mortality for the effect of 'natural' mortality (as determined by the survival of untreated larvae - mean of three replicates). GLIM analysis (Chapter 3) was used to assess differences between treatments.

Experiment 2: Comparison of *M. anisopliae* DAT F-001 and F-054

DAT F-054 was not included in the first experiment due to initial difficulties in the spore production of the inoculant. This isolate was tested 8 weeks later using the same procedure as outlined above, and included 3 replicates of both DAT F-001 inoculated and untreated control as checks. Both experiment 1 and 2 were conducted in the same incubator and consequently the first part of the experiment was still in progress when the second part commenced. However, the L3 larvae had been maintained at 10°C for an additional 8 weeks before use in this experiment.

Table 4.2 The origin, isolation date and host stage of fungal isolates

Fungal isolate	date isolated	*A. couloni* life stage	origin
Metarhizium anisopliae			
DAT F-001 (strain 3)*	10 Jul. '81	larva	Ballarat, Vic
DAT F-054 (strain 16)*	18 Nov. '87	adult	Woodbury, Tas
DAT F-141 (strain 3)*	May '89	larva	Wilmot, Tas
Metarhizium flavoviride			
DAT F-133	1 Sep. '88	adult	Mt.Morriston, Tas
Beauveria bassiana			
DAT F-140	1 May '89	adult	Woodbury, Tas

* From the classification system of Yip, Rath and Koen (1992)

4.4.3 Results

Experiment 1: Comparison of *M. anisopliae* DAT F-001, F-141, *M. flavoviride* DAT F-133 and *B. bassiana* DAT F-140

Survival of untreated larvae

There was 92.2 ± 0.41% of larvae surviving at day 56. The larvae started to pupate by 70 days and by 98 days, 60% (84/142) were prepupae and pupae. Total survival was 63.3 ± 3.5% after 98 days.

Survival of treated larvae

Fungi isolated from adult *A. couloni* (*M. flavoviride*, DAT F-133 and *B. bassiana*, DAT F-140) were non-pathogenic ($P<0.0001$) to larvae (Fig. 4.2). There were no differences ($P>0.05$) in the numbers of larvae/prepupae/pupae surviving in the untreated replicates or either of these two treatments.

Both larval isolates (*M. anisopliae*, DAT F-001 and F-141) were highly virulent to L3 larvae (Fig 4.2) and had LT_{50}'s of 24.5 and 21.0 days (respectively). GLIM analysis indicated that these two isolates differed in their virulence (Table 4.3).

Experiment 2: Comparison of *M. anisopliae* DAT F-001 and F-054

Survival of untreated larvae

There was 72.4 ± 5.4% survival at day 56. The larvae had started to pupate by 56 days and by 98 days, 73% (54/74) were prepupae or pupae. Total survival was 32.9 ± 7.7% after 98 days.

Survival of treated larvae

The survival of treated larvae was different ($P<0.05$) to the survival of untreated larvae at all times from 14 days to 98 days. *M. anisopliae* DAT F-001 was more virulent than *M. anisopliae* DAT F-054 (Fig. 4.3) and the LT_{50}'s were 13.6 and 58.6 days respectively (Table 4.3). The high standard errors for the DAT F-001 curve (Table 4.3) are due to the fitting of a 2-parameter model to just 3 data points (Fig. 4.3), rather than a poor fit of the data.

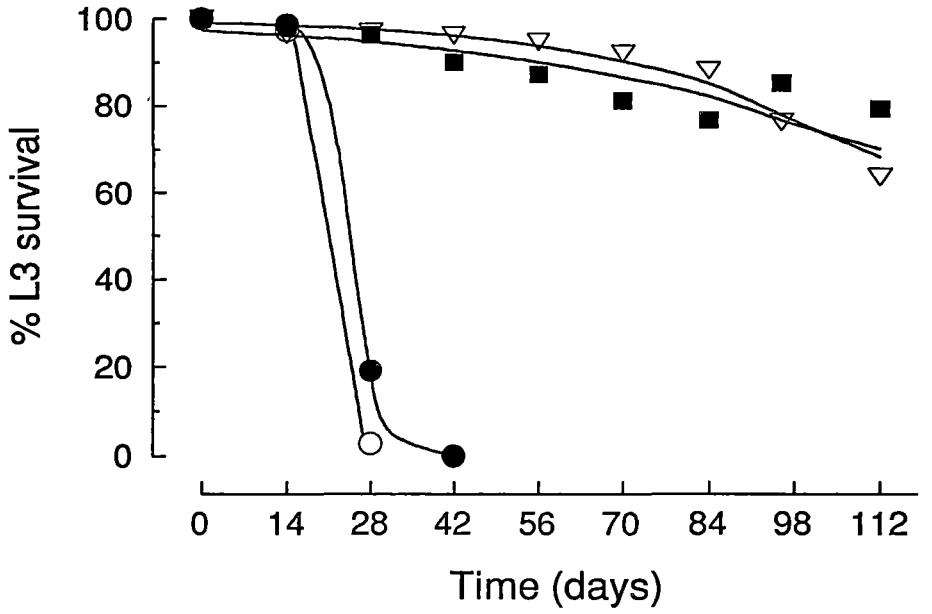

Figure 4.2 Regression curves of survival (corrected) of *A. couloni* L3 larvae versus time, after exposure to 2 x 10^6 spores/g of (●) *M. anisopliae* DAT F-001, (○) *M. anisopliae* DAT F-141, (▽) *M. flavoviride* DAT F-133 and (■) *Beauveria bassiana* DAT F-140 in sand-peat at 20° ± 2°C.

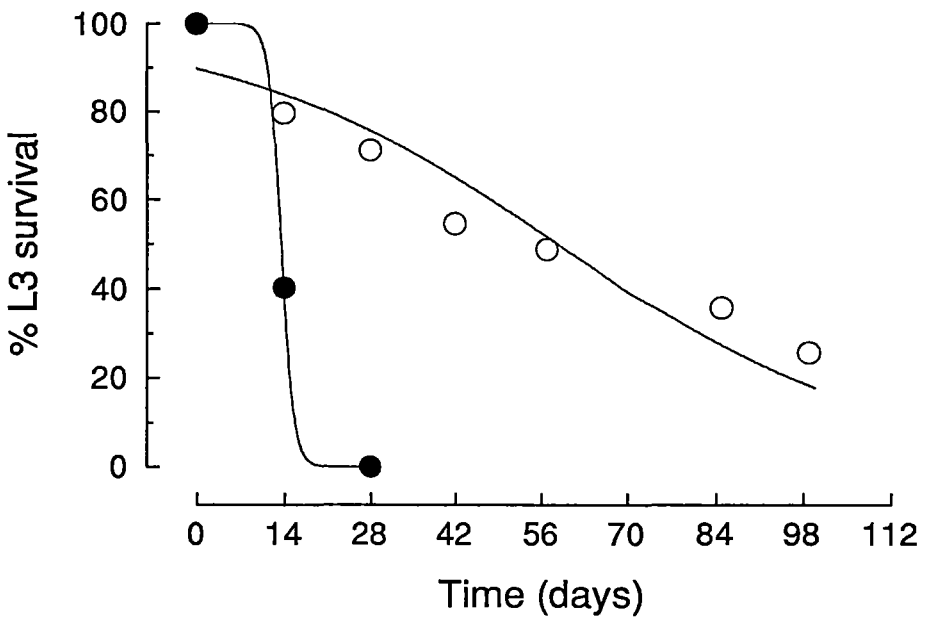

Figure 4.3 Regression curves of survival (corrected) of *A. couloni* L3 larvae versus time, after exposure to 2 x 10^6 spores/g of (●) *M. anisopliae* DAT F-001, and (○) *M. anisopliae* DAT F-054 in dosed sand-peat at 20° ± 2°C.

Table 4.3 **Regression equations (using GLIM procedures) for the survival of L3 larvae of *A. couloni* exposed to fungal inoculated sand-peat at a concentration of 2 x 10^6 spores/g.**

		Parameters*						
		integration constant (*a*)		intrinsic rate of increase (*r*)				
Fungal isolate		estimate	SE	estimate	SE	sigt†	LT_5	LT_{50}
Experiment 1								
M. anisopliae	DAT F-001	10.15	1.16	-0.414	0.042	a	17.4	24.5
"	DAT F-141	10.75	1.48	-0.511	0.059	b	15.3	21.0
M. flavoviride	DAT F-133	4.70	0.34	-0.035	0.0042	c	50.1	(134.0)‡
B. bassiana	DAT F-140	3.59	0.20	-0.024	0.0026	c	26.3	(147.0)
Experiment 2								
M. anisopliae	DAT F-001	13.53	29.85	-0.9941	2.132	a	10.6	13.6
"	DAT F-054	2.17	0.13	-0.0370	0.0026	b	-	58.6

* Where the proportion surviving = $(e^X) / (1+e^X)$

where x = $a + r$ time (days)

† In each experiment equations followed by the same letter do not differ significantly (P>0.05). Those labelled 'c' do not differ from the untreated controls.

‡ figures in brackets represent extrapolated data.

4.4.4 Discussion

These experiments have shown that only the two fungi originally isolated from *A. couloni* larvae (DAT F-001 and F-141) were pathogenic to L3 larvae of the host. The adult isolates in the first experiment (*M. flavoviride* and *B. bassiana*) were non-pathogenic to L3 larvae whereas *M. anisopliae* DAT F-054 was of low virulence.

M. anisopliae DAT F-001 was more virulent to L3 larvae in the second experiment than in the first (Table 4.3). This was probably due to increased susceptibility of the larvae as a result of a physiological effect induced by either the longer storage at 10°C or by the increased larval age, rather than an increase in the virulence of the fungus with storage. This effect probably also increased the virulence of *M. anisopliae* DAT F-054.

These results which show that (i) adult isolates have little or no virulence to larvae, and (ii) that larval isolates are highly virulent to larvae are consistent with those of Boucias and Pendland (1984) and others (reviewed by McCoy *et al.* 1988) who report that fungi may infect only one particular life stage (ie. egg, larval, pupal or adult). The virulence of these isolates for adult *A. couloni* is reported in the next section.

4.5 EFFECT OF *METARHIZIUM ANISOPLIAE, M. FLAVOVIRIDE* AND *BEAUVERIA BASSIANA* ON THE SURVIVAL OF ADULT *A. COULONI.*

4.5.1 Introduction

Cultures of three species of entomogenous fungi, originally isolated from adult *A. couloni*, were not pathogenic to L3 larvae of *A. couloni* (section 4.4). If these fungi are highly virulent to adult *A. couloni*, a mixture of fungal species, which gives both larval and adult control, may be useful in the total suppression of *A. couloni* populations.

This experiment reports the virulence of these adult isolates in comparison with the larval isolate *M. anisopliae* DAT F-001.

4.5.2 Materials and Methods

The experimental procedure for this experiment was similar to that described in section 4.4, except that three replicates of three concentrations (1×10^4, 1×10^5, 1×10^6 spores/g sand-peat mix) of each isolate (*M. anisopliae* DAT F-001, F-054, *Metarhizium flavoviride* DAT F-133, *Beauveria bassiana,* DAT F-140), and an untreated control, were used. Males (25) and females (25) were randomly assigned to each tray. The number of eggs laid/tray was recorded in addition to the number of surviving males and females. Eggs were removed from the trays at each sampling occasion.

This experiment was conducted 12 months after the larval experiment. The adults were collected from the same paddock as the larvae, and consequently they were from the same genetic pool.

4.5.3 Results

Survival of untreated adults

Male and Female survival was about 80% after 42 days but then declined rapidly over the next 28 days to 10% and 35% respectively (Fig. 4.4).

Survival of treated adults

Specific criteria were set for the significant reduction of survival of the male and female adults by the fungi because the life span of the untreated adults was short (Table 4.4): The fungi had to kill the adults before the rapid decline in survival which occurred in males and females after 42 and 56 days respectively (Fig. 4.4). Therefore, (1) For males, a reduction in survival equal to, or less than 50%, would have to be achieved between 28 and 42 days, while, (2) For females, this time was between 42 and 56 days (Fig. 4.4). Reduction at these times or earlier may limit mating and subsequent egg production.

M. anisopliae DAT F-001 reduced the survival of both sexes at concentrations of 10^5 and 10^6 spores/g sand-peat, but was not pathogenic at 10^4 spores/g sand-peat (Table 4.4). All concentrations of *M. flavoviride* DAT F-133 reduced the male and female survival to less than or equal to 50% within the appropriate time but this isolate was less virulent than DAT F-001 at 10^6 spores/g sand-peat (Table 4.4).

M. anisopliae DAT F-054 and *B. bassiana* DAT F-140 were non-pathogenic to adults at the concentrations and times studied (Table 4.4).

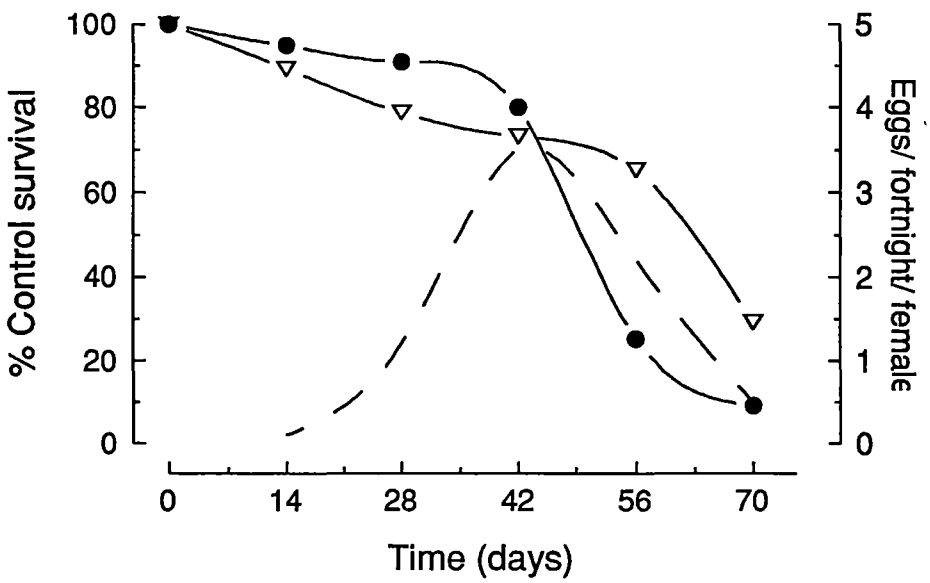

Figure 4.4 **Survival of untreated (●) male and (∇) female *A. couloni* and (— — —) egg production/fortnight/surviving female in sand-peat at 20° ± 2°C.**

Table 4.4 **Survival of male and female *A. couloni* (corrected data) and reduction in egg production after 28, 42 or 56 days following exposure at 20°C to three concentrations (10^4, 10^5, 10^6 spores/g sand-peat) of four entomogenous fungi (*M. anisopliae* DAT F-001, DAT F-054; *M. flavoviride*, DAT F-133 and *B. bassiana* DAT F-140).**

		% Survival of adult *A. couloni*			
		Male		Female	
Fungal isolate	concn (spores/g sand-peat)	28 days	42 days	42 days	56 days
M. anisopliae					
DAT F-001	10^4	94.2 ± 3.7*	103.3 ± 6.0	102.0 ± 16.2	104.3 ± 12.3
	10^5	64.7 ± 1.4	51.7 ± 8.3[a]	89.3 ± 4.8[b]	36.8 ± 7.1[ab]
	10^6	19.7 ± 6.1[a]	3.3 ± 3.3[a]	18.2 ± 1.8[ab]	0.0[ab]
DAT F-054	10^4	97.1 ± 4.2	83.3 ± 4.4	122.0 ± 4.8	98.2 ± 9.4[b]
	10^5	101.3 ± 2.4	76.7 ± 6.0	91.1 ± 13.2	65.4 ± 4.1
	10^6	91.4 ± 7.8	86.7 ± 18.6	100.2 ± 12.8	73.6 ± 12.8
M. flavoviride					
DAT F-133	10^4	83.0 ± 10.0	73.3 ± 8.3[a]	96.5 ± 9.7	65.4 ± 10.8[a]
	10^5	67.5 ± 10.6[a]	51.7 ± 13.0[a]	63.8 ± 14.9	47.0 ± 16.0[ab]
	10^6	47.8 ± 9.9[a]	38.3 ± 7.3[a]	58.3 ± 10.2[ab]	24.5 ± 3.5[ab]
B. bassiana					
DAT F-140	10^4	97.1 ± 6.5	103.3 ± 7.3	89.3 ± 14.2	67.5 ± 3.5[b]
	10^5	84.4 ± 2.4	66.7 ± 4.4	107.5 ± 4.8[b]	45.0 ± 14.8[ab]
	10^6	85.8 ± 5.1	71.7 ± 1.7	105.7 ± 13.2[b]	87.9 ± 20.5

* mean ± SE; n = 3

a values annotated are significant ($P<0.05$) such that 100% > mean survival ≤ 50%.

b values annotated (females only) indicate fungal concentrations in which cumulative egg production was significantly less ($P<0.05$) than control egg production.

Table 4.5 **Mean egg production per replicate tray after 42 and 56 days exposure to three concentrations (10^4, 10^5, 10^6 spores/g sand-peat) of four entomogenous fungi (*M. anisopliae* DAT F-001, DAT F-054; *M. flavoviride*, DAT F-133 and *B. bassiana* DAT F-140) at 20°C.**

Treatment	concn.	Eggs/replicate tray	
		42 days	56 days
Untreated		119 ± 21	157 ± 13
DAT F-001	10^4	66 ± 15	93 ± 21
	10^5	36 ± 15*	52 ± 7*
	10^6	40 ± 16*	42 ± 17*
DAT F-054	10^4	85 ± 19	88 ± 21*
	10^5	114 ± 27	124 ± 30
	10^6	65 ± 17	97 ± 25
DAT F-133	10^4	160 ± 17	175 ± 22
	10^5	71 ± 9	76 ± 9*
	10^6	28 ± 6*	36 ± 8*
DAT F-140	10^4	110 ± 7	122 ± 12
	10^5	54 ± 6*	60 ± 8*
	10^6	33 ± 10*	37 ± 11*

* Values annotated are different ($P<0.05$) from the untreated at either 42 or 56 days; mean ± SE, n=3.

Egg Production

Cumulative egg production/tray was reduced by 75% after 56 days exposure of adults to the 10^6 concentration of all fungal isolates except *M. anisopliae* DAT F-054 (Table 4.4; Table 4.5). After 42 days, 55 to 75% less eggs had been produced in the 10^5 and 10^6 DAT F-001 (*M. anisopliae*) and F-140 (*B. bassiana*) treatments, while egg production was only lessened by DAT F-133 (*M. flavoviride*) at 10^6 spores/g sand-peat (75% reduction, Table 4.5). Where egg production was reduced (annotated values, Table 4.5), the egg production did not differ with increasing fungus concentration ($P>0.05$). Egg production was not reduced at a concentration of 10^4 spores/g sand-peat by any fungus except *M. anisopliae* DAT F-054 (at 56 days, Table 4.5).

4.5.4 Discussion

This experiment has shown one larval isolate (*M. anisopliae* DAT F-001) and one adult isolate (*M. flavoviride* DAT F-133) were pathogenic for adults at concentrations of 10^5 spores/g sand-peat or higher. Both these fungi and a non-pathogenic isolate (*B. bassiana* DAT F-140) reduced the number of eggs laid per tray by up to 75%.

Reduction in adult numbers is only important if there is a resultant reduction in the number of eggs laid because the adults are non-feeding and cause no damage. Reduction of the eggs laid by a population could lead subsequently to a smaller population of larvae. This is particularly true for an insect with such low fecundity as *A. couloni* ('*K* strategists' as defined by Matthews 1976, p.3 ff.). In this experiment, untreated females only laid 6 - 8 eggs each over an 8 wk period. Even under ideal laboratory conditions, McQuillan (personal communication, 1992) has found that 80% of females only lay 13 - 25 eggs each. Reduction in egg production is not only a result of decreased female numbers as egg production was reduced in the *B. bassiana* treatments even though female survival was near or equal to 100%. In these instances the reduction (from 100%) in male survival between 28 and 42 days (though not statistically significant) may have been responsible (Table 4.4).

The lack of virulence of both *M. anisopliae* DAT F-054 and *B. bassiana* DAT F-140, suggests that these two isolates are either saprophytic on *A. couloni* adults or are pathogenic only when the host is close to natural death. *M. anisopliae* DAT F-054 was found very late in the adult life stage, whereas *B. bassiana* DAT F-140 was found several months after all the adults had died. In contrast, *M. flavoviride* DAT F-133 was found in adults immediately after mating flights.

4.6 GENERAL DISCUSSION

These experiments examined the pathogenicity of nine isolates of fungi originally isolated from *A. couloni*. Only two of these isolates (*M. anisopliae* DAT F-001 and *M. flavoviride* DAT F-133) could be regarded as pathogenic. The other isolates, if pathogenic, had very low virulence at the concentrations tested. These studies show that neither isolation of a fungus from a cadaver nor mortality induced after rolling insects in fungal spores, offer proof of virulent isolates. However, the technique of rolling insects in spores proved to be useful in limiting the number of isolates in subsequent bioassays. This technique was also used by Milner (1990, 1992) and Yip, Rath and Koen (1992) to screen *M. anisopliae* isolates for pathogenicity to scarabs.

M. anisopliae DAT F-001 was the most promising isolate tested, being able to infect L3 larvae at concentrations as low as 10^3 spores/g sand-peat (section 4.3), and adults at 10^5 and 10^6 spores/g sand-peat (section 4.5). In both experiments the virulence of the isolate increased with increasing spore concentration. The effectiveness of the low spore concentrations in the larval bioassay contrasts with the accepted requirement of concentrations of 10^5 to 10^8 spores/g soil (Ferron, 1981) and suggests that the influence of time on the virulence of low spore concentrations in soil should be further investigated. This is examined in the next chapter.

Egg production was also reduced by DAT F-001. In the field, the eggs and hatching larvae would also be exposed to the fungus in the soil. A cumulative mortality should occur if eggs and early instar larvae were susceptible to the fungus. A preliminary experiment is reported in the next chapter.

M. flavoviride DAT F-133 was pathogenic for adults at all spore concentrations tested but was not pathogenic for L3 larvae. This isolate was not intensively studied in later experiments, even though it may be of greater use in the control of adult *A. couloni* (due to its greater virulence at low concentrations) than *M. anisopliae* DAT F-001, because registration of a microbial insecticide containing two different species of fungus would be much more costly than a single species product. However, this isolate, and the two other adult isolates found in Tasmania may be a factor in the natural regulation of *A. couloni*, and some further studies are warranted. The influence of temperature on the germination of spores of these isolates is reported in Chapter 6.

Chapter 5

5.0 *M. ANISOPLIAE* DAT F-001 DOSAGE EXPERIMENTS

5.1 GENERAL INTRODUCTION

M. anisopliae DAT F-001 was the most promising fungal pathogen of those examined in Chapter 4. No isolate was found to be more virulent for L3 larvae and the fungus was additionally pathogenic to adults. Total egg production was reduced when adults were exposed to high concentrations. These studies did not address, 1. the relationship between concentration and exposure time, and the effectiveness of low concentrations for L3 larvae; 2. the subsequent effect on eggs and L1 larvae, after adults had been exposed to concentrations of *M. anisopliae* DAT F-001; and 3. the effect on larval feeding of a DAT F-001 infection in respect to whether infected larvae reduce their food consumption. This chapter examines these three areas.

5.2 EFFECT OF DAT F-001 CONCENTRATION AND EXPOSURE TIME ON SURVIVAL OF L3 LARVAE OF *A. COULONI.*

5.2.1 Introduction

Ferron (1981) in a review of laboratory studies on the effectiveness of the insect pathogenic fungi *Metarhizium* and *Beauveria* reported that a concentration of between 10^5 and 10^8 spores/g soil was needed to induce disease development in soil insects. A study of the relationship between concentration of DAT F-001 and exposure time on the survival of *A. couloni* is essential if a balance is to be achieved between population reduction, limitation of pasture damage, fungal application rates and product (pathogen) cost.

This experiment assesses the virulence of seven concentrations (10^1 - 10^7 spores/g sand-peat) of *M. anisopliae* DAT F-001 for L3 larvae of *A. couloni.*

5.2.2 Materials and Methods

A concentration of $1.13 \pm 0.13 \times 10^7$ (mean ± SD.) spores/g sand-peat mix was obtained by combining 833 g of the DAT F-001 inoculant with 25 kg of sand-peat (1:1) and sufficient tap water to give a moisture content of about 20% (w/w) followed by mixing in a concrete mixer for 10 minutes. Serial dilutions (1:9) were made using 2.5 kg of the dosed sand-peat and adding 22.5 kg of undosed

sand-peat with a 20% moisture content. This procedure was repeated until concentrations from 10^7 to 10^1 spores/g sand-peat were prepared. The accuracy of the concentrations from 10^3 to 10^7 were checked using the plate-count method described in Chapter 3 and were found to be within 5% of the stated concentration. 7.5 kg of each treatment (concentration) was placed in each of three galvanized iron trays with lids (400x300x80 mm). Three trays with 7.5 kg of non-dosed sand-peat were used as a control treatment.

L3 larvae (75) were placed onto the top of the sand-peat for each of the three replicate trays used for each concentration (7 DAT F-001 concentrations and 1 nil dose control = 1800 larvae) and allowed to burrow into the mix. The trays were incubated at 20° ± 2°C for up to 112 days. Every 14 days the number of surviving and mummified larvae were recorded. Mummified larvae were removed at the time of counting. 'Abbott's formula' (Finney, 1971) was used to correct treatment mortality for the effect of 'natural' mortality. Statistical analysis used the GLIM procedure and *t*-tests as outlined in Chapter 3.

5.2.3 Results

Survival of Untreated Larvae

After 84 days, 86.7 ± 2.39% (mean ± SE) of larvae were still alive. After this time, the larvae started to pupate and total survival declined to 44.7 ± 2.4% at 112 days when the experiment was terminated. Only 6% of these larvae or pupae were mummified by 112 days.

Survival of Treated Larvae

Survival of L3 larvae was dependent ($P<0.0001$) on concentration and correlated ($P<0.001$) with time (Fig. 5.1). The survival of treated larvae at the different concentrations was significantly different ($P<0.05$) from the untreated (control) survival when the corrected survival (Fig. 5.1) was between 40 and 60%. The survival of treated larvae in all concentrations was significantly different ($P<0.05$) from the untreated larvae by 112 days.

All concentration regression curves except for 10^2 and 10^3 spores/g sand-peat differed ($P<0.05$) from each other (Table 5.1). The intrinsic rate of population

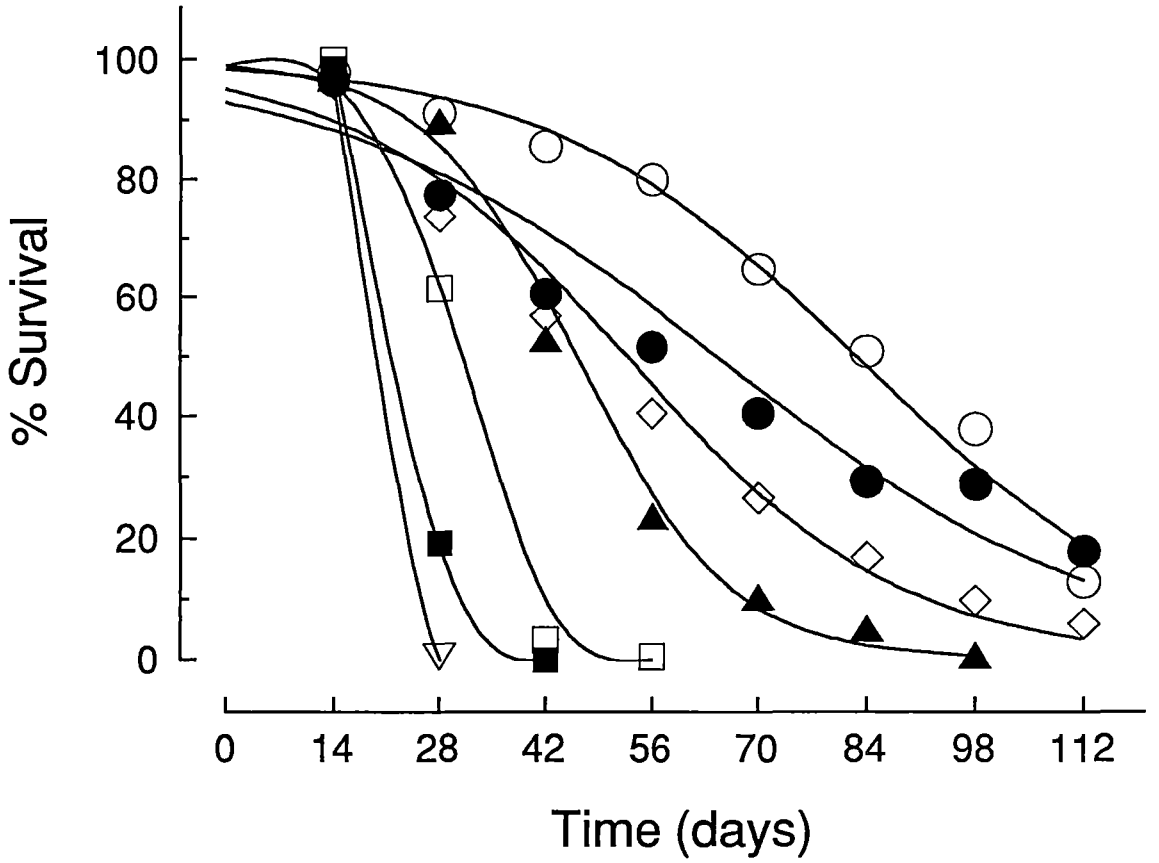

Figure 5.1 **Survival-time regression curves and mean values (corrected data) for L3 *A. couloni* exposed to various concentrations of *M. anisopliae* DAT F-001 treated sand-peat and incubated at 20° ± 2°C (▽ 10^7; ■ 10^6; ❑ 10^5; ▲ 10^4; ◊ 10^3; ● 10^2; ○ 10^1).**

increase (*r*, as used by many ecologists including Yanagita, 1990 where *r* = births - deaths), or more specifically here (because there were no 'births'), the intrinsic rate of mortality (the absolute value of *r*) increased as the concentration was increased (Table 5.1). However, the time required before the onset of any fungal-induced larval mortality or 'lag phase', was equated here to the LT_5 and varied only slightly in response to concentration. By comparison the LT_{50} values increased nearly 4.5-fold over the concentration range (Table 5.1). The ratio of the LT_5 to the LT_{50} was quantified as the %Delay Time (%DT), and gave a measure of the speed of the 'population' collapse after disease initiation (Table 5.1). In these experiments the slow rate of kill at a 10^1 concentration was indicated by the low %DT figure (28.5%) while the %DT for higher concentrations, which killed at a faster rate, range from 65.4% (10^5) to 75.1%. (10^7).

Over the 112 day study period, concentrations of DAT F-001 from 10^3 to 10^7 spores/g sand-peat reduced L3 survival to zero. The maximum mummification level for these concentrations did not differ significantly (P=0.25) and averaged 86.8%. By 112 days, 92.0 ± 3.5% of larvae had died (uncorrected) at the 10^2 concentration, of which 75% had mummified. At the 10^1 concentration, 94.2 ± 3.8% of larvae had died (uncorrected) of which 65% were mummified.

5.2.4 Discussion

This study has shown that all concentrations of DAT F-001 (10^1 - 10^7 spores/g sand-peat) are pathogenic to L3 larvae. While only concentrations from 10^3 - 10^7 killed 100% of larvae within the time frame of the experiment, it is likely that the two lower concentrations (10^1, 10^2) would have done likewise if the time

Table 5.1 Survival-time regression equation, LT_5, LT_{50} and %DT values (corrected data) for L3 larvae of *A. couloni* exposed to sand-peat treated with doses of *M. anisopliae* DAT F-001.

Concn (spores/g)	parameter* integration constant (*a*) estimate	SE.	intrinsic rate of increase (*r*) estimate	SE.	sigt.†	LT_5 (days)	LT_{50} (days)	%DT‡
10^1	4.12	0.16	-0.0498	0.0020	a	23.6	82.7	28.5
10^2	2.57	0.19	-0.0399	0.0025	b	-	64.5	-
10^3	2.96	0.20	-0.0563	0.0028	b	-	52.6	-
10^4	4.57	0.25	-0.0992	0.0044	c	16.4	46.1	35.6
10^5	8.47	0.61	-0.2838	0.0201	d	19.5	29.8	65.4
10^6	10.76	1.17	-0.4364	0.0423	e	17.9	24.7	72.5
10^7	11.89	1.11	-0.6279	0.0705	f	14.2	18.9	75.1

* Where the proportion surviving (y) = $e^x/(1 + e^x)$

where x = $a + r$time(days)

† Equations followed by the same letter do not differ significantly (P>0.05)
‡ %Delay Time (%DT = LT_5/LT_{50} x 100)

had been further increased (Fig. 5.1).

These results are comparable with two previous pilot experiments which used DAT F-001 (Chapter 4). The first of these experiments indicated that after 51 days at 20°C the LC_{50} was around 10^3 spores/g sand-peat (Chapter 4, Fig. 4.1), while the results presented here show that the 10^3 concentration has an LT_{50} of 52.6 days. Rath (1989) (Appendix 1) has shown that at 28 days the LC_{50} in the first pilot experiment (Chapter 4) was approximately 9×10^4 spores/g sand-peat, and this equates with the LT_{50} of 29.8 days of the 10^5 concentration tested here. The second pilot experiment which used a concentration of 2×10^6 spores/g sand-peat had an LT_5 of 17.4 days and an LT_{50} of 24.4 days (Chapter 4, Table 4.3) which is almost identical to the 1×10^6 concentration used here (Table 5.1). The first pilot experiment was established in September 1987, while the second pilot experiment and this current experiment were set up in September 1989. These data highlight the consistent efficacy of DAT F-001.

Concentrations of between 10^5 and 10^8 spores/g soil are commonly used in pathology studies of soil insects (Muller-Kogler and Stein, 1970, 1976; Ferron, 1978, 1981), though Ferron (1981) states that low, sublethal, concentrations have been insufficiently studied. This study with L3 larvae of *A. couloni* has shown that there was no concentration tested that did not cause significant mortality provided the observation time was of sufficient duration. The lowest concentrations tested, 10^1 and 10^2 spores/g sand-peat, are not quantifiable by standard plate-count techniques (Milner and Lutton, 1976) or immuno-assay techniques (Guy and Rath, 1990). While the effectiveness of these concentrations in the experiment may have been enhanced by the high level of organic matter in the sand-peat mixes (Milner, 1989), the effectiveness of low doses is supported by Doberski (1981b) who has shown that cuticle contact of as few as 11 *M. anisopliae* spores/larva can cause up to 90% mortality of *Scolytus scolytus* in 30 days.

The LT_5 was used to measure the time required before the onset of fungal-induced larval mortality. This 'lag-phase' is a combination of all the factors required to kill the larvae. This includes larval-spore encounter, germination, penetration, toxin production, etc., and should not be confused with an 'incubation period'. While there is somewhat of a trend to increase the lag-phase duration as concentrations are lowered (Table 5.1), the narrow range of

lag-phase durations (14.2 - 23.6 days) contrasts with the broad range of LT_{50}'s (18.9 - 82.7 days; Table 5.1). This suggests that larvae at all concentrations can encounter and be killed by the fungus in the same time period, but that as the concentrations are lowered the spatial distribution of the fungus means that on average a greater period of time is required for larvae to encounter a lethal dose.

5.3 PRELIMINARY STUDIES OF THE VIRULENCE OF DAT F-001 FOR EGGS, L1 AND ADULT *A. COULONI*.

5.3.1 Introduction

While L3 larvae are the damaging stage of *A. couloni*, effective biocontrol is likely to be dependent on reduction of the population before this stage. *M. anisopliae* DAT F-001 has been shown to reduce egg production and to be pathogenic to both male and female adult *A. couloni* at concentrations of 10^5 and 10^6 spores/g sand-peat (Chapter 4; Table 4.4). This experiment further investigates the effect of DAT F-001 concentrations on adults and the subsequent effect on eggs and L1 larvae.

5.3.2 Materials and Methods

Experiment 1a Effect of concentration on adult survival.

The experimental procedure was similar to that used for L3 larvae (section 5.2.2) with the exception that two replicate trays of 50 adults were used for each of the four concentrations and one non-dosed control (500 beetles). The results were recorded after 21 days incubation at 20° ± 2°C. Adults were not sexed in this experiment. The data were examined using linear regression analysis after being corrected (Abbott's formula) for control mortality. Dose data was log transformed.

Experiment 1b Effect of concentration on egg and L1 survival.

Eggs, and the dosed sand-peat in which they were laid, were removed from the adult experiment after 21 days and placed in petri-dishes (25 eggs/dish; 4 dishes/concentration and non-dosed control - 500 eggs). When there was insufficient eggs produced at a particular dose, eggs from the nearest lower dose were used to supplement the experiment. The survival and mummification for both eggs and L1 larvae were recorded after a further 28 days incubation at 20° ± 2°C. The data were examined using linear regression analysis after being corrected (Abbott's formula) for control mortality. Dose data was log transformed.

5.2.3 Results

(a) Effect of concentration of DAT F-001 on adult survival.

Adult survival declined (P=0.0009) with increasing concentration (Fig. 5.2) and the LC_{50} was 4.03×10^5 spores/g sand-peat (Table 5.3). Only 16.2 ± 2.6% of untreated adults died. Mummification increased with concentration, reaching

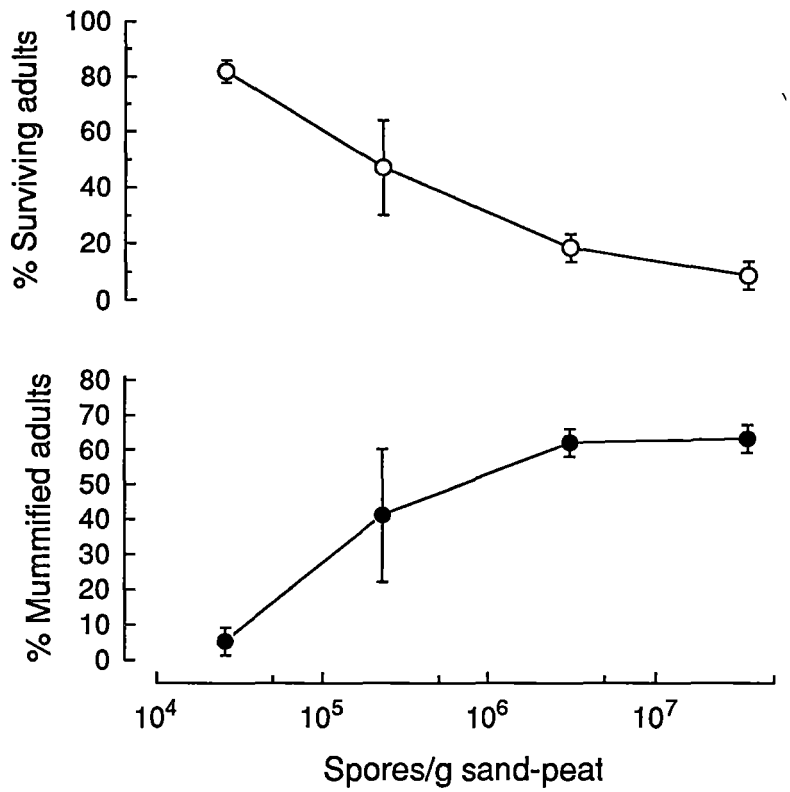

Figure 5.2 **The effect of concentration of *M. anisopliae* DAT F-001 on (○) survival (corrected data) and (●) mummification of adult *A. couloni* after 21 days at 20° ± 2°C.**

60.5% at the highest concentration of 3.5 x 10^7 spores/g sand-peat after 21 days incubation (Fig. 5.2). Total egg production was reduced (P=0.001, r^2=75.3%) as DAT F-001 concentration increased (Table 5.2), though production at the 10^4 concentration was not different from the control.

(b) Effect of concentration of DAT F-001 on egg and L1 survival.

Increasing the DAT F-001 concentration (in sand-peat) to which *A. couloni* eggs were exposed, increased the mummification of eggs and reduced (P=0.0003) the survival of L1 larvae (Fig. 5.3). After 28 days incubation a combined total of 45% of eggs and larvae were mummified by the two highest concentrations. The LC_{50} for L1 larvae treated in this manner was 1.05 x 10^6 spores/g sand-peat.

(c) Comparison of survival of treated L1, L3 and adult *A. couloni.*

Analysis of the above experiments combined with the 28 day data from a previous experiment (section 5.2.3; Fig. 5.1), showed that L1 and adult regression lines (Fig. 5.4) did not differ (P=0.27). However, they are both different (P<0.022) from L3 larvae (Table 5.3).

Table 5.2 **Egg production by *A. couloni* in response to increasing concentrations of *M. anisopliae* DAT F-001 (50 adults/tray).**

M. anisopliae DAT F-001 concn (Spores/g sand-peat)	Egg production/replicate tray (mean ± SE., n=2)
2.6×10^4	142.5 ± 22.5
2.3×10^5	58.5 ± 6.5
3.1×10^6	42.5 ± 2.5
3.5×10^7	25.5 ± 0.5

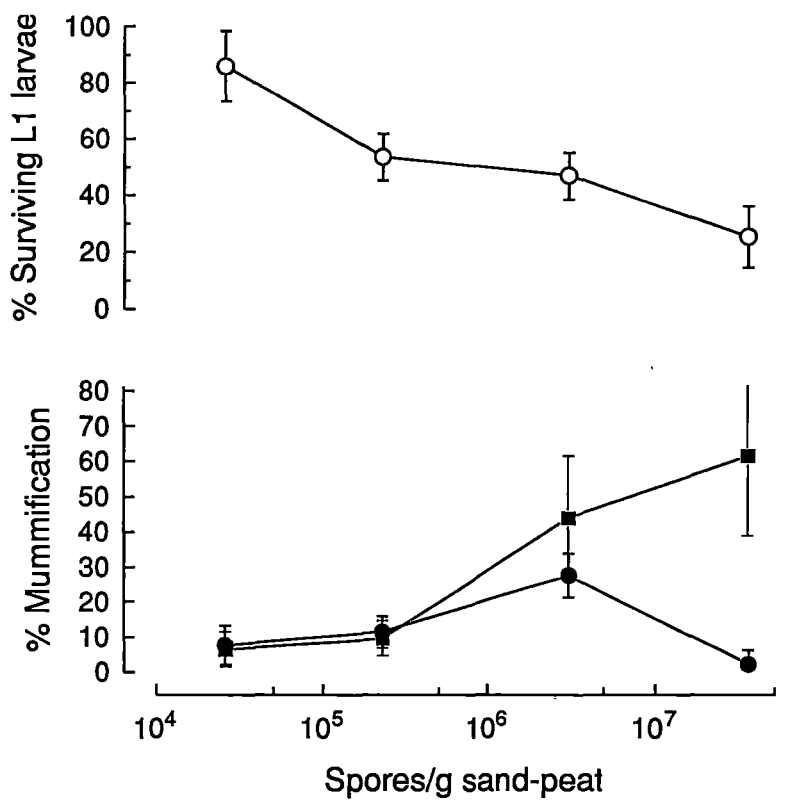

Figure 5.3: **The effect of concentration on (○) L1 larval survival (corrected data) and mummification of (■) eggs and (●) L1 larvae after exposure of eggs and hatching larvae to *M. anisopliae* DAT F-001 for 28 days at 20° ± 2°C.**

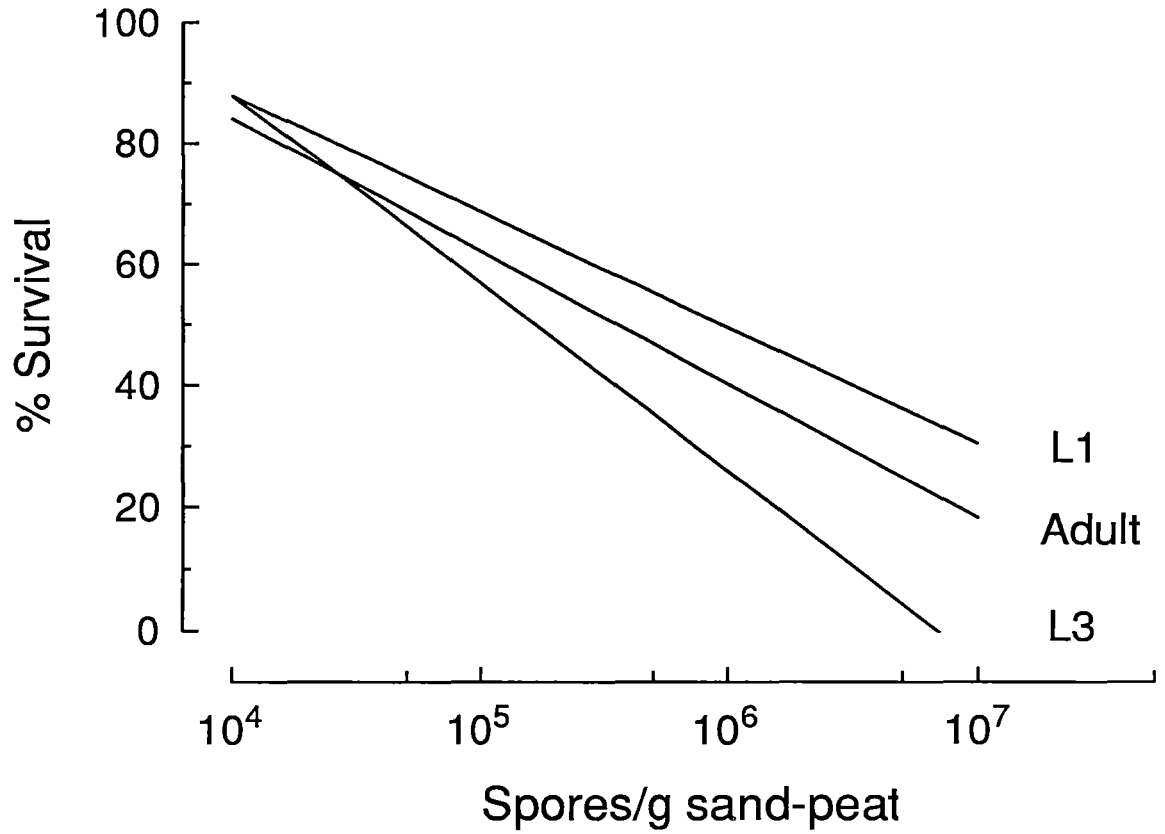

Figure 5.4 **Regression lines of survival versus *M. anisopliae* DAT F-001 concentration for L1 larvae and adults after 21 days and L3 larvae after 28 days at 20°C.**

Table 5.3 **Regression equations of survival of L1 larvae and adults after 21 days, and L3 larvae after 28 days at 20°C.**

A. couloni life stage	Regression	r^2	LC_{50} (spores/g sand-peat)
L1	y = 165.6 - 19.2 (log concn) [a*]	57.0	1.05×10^6
L3	y = 213.1 - 31.0 (log concn) [b]	97.0	1.83×10^5
adults	y = 172.1 - 21.9 (log concn) [a]	86.0	4.03×10^5

* Equations followed by the same letter do not differ significantly (P>0.05).

5.3.4 Discussion

These experiments have shown that *M. anisopliae* DAT F-001 was pathogenic for eggs, L1 and adult *A. couloni*. This study confirms the earlier study (Chapter 4) which showed that concentrations around 10^4 spores/g sand-peat have little effect on adult survival over 28 days. Concentrations of 10^5 spores/g sand-peat or higher are required to significantly reduce adult survival and reduce total egg production.

L1 larvae and adults were equally susceptible to DAT F-001, while L3 larvae were more susceptible (Fig. 5.4), though it is likely that if the L3 larval regression could have been calculated at 21 days rather than at 28 days, this difference may have disappeared. However, in either case, this contradicts the hypothesis of an age maturation immune response of insects to pathogens (McCoy *et al.*, 1988). An explanation might be that L1 larvae encounter fewer fungal spores in the soil than L3 larvae, since L1 larvae move much less than L3 larvae. Boucias and Pendland (1984) concluded that the 'age maturation response' in some cases was no more than the specificity of a pathogen for a particular life stage. Like the study reported here, Doberski (1981a, 1981b) found that *M. anisopliae* could infect both larvae and adults of *Scolytus scolytus*.

5.4 EFFECT OF DAT F-001 ON FEEDING BY L3 LARVAE.

5.4.1 Introduction

A crucial component of these pathogenicity studies is the examination of the effect of a *M. anisopliae* infection on feeding of larvae of *A. couloni*. The LT_{50} of L3 larvae exposed to varying concentrations of DAT F-001 (10^1 to 10^7 spores/g) range from 83 to 19 days (respectively) at 20°C (Table 5.1). If the larvae are actively feeding for much of this time, considerable pasture damage will continue to occur after application of the fungus. However, if feeding activity declines after infection then it may be possible to use low field application rates. This experiment examines the frass production of L3 larvae following contamination with *M. anisopliae* DAT F-001.

5.4.2 Materials and Methods

L3 larvae (45) were placed in individual petri dishes of sand-peat at 20°C for 10 days prior to experimentation. After this time larvae were rolled in spores of DAT F-001 (15 larvae) or DAT F-054 (*M. anisopliae* weakly pathogenic to L3 *A. couloni*; Chapter 4, Tables 4.3, 4.4) (15 larvae). The remaining 15 larvae were used as an untreated control. All larvae were then placed singly into 90 mm petri dishes filled with sand-peat and incubated at 20°C. The larvae were removed from their petri dishes every three days and placed into fresh dishes of sand-peat mix. All frass pellets were removed individually with tweezers from the old dishes. The effect of DAT F-001 and DAT F-054 on L3 larval feeding was determined by recording the number and weight of frass pellets produced by individual insects over each three day period for up to 30 days. GLIM procedures (Chapter 3) were used to analyze the data.

5.4.3 Results

There was no linear correlation ($r^2<0.085$) between mean weight of frass pellets/day and the number of pellets produced/day. Additionally, the number of pellets produced/day was not related ($P=0.19$) to the weight of the larvae.

Control feeding: There was no difference in feeding between the untreated control larvae and larvae treated with *M. anisopliae* DAT F-054 over the 30 day experimental period (Table 5.4, Fig. 5.5). For both these treatments frass

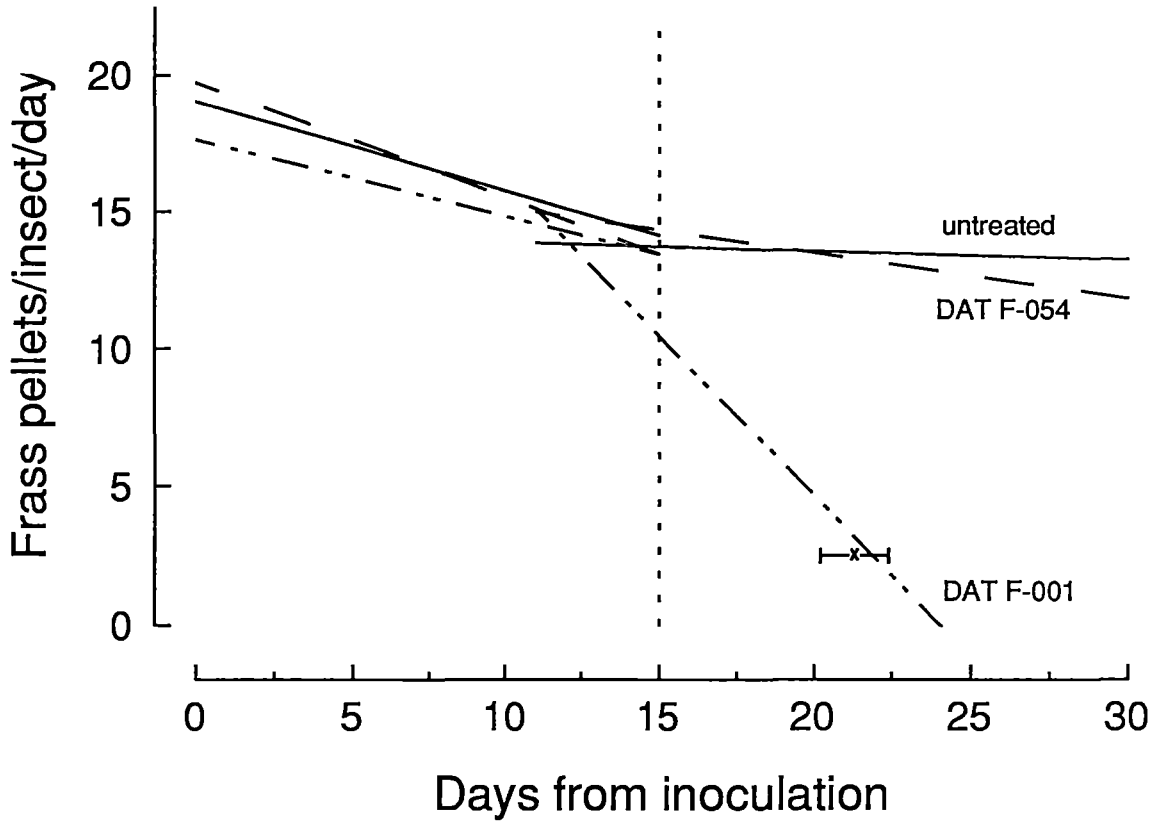

Figure 5.5 **Regression lines showing the effect of (— - - —) *M. anisopliae* DAT F-001 and (— —) DAT F-054 and a (——) non-dosed treatment on average frass production/larvae/day. Average time to death of DAT F-001 treated larvae is indicated (mean ± SE., n = 15)**

production declined (P<0.0001) about 20% initially, but from 11 to 30 days frass production remained constant (P=0.44). All DAT F-054 and untreated control larvae were still alive after 39 days when the experiment was terminated.

DAT F-001 feeding: There was no difference (P=0.39) in the frass production of larvae in the DAT F-001, DAT F-054 treatments or the untreated control from zero to 15 days (Fig. 5.5, Table 5.4). After 15 days DAT F-001 treated larvae produced less pellets (P<0.0001) than the two control groups. Data from this later period were analyzed from 11 days to enable the regression analysis of DAT F-001 to include healthy as well as diseased and dead larvae (Fig. 5.5, Table 5.4). The mean death time for DAT F-001 treated larvae was 21.3 ± 0.87 days (mean ± SE.).

Table 5.4 **Regression equations for the frass production/day by L3 larvae of *A. couloni* treated with *M. anisopliae* DAT F-001, DAT F-054 and an untreated control.**

Insect Treatment	Regression	
	0-15 days	11-30 days
untreated control	$y = 19.01 - 0.327x^{a*}$	$y = 13.56 - 0.022x^{a}$
DAT F-054	$y = 19.71 - 0.421x^{a}$	$y = 16.83 - 0.167x^{a}$
DAT F-001	$y = 17.60 - 0.177x^{a}$	$y = 27.91 - 1.161x^{b}$

* Within a period, equations followed by the same letter do not differ significantly (P>0.05).

5.4.4 Discussion

The study of the effect of DAT F-001 on L3 larval feeding has shown that the larvae continued to feed at normal rates for 70% of the time from application to death. This result was similar to that obtained by Tyrrell (1990), however, he injected protoplasts of the fungus *Entomophaga aulicae* into the spruce budworm, *Choristoneura fumiferana*, and as a consequence his results are one step further removed from a field situation. Also McDowell *et al.* (1990) found that pyralid larvae treated with *B. bassiana* continued to develop and consume food at normal rates until they died or pupated. From the present results, it is clear that in the field, damage caused by *A. couloni* would not be lessened until the larval population was significantly reduced. This finding throws doubt on the field efficacy data of Coles and Pinnock (1982, 1984), who recorded *Aphodius tasmaniae* in any stage of *M. anisopliae* infection, as dead, because while the larvae will succumb to the disease they are likely to still be actively eating. Consequently, live infected larvae would still be damaging pasture and the infection may not kill them until considerable pasture damage has occurred.

5.5 GENERAL DISCUSSION

This study has shown that *M. anisopliae* DAT F-001 is pathogenic for eggs, L1, L3 and adult *A. couloni* over a wide range of concentrations. Concentrations tested against L3 larvae reduced survival to zero or near zero and these concentrations ranged from 10^1 and 10^2 spore/g sand-peat (which are impossible to accurately detect using standard plate count methods) to very high concentrations of 10^6 and 10^7 spores/g sand-peat. While all concentrations used were effective, the time taken to kill the larvae increased as the concentration was reduced. The increase in LT_{50} as *M. anisopliae* concentrations were reduced has been shown for the elm bark beetle (*S. scolytus*; Doberski, 1981a) and the black vine weevil (*O. sulcatus*; Soares *et al.*, 1983). Quintella *et al.* (1990) have shown that the same response is present in the weevil, *Chalocodermus bimaculatus*, infected with *B. bassiana.*

The study of the effect of DAT F-001 on L3 larval feeding showed that exposed/infected larvae did not have reduced activities until late in the infection process and consequently there was little prospect of reduced pasture damage until the larvae were killed by the fungus.

The agricultural implication of this study is that with a long-lived insect such as *A. couloni* there may be the opportunity to use one of a range of concentrations. The increase in LT_{50} that occurred as the concentration was reduced may not effect the overall rate of field control provided that mortality timing and application are not critical. This is critical in commercial development of *M. anisopliae* DAT F-001 as it will allow a manufacturer to more successfully balance field efficacy against product cost. As an example, Rath *et al.* (1990) and Moscardi (1988) have described large-scale production systems for *M. anisopliae based* on cereal grains. Using their systems, a soil concentration of 10^6 spores/g soil would equate to 1,000 kg of product/ha. This amount could not be applied using currently available machinery, and, at a cost of approximately US$1.5/kg (Rath *et al.*, 1990), would be too expensive in a low value, broad acre crop such as Australian sheep pastures.

The data in this chapter showed that concentrations of 10^4 - 10^5 spores/g soil (or lower) control *A. couloni* in the laboratory tests. The field efficacy of these concentrations is studied in Chapter 8.

Chapter 6

6.0 EFFECT OF TEMPERATURE ON FUNGAL VIRULENCE

6.1 General Introduction

Most strains of *M. anisopliae* are mesophiles which grow well over a broad temperature optimum from 20° to 30°C but grow little or not at all outside the 15° to 35°C range (Milner, 1989). While no soil temperatures are recorded for the Midlands sheep grazing areas of Tasmania (145°30'E, 42°S) the mean air temperatures are much lower than this, ranging from 15.1°C in summer to 5.3°C in winter (Fig. 6.1). *A. couloni* are active in pastures throughout the year but may enter a facultative diapause when temperatures fall below 8°C (McQuillan, 1990). The rate of germination of *M. anisopliae* has been found to correlate with virulence (Milner *et al.*, 1991), and, if *M. anisopliae* is to effectively control *A. couloni* in south-eastern Australia, growth, infectivity and insect death must occur at these low temperatures. These experiments investigate the effect of temperature on the virulence of *M. anisopliae* DAT F-001 for L3 larvae of *A. couloni*.

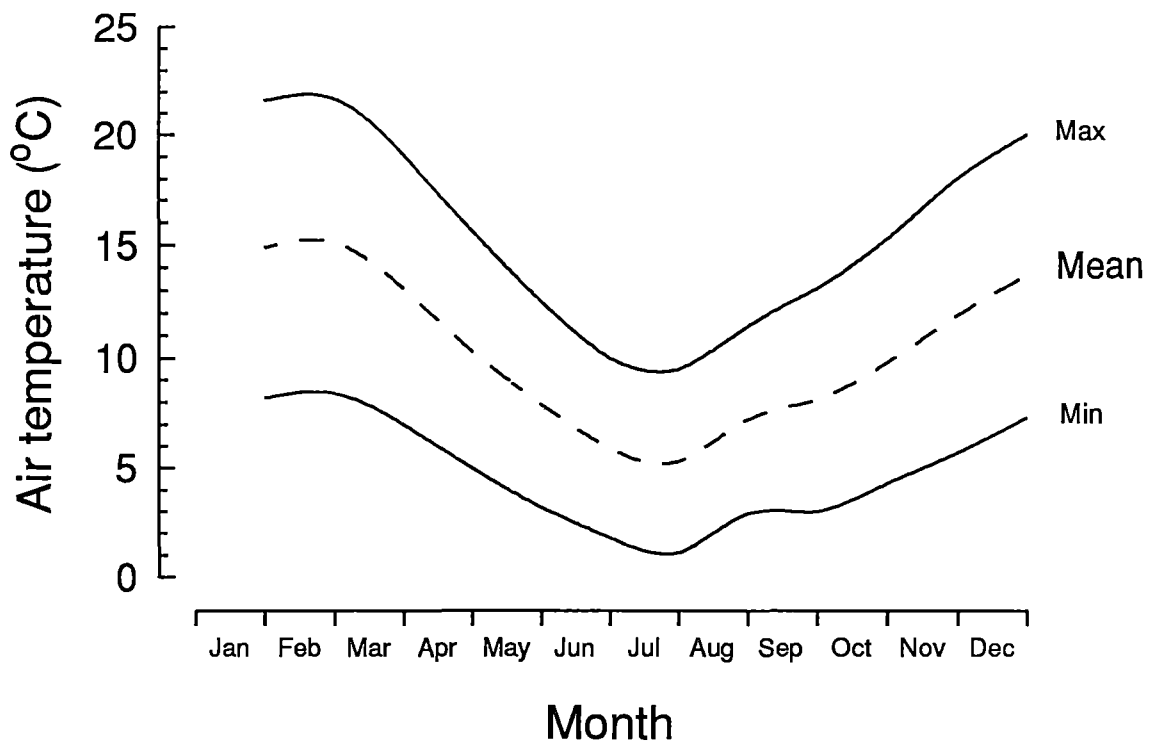

Figure 6.1 **Maximum, minimum, and mean air temperatures in the central sheep grazing area (Oatlands) of Tasmania (100 year average data from Davies, 1988).**

6.2 FUNGAL GERMINATION

6.2.1 Introduction

The experiments conducted to date (Chapters 4 and 5) have been aimed at assessing either the virulence of a range of fungal isolates or the dose related virulence of *M. anisopliae* DAT F-001. These experiments have shown that virulence is both fungal isolate and concentration specific, but these experiments were conducted at either 20° or 25°C.

If entomogenous fungi are going to be of use as biological control agents in Tasmanian pastures, the virulence of the fungi at ambient temperatures (5° - 15°C) must be examined. The first step in this process is to determine the temperature range over which the fungi can grow.

These experiments examine the effects of temperature on the germination of spores of *M. anisopliae* DAT F-001, and as a comparison, the germination rates of selected isolates of *M. anisopliae*, *B. bassiana* and *M. flavoviride* studied in Chapter 4.

6.2.2 Materials and Methods

Experiment 1 Comparison of the germination of five fungal isolates.

For each isolate (Table 6.1), spores were harvested from SDA + YE plates which had been point inoculated and allowed to grow for 14 days at 25° ± 1°C. Harvested spores were suspended in 0.01% Triton X-100 solution and serially diluted until a suspension containing about 1 x 10^7 spores/ml was obtained. Five petri dishes (90 mm dia.) containing 15 ml of Czapek-dox agar (Oxoid CM97) were inoculated with 0.1 ml of the fungal spore suspension (spread-plate technique), giving five replicate plates to be incubated at 5°, 10°, and 15°C (± 1°C). Czapek-dox agar was used as it was a commonly used mycological agar and it had the desirable attribute of being highly translucent, which allowed the spores to be visible on the plate under the light microscope. No attempt was made to accelerate or synchronize the germination of the spores (Dillon and Charnley, 1985). The proportion of germinated spores (when the germ tube was half the length of the conidia) was counted for one field of view on each plate under the microscope (400x) after 7 days incubation.

Experiment 2 ***M. anisopliae*** **DAT F-001 germination rates.**

M. anisopliae DAT F-001 germination rates were determined using a similar method to Experiment 1, except that 5 replicate plates were incubated at 2°, 5°, 8°, 10°, 16°, 20° and 25°C. The proportion of germinated spores was counted as described above at intervals between 18 hours and 60 days. Statistical analysis utilized the GLIM procedure outlined in Chapter 3.

6.2.3 Results

Experiment 1 **Comparison of the germination of five fungal isolates.**

Isolates of DAT F-001, F-141 and F-140 germinated at 5°, 10° and 15°C and were near to or over 95% germination by the time of recording (7 days). *M. flavoviride* (DAT F-133) was also able to germinate at these temperatures but was slower to do so (Table 6.1). *M. anisopliae* DAT F-054 was unable to

Table 6.1 **% Germination of fungi on Czapek-dox agar after 7 days incubation at 5°, 10° and 15°C (mean ± SE., n=5).**

	Temperature (°C)		
Fungal isolate	5	10	15
M. anisopliae			
F-001 (strain 3)*	93.6 ± 2.7	>95	>95
F-054 (strain 16)*	0.0	0.0	94.9 ± 3.7
F-141 (strain 3)*	89.6 ± 2.4	>95	>95
M. flavoviride			
F-133	24.7 ± 6.1	79.3 ± 3.9	88.4 ± 6.0
B. bassiana			
F-140	93.4 ± 2.0	>95	>95

* using the key of Yip, Rath and Koen (1992)

germinate at 5° or 10°C.

Experiment 2 ***M. anisopliae*** **DAT F-001 germination rates.**

The intrinsic rate of spore germination (*r*) increased as temperatures were increased from 2° to 10°C whereas at 16°C, *r* was similar to that at 10°C. However, all curves were significantly different (P<0.0001) from one another (Table 6.2). This is shown graphically for temperatures of 16°C and below (Fig 6.2). Germination was too fast at temperatures above 16°C to allow the same analyses, however, there was 92% germination at 25°C and 78% germination at 20°C after 21 hours incubation.

Germination was greatly retarded at 2°C, taking six times as long as 5°C to reach 50% germination (Table 6.2). Plates held at 5°C were sporulating profusely by the time 80% germination had occurred at 2°C.

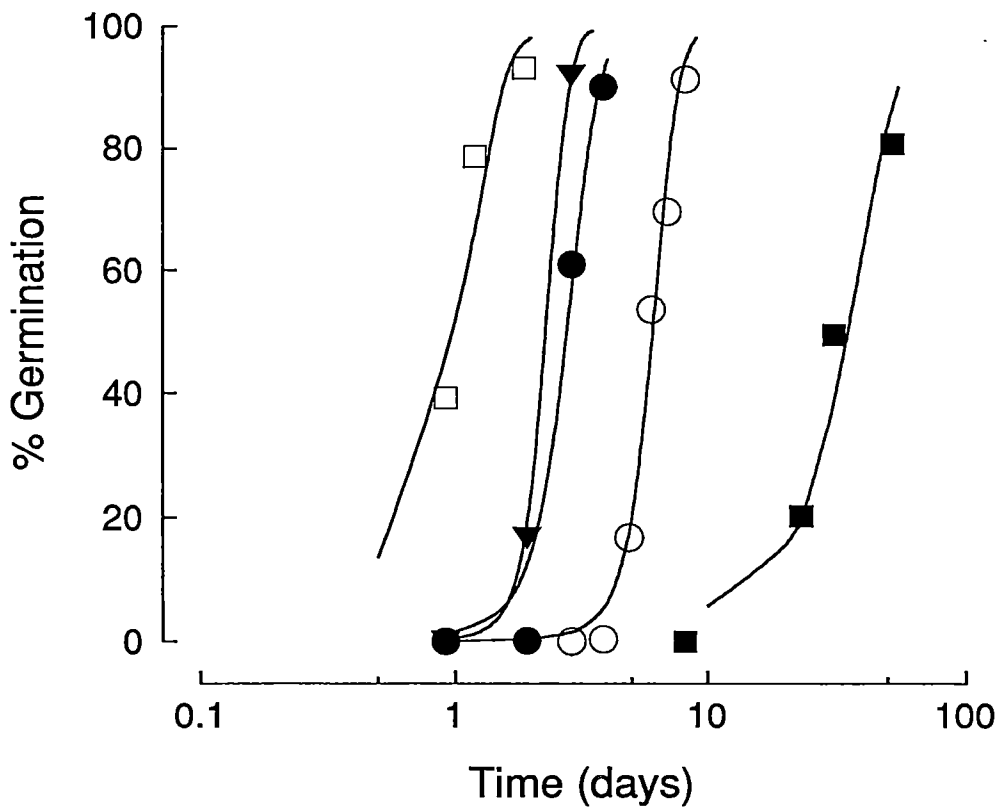

Figure 6.2 **Germination vs time regression curves and mean values for *A. couloni* DAT F-001 incubated on Czapek-dox agar at (❑) 16°, (▼) 10°, (●) 8°, (○) 5° and (■) 2°C.**

Table 6.2 **Regression equations, standard errors and 50% germination of *M. anisopliae* DAT F-001 on Czapek-dox agar at different temperatures.**

Temperature (°C)	parameter* integration constant (*a*) estimate	SE.	intrinsic rate of increase (*r*) estimate	SE.	50% Germination time (days)
16	-3.790	0.227	3.872	0.173	1.0
10	-9.132	0.456	3.956	0.186	2.3
8	-6.261	0.413	2.289	0.128	2.7
5	-7.992	0.288	1.325	0.043	6.0
2	-3.740	0.127	0.106	0.004	35.3

* Proportion germinated $= (e^X)/(1+e^X)$

where x $= a + r$ time (days)

6.2.4 Discussion

Amongst the isolates tested here there was considerable variation in the temperatures required for germination, with some isolates being able to germinate at temperatures as low as 5°C, while *M. anisopliae* DAT F-054 was not able to germinate below 15°C. Comparison of the temperature growth ranges of the fungi and their virulence at 20°C (Chapter 4) aids in the selection of suitable isolates for *A. couloni* control. These temperature studies confirm the appropriateness of selection of DAT F-001 for further biocontrol studies. The variation in germination response to temperatures of different isolates of *M. anisopliae* has been shown by Latch (1965) and Hywel-Jones and Gillespie (1990), and is a common response by fungi (Gottlieb, 1978). Gottlieb (1978) suggests that these variations are due to differences in specific biochemistry. Temperature affects biochemical reactions by hastening them at high temperatures and slowing them at low temperatures. Yip *et al.* (1992) used growth temperature as a major separating trait of *M. anisopliae* strains.

M. anisopliae DAT F-001 was pathogenic to eggs, L1, L3 and adult *A. couloni* over a range of concentrations (Chapter 5) and germinated at temperatures

from the minimum tested (2°C) to the maximum tested (25°C) though the rate of germination varied considerably (Table 6.2). This was also shown for this isolate by Rath (1989, Appendix 1).

The germination rate can be seen as a measure of the temperature related growth of DAT F-001 as the germination rate compares favourably with both the vegetative growth and spore production data of DAT F-001 recorded by O. Goebel and G. Riba (unpublished data). Germination, growth and spore production are a part of the overall disease process. Variation in the rates of these factors will vary the virulence (LT_{50}) of the fungus. The data presented here suggests that the virulence of DAT F-001 for *A. couloni* will be markedly affected by temperature. Further, it is likely that the biologically 'useful' minimum temperature is 5°C, as the 6-fold increase in 50% germination time as the temperature is reduced from 5° to 2°C (Table 6.2) tends to preclude any agricultural relevance at 2°C.

6.3 L3 LARVAL SURVIVAL

6.3.1 Introduction

Investigations on the pathogenicity of *M. anisopliae* DAT F-001 have all been conducted at 20°C (Chapter 3 and 4), yet, the L3 larvae, the major damaging stage of *A. couloni*, are present from mid autumn to early summer when the mean air temperatures range from 5° to 15°C (Fig. 6.1) (these temperatures are closely related to soil temperatures - Chapter 8). The germination and growth of DAT F-001 is reduced as temperatures are lowered and it could be expected that the virulence would be reduced correspondingly.

This experiment examines the effect of DAT F-001 incubation temperature and exposure time on survival and mummification of L3 larvae of *A. couloni.*

6.3.2 Materials and Methods

The concentration of 4.1 ± 0.45 x 10^6 (mean ± SD.) spores/g sand-peat mix was obtained by combining 200 g of the *M. anisopliae* DAT F-001 inoculant with batches of 22.5 kg of sand-peat (1:1) and sufficient tap water to give a moisture content of about 20% (w/w) followed by mixing in a cement mixer for 10 minutes. Each batch was divided between three trays so that each tray contained 7.5 kg of dosed (16 trays in total) or untreated (non-dosed) sand-peat (16 trays). Seventy five L3 larvae were randomly selected and allocated to each tray. Larvae were placed on the surface and allowed to burrow into the sand-peat mix. Treated and untreated trays were randomly allocated to each of the four temperature regimes studied (constant 5°, 10° and 15°C and a fluctuating 15°/5°C temperature on a 12:12 hour cycle). There were four replicate treated and untreated trays at each temperature. The temperature of the sand-peat in the 15°/5°C cabinet was recorded at 5 minute intervals using an Environdata® computerized datalogger (Fig. 6.3). The constant temperature incubators were monitored using calibrated digital thermometers and chart-recorders. The temperatures in these incubators fluctuated less than ±1°C.

Every 14 days the number of surviving and mummified larvae as well as the total weight of larvae in the untreated trays were recorded. Mummified larvae were removed to reduce the possibility of increasing the *M. anisopliae* concentration in the sand-peat. Recording of larval survival in the untreated

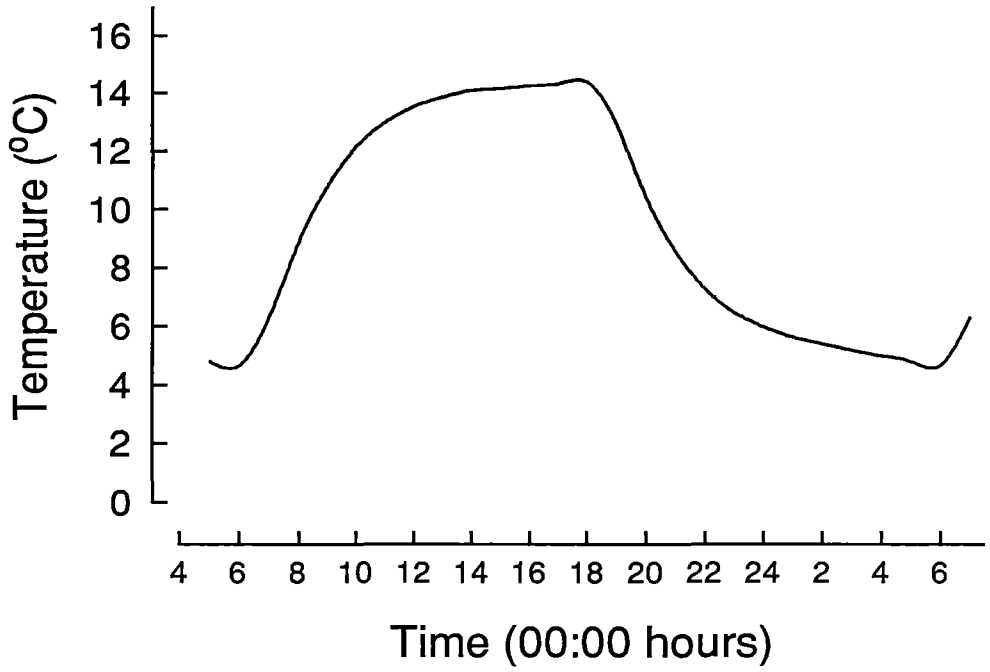

Figure 6.3 **Temperature Profile in trays of sand-peat incubated in a controlled environment cabinet set at 15°/5°C for a 12:12 hour daily cycle (av. SE. = 0.17°C).**

trays at each temperature ceased 14 days after all the larvae in the *M. anisopliae* treated trays had died. 'Abbott's formula' (Finney, 1971) was used to correct treatment mortality for the effect of 'natural' mortality. GLIM analysis and *t*-tests (Chapter 3) were used to discern differences between the treatments.

6.3.3 Results

Survival of Untreated Larvae

Survival of untreated larvae declined ($P<0.0001$) with time (Fig. 6.4A). Over the course of the experiment there was no difference ($P=0.11$) in survival between larvae incubated at 10°C constant or incubated at the fluctuating 15°/5°C temperature regime. The survival of larvae at 5°C was less ($P<0.0001$) than at 10°C.

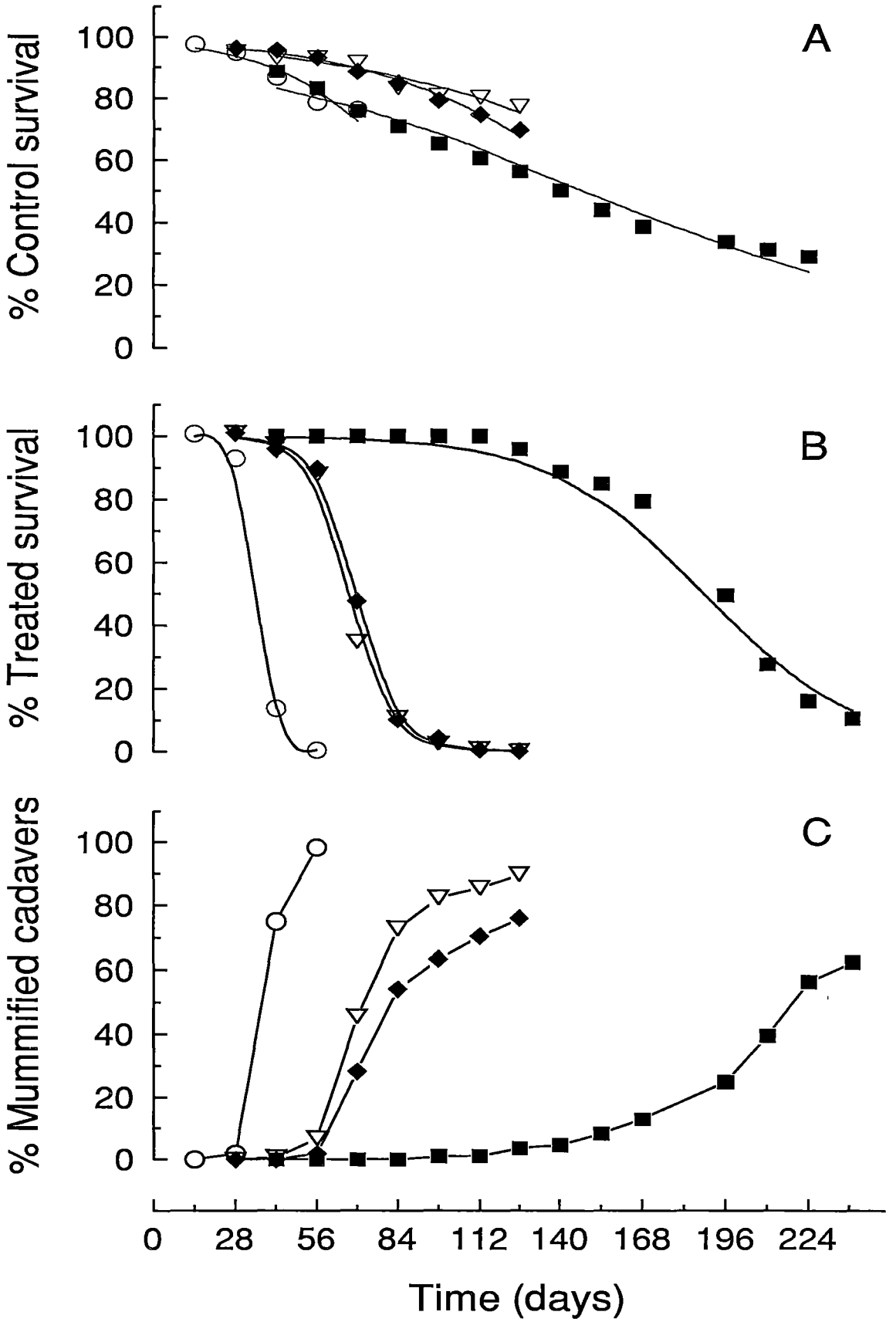

Figure 6.4 The effect of temperature on (A) survival of control L3 larvae, and (B) the survival (corrected data) of treated L3 larvae, and (C) the percentage mummified cadavers of treated L3 larvae (○ 15°; ▽ 10°; ◆ 15/5°; ■ 5°C)

Table 6.3 **Temperature-time regression equation, standard errors and LT_{50}'s for the survival of L3 larvae of *A. couloni* exposed to 4.1 x 10^6 spores DAT F-001/g sand-peat mix.**

	parameter*							
Temp	integration constant (a)		intrinsic rate of increase (r)			LT_5	LT_{50}	
(°C)	estimate	SE.	estimate	SE.	Sigt†	(days)	(days)	%DT‡
15	10.83	0.57	-0.2995	0.0152	a	26.3	36.1	72.9
10	8.81	0.34	-0.1309	0.0050	b	44.8	67.3	66.6
15/5	9.15	0.35	-0.1313	0.0050	b	47.3	69.7	67.9
5	7.18	0.19	-0.0388	0.0010	c	111.5	188.9	59.0

* Proportion surviving = $(e^X)/(1+e^X)$

where $x = a + r$ time (days)

† Significance: equations followed by the same letter do not differ significantly ($P<0.05$)

‡ % Delay Time (%DT = LT_5 / LT_{50} x 100)

Larval weight

At 15°C untreated larvae gained weight ($P=0.005$) during the experiment. At all other temperatures larvae only maintained their initial body weight ($P>0.27$).

Survival of DAT F-001 Treated Larvae

Survival of treated larvae was significantly different ($P<0.05$) from untreated larval survival, at all temperatures except 5°C ($P=0.061$), when the corrected treated survival was equal to or less than 50% (Fig. 6.4B). At 5°C, the survival of treated larvae was different ($P<0.05$) from untreated larvae at, and after, 210 days when corrected survival was 31.3 ± 4.5% (Fig. 6.4B).

Decreasing the temperature increased the time required to kill larvae. This is shown by the magnitude and the negative value of the intrinsic rate of increase (r) as well as the LT_{50} values (Table 6.3) and the response curves (Fig. 6.4B). Larval survival at the fluctuating 15°/5°C regime (Fig. 6.3) was identical (P=0.89) to that at the constant 10°C regime (Fig. 6.4B). All other temperature-survival regressions were different (P<0.0001) from one another (Table 6.3).

As temperature was lowered there was an increase in the lag phase, ie., the time required before any fungal-induced larval mortality could occur (Fig. 6.4B). As in Chapter 5, the LT_5 is used as the determinant of the lag phase (Table 6.3). The ratio of the LT_5 to the LT_{50} was quantified as the %Delay Time (%DT) and gave a measure of the relative speed at which a population of larvae will die after disease initiation. In these experiments the %DT increased with increasing temperature, ranging from 59.0% at 5°C to 72.9% at 15°C.

DAT F-001 Mummification

The maximum mummification of L3 larvae treated with DAT F-001 decreased with decreasing temperature (Table 6.4). Mummification was higher at 10°C than it was at the fluctuating 15°/5°C temperature regime (Fig. 6.4C, Table 6.4).

Table 6.4 Maximum mummification of L3 larvae of *A. couloni* exposed to 4.1 x 10^6 spores DAT F-001/g sand-peat at 15°, 10°, 15/5° and 5°C

Temp (°C)	Maximum mummification* expressed as	
	% of control survival	% of initial larvae/rep
15	98.3 ± 6.38	77.3 ± 5.02
10	89.7 ± 8.62	69.3 ± 6.70
15/5	76.1 ± 5.09	53.0 ± 7.10
5	66.6 ± 13.48	16.3 ± 4.80

* mean ± SE., n=4.

Expression of maximum mummification as both a percentage of control survival and as a percentage of the initial 75 larvae per replicate made no difference to the relative effect of temperature (Table 6.4). At 5°C, 67.6% of the fungus-killed larvae were mummified but the slow decline in control survival combined with the slow rate of disease development at 5°C meant that only 16.3% of the initial 75 larvae per replicate were mummified (Table 6.4).

6.3.4 Discussion

M. anisopliae DAT F-001 was pathogenic for L3 larvae of *A. couloni* at temperatures from 5° - 15°C, though the virulence, as determined by either the LT_5 or the LT_{50}, decreased with decreasing temperature (Fig. 6.4; Table 6.3). This result was similar to that shown by Rath and Yip (1989, Appendix 2), however their experiment terminated before the efficacy of DAT F-001 at 5°C could be determined. Percentage mummification of L3 larvae was also reduced as temperatures were lowered. The decline in virulence of *M. anisopliae* and other entomogenous fungi, due to declining temperatures, has been shown by other authors (including Marchal, 1976; Doberski, 1981b; Soares *et al.* 1983; Eilenberg, 1987; McCoy *et al.*, 1988; Goh *et al.*, 1991). The data presented here supports the view of Ferron (1978) that temperatures lower than optimal retard the development of mycosis without necessarily affecting the total mortality.

The significance of these findings resides in the relationship between the temperature-survival regressions and Tasmanian field temperatures. Mean annual air temperatures in the Midlands range from a minimum of 5.3°C in July to a maximum of 15.1°C in February. At the concentration used in this experiment (4.1×10^6 spores/g sand-peat) there would be sufficient time to significantly reduce populations of one generation of L3 larvae at field temperatures. As an example, L3 larvae are present in pastures for approximately 270 days, and based on an average temperature of only 5°C, the LT_{50} would be 189 days. However, the field temperature only averages as low as 5°C for about 70 days per year (Fig. 6.1) and this occurs in the middle of the L3 development stage.

6.4 GENERAL DISCUSSION

There was close correlation between the results from the germination studies which showed an increase in germination rate as temperatures were raised from 2° to 25°C and the virulence study. Specifically, there was close agreement between the increases in 50% germination times (Table 6.2) and the increases in LT_{50} at 5°, 10° and 15°C. For example as the temperature was lowered from 10°C to 5°C, 50% germination took 2.6 times as long, while the time to reach the LT_{50} took 2.8 times as long. The correlation between growth and virulence of *M. anisopliae* to a specific host, at various temperatures, has also been shown by Doberski (1981b), Latch and Kain (1983) and Samuels *et al.* (1989). Georgis and Gaugler (1991) have shown that the failure of nematodes to control the Japanese beetle (*P. japonica*) was due to using strains of nematodes whose biological activity was not matched to the temperatures of the environment.

Below 5°C, DAT F-001 may be avirulent for L3 larvae, as the time required for 50% germination at 2°C (35.2 days) was nearly six times longer than that required at 5°C (6.0 days). This suggests that the LT_{50} at 2°C would be over three years (1133 days). As *A. couloni* has only a two-year life-cycle, such an LT_{50} value would render the fungus avirulent.

Rath and Yip (1989) have shown that the virulence of DAT F-001 for L3 larvae of *A. couloni* continues to increase as temperatures are raised from 15° to 25°C. Maximal growth temperature is important as McGuire *et al.* (1987) were unable to establish the fungus *Erynia radicans* in field populations of the host when the temperatures were above the threshold determined in the laboratory. The maximal temperature for *M. anisopliae* DAT F-001 was not determined here, though O. Goebel and G. Riba (unpublished data) have shown that DAT F-001 mycelial growth ceases at 33°C. A.C. Rath (unpublished registration data) has shown that DAT F-001 does not grow at 35° - 37°C. These temperatures are very much higher than the field temperatures applicable for *A. couloni* control (Fig. 6.1) and consequently, DAT F-001 should not be limited by the temperatures experienced in Tasmania.

The increase in the LT_5 (a measure of the mortality 'lag phase') as temperature was reduced was probably the result of both the reduction of larval movement, and hence lessening larval contact with DAT F-001 spores, and the slowing

down of fungal germination, growth and disease development (McCoy *et al.*, 1988). The slowing down of the fungal germination with decreasing temperature (Fig. 6.2, Table 6.2) has already been discussed. Active larval movement must occur at 15°C as at this temperature the larvae gained in weight. At temperatures of 10°C or less there was no weight gain. McQuillan (1990) has determined that a facultative diapause occurs in L3 larvae of *A. couloni* at temperatures of 8°C or less, consequently, larval movement must have been less at 5° than at 10°C. Therefore, at each of the temperatures tested larval movement was reduced as temperatures declined.

The significance of these findings and those of Chapter 5, was that temperature was more important than fungal concentration in determining virulence. In the experiments reported here, the LT_{50} of L3 larvae exposed to 4.1×10^6 spores/g at 10°C was 67.3 days while at 20°C a concentration of only 1×10^2 spores/g was required to reach the same LT_{50} (Chapter 5). This was further illustrated by comparing the lag phases, which are the time periods required before any mortality will occur and where estimated by the LT_5. The LT_5 increased considerably as the temperatures was dropped from 15° (26.3 days) to 5°C (111.5 days), however, at 20°C the LT_5 increased relatively less as the concentration was reduced from 10^7 spores/g (14.2 days) to 10^1 spores/g (23.6 days).

If the fungus were applied to a field population of *A. couloni* larvae, the LT_5 and the %Delay Time (%DT - a measure of the speed of the population collapse following the onset of disease symptoms, Table 6.3)) would be useful determinants of the progress of the epizootic. For example, at 15°C the data suggest that with an LT_5 of 26 days, fungus-induced mortality would not occur until about four weeks after application while the %DT of 73% meant that 26 days was already 73% of the time required to kill 50% of the larvae. By comparison, at 5°C the LT_5 was estimated to be 112 days, and the %DT to be 59%. The data in Figure 6.4B would need to be modelled to include only the gross cyclic field temperatures (Fig. 6.1), as the data showed that larval mortality under a daily fluctuating temperature regime was not different from that under a constant daily temperature.

The development of a simple model on the temperature data of Rath and Yip (1989) is reported in Chapter 7 and the accuracy of model for field populations of L3 larvae of *A. couloni* is discussed in Chapter 8.

Chapter 7

7.0 PREDICTING THE TIMING OF FIELD MORTALITY USING A SIMPLE MODEL.

7.1 INTRODUCTION

One of the most important attributes of a microbial control agent is that it should provide predictable pest control (Burges and Hussey, 1971). Pinnock and Brand (1981) believe that field experiments conducted without adequate attention to dose-response are usually poorly designed and fail to yield reliable data. Laboratory experiments which mimic varying field conditions give quantitative data which can be used to help predict field results (Hall, 1982).

Mathematical modelling provides a means for organizing and synthesizing experimental data (Onstad and Carruthers, 1990). Modelling has the potential to indicate certain pathogens or strategies that will not meet the requirements of a given pest control situation (Pinnock and Brand, 1981; Anderson, 1982), and allows researchers to evaluate alternative hypotheses quickly and can more efficiently direct research towards desired goals (Onstad and Carruthers, 1990).

Anderson (1982) distinguishes between the two distinct ways in which pathogens can be used in pest control:

1. As a single introduction in which the pathogen becomes enzootic in the pest population and can control the population by the development of epizootics, or

2. as a repeated application where the pest population is exposed to an applied control agent as often as necessary to achieve reductions in the population. Here the microbial agent is used in the same manner as a chemical insecticide.

General and pest specific models of the single introduction strategy have been developed by many authors including Pinnock and Brand (1981), Anderson (1982), Fuxa (1987) and Onstad and Carruthers (1990). These models are defined as epizootiological or host-density dependent models (Pinnock and Brand, 1981) because it is most important that the pathogen persists within the host population and affects the host density. Additionally, the density of the pathogen is affected by the host density.

Host-density independent modelling is used in studies of repeated applications. In these situations the host does not influence the pathogen (Pinnock and Brand, 1981).

Control of *A. couloni* uses the host-density independent strategy even though the pathogen is only applied once. In the year in which *M. anisopliae* is applied to the soil, initial control relies on each larva encountering a lethal dose of fungal spores. Horizontal or vertical transmission of the fungus in the insect population, essential to the host-density dependent strategy, is unimportant in this situation. In subsequent years, control relies on the maintenance of the fungus in the soil, not in the host population. In this way, an immigrant host population will be controlled as effectively as the endemic population. As the level of fungus particles declines in the soil, control may rely more on horizontal transmission and consequently epizootiological control may become dominant.

This chapter reports the development of a simple host-density independent model for determining the timing of field mortality of L3 larvae of *A. couloni*.

Chapter 8

8.0 FIELD EFFICACY

8.1 GENERAL INTRODUCTION

Laboratory experiments have shown that *Metarhizium anisopliae* DAT F-001 is effective against *Adoryphorus couloni* eggs, larvae and adults over a wide range of concentrations (10^1 - 10^7 spores/g soil) and at temperatures from 5° to 25°C (Chapters 5 and 6). These data enabled the development of a simple model to predict the timing of field mortality following application of the fungus (Chapter 7). The model proved to be accurate when tested against a small preliminary field experiment conducted in 1987. In that experiment (Rath, 1989, Appendix 1), there was a 60% reduction in the survival of L3 larvae in the *M. anisopliae* treated plots when the fungus was incorporated into the soil. Rath (1989) found that surface application or deposition of the fungus in cuts in the soil at 10 cm x 10 cm intervals had little or no effect on larval survival.

Successful biocontrol of *A. couloni* is likely to result from a product which can be easily applied to pasture soils, gives acceptable control the first year but is able to control the pest population for a longer period of time (Chapter 3). This chapter reports the results of three field experiments aimed to generate data on the effects of application time and concentration of the fungus, as well as the prospects for long-term control of *A. couloni* and the subsequent saving of autumn-winter pasture productivity. The field accuracy of the mortality timing model was also examined.

8.2 GENERAL MATERIALS AND METHODS

M. anisopliae production and application

Kibbled grain of Japanese Millet *(Echinochloa utilis)* (2 kg) was mixed with tap water (600 ml) in a 20 L expoxy-lined cylindrical drum. The drum had an opening (200 mm x 150 mm) in the side to enable mixing and later harvesting. The opening was covered with autoclavable plastic and a steristopper® was placed in the neck (50 mm dia.) of the drum. Each drum was autoclaved at 121°C for a total time of 60 minutes. After cooling each drum was inoculated with 30 pieces (15 x 15 mm) of 14 day-old sporulating cultures of DAT F-001 grown on Sabouraud Dextrose Agar. The grain/fungus mixtures (inoculant) were shaken every two to three days to avoid clumping of the grain during the

three week incubation period at 20° ± 2°C. The number of spores per gram of the inoculant was determined using Kova® slides. The inoculant was then bagged into 10 kg lots and stored at 10°C for an average period of three months before use. Prior to use the spore concentration in each 10 kg lot was re-assessed using Kova® slides. All inoculant bags were approximately 10% a.i.

Description of Experimental Sites

Experiments were established in 1989 on three sheep farms ('Glenmorey', 'Warringa' and 'Inverell') located close to one another in the Tasmanian Midlands (147°30'E, 42°15'S). The 'Glenmorey' and 'Warringa' experimental sites had a previous history of *A. couloni* infestations (1986-1989) while the 'Inverell' site was infested for the first time in 1989. The 'Warringa' and 'Inverell' sites were flat but the 'Glenmorey' site was on a north-facing hill with a 15° slope.

Environdata Easidata® computerized data loggers were stationed on each experimental site and recorded the daily mean, maximum and minimum soil temperatures (50 mm and 100 mm deep) and daily rainfall over the experimental period. These data were compared with the long-term average temperature and rainfall data from the Oatlands weather recording station (Davies, 1988) except for the rainfall data from 'Glenmorey' and 'Warringa'. These two properties were closer to the Tunbridge weather station and Davies (1988) has published the average rainfall data from this station.

General Experimental Design

The design consisted of paired inoculant treated and kibbled millet (nil-dose) control plots which were randomly assigned within each of four replicate blocks. Where treatments comprised different application times of *M. anisopliae*, each treatment had its own neighbouring control plot. Each plot (Fig. 8.1) was divided into five strata (10 x 5 m) from which one randomly located soil sample (1.0 x 0.2 x 0.3 m; w x l x d, Fig. 8.2) was taken at any sampling occasion (20 soil samples/treatment/sample time). Sampling co-ordinates within each strata were randomly chosen without replacement, and were identical for that specific treatment and control stratum. The strategy of sample pairing, both between strata and between plots within blocks, was adopted to minimize the influence of natural changes in insect abundance upon the results of the experiment The rationale for this method has been described by Koen and Rath (1990, Appendix 4). Their method of analysis was modified such that the paired t-test was applied to the block means (hence reducing the degrees of freedom from 19 to 3). This approach gave a more conservative analysis than that proposed by Koen and Rath (1990). All experimental sites were unfenced allowing grazing livestock free access to the plots.

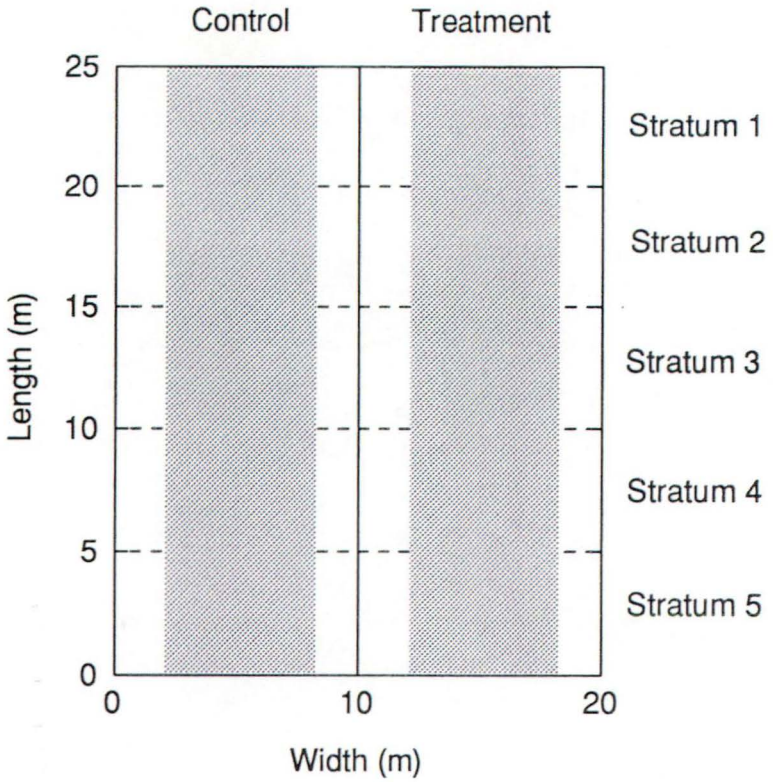

Figure 8.1 General design of each experimental block. The shaded area is the 6 m wide sampling zone.

Figure 8.2 Typical soil sample excavation using sorting boards and a galvanized iron template (1.0 x 0.2 x 0.3 m; w x l x d).

Samples were taken immediately prior to, and immediately post *M. anisopliae* application. In 1989, while L3 larvae were present in the field plots, sample periods were either at three or six weekly intervals. When the larvae developed into pupae and then adults (1990), samples were taken bimonthly. Samples were taken when appropriate during 1991.

At each sampling period the soil was sifted by hand and the numbers of alive, dead and mummified larvae, pupae and adults were recorded from each soil sample. Occasionally, counts were also made of the numbers of non-target invertebrates in the samples.

Equipment for application of *M. anisopliae*

The pasture drills were designed to apply seed and fertilizer into the soil at a depth of around 20 - 25 mm. Most drills had separate storage bins for both the seed and the fertilizer. Gear wheels at the base of the bins determined the rate of flow of material from each bin. The flow rate was modified to allow for varying application rates of different sized or weighted product. As the tractor moved along material flowed down tubing to the drill tip and into the soil. The drill tips were usually spaced at 150 - 155 mm intervals.

In these experiments two types of drill were used. The first was a cultivation drill which required the ground to be loosened by ploughing prior to running the tube-like drill across the pasture. The second type of drill was a direct-drill which was designed to cut directly into the soil/pasture without the need for ploughing. At 'Warringa' a triple disk drill was used (Fig. 8.3) while a spring tyne and coulter drill was used at 'Inverell'.

Three to five bags (10 kg) of fungus inoculant were used to dose the pastures. Bags were emptied directly into both the seed and fertilizer bins on the drills (Fig. 8.4). The drills were calibrated by collecting inoculant material running down the tubes in plastic bags during preliminary drill 'runs'. The bags were weighed and the rate/ha calculated.

Figure 8.3 Application of *M. anisopliae* DAT F-001 by undersowing the established pasture at 'Warringa' with the fungus using a Duncan Triple Disk Drill (April, 1989).

Figure 8.4 *M. anisopliae* inoculant in the seed and fertilizer bins of a pasture seed drill at 'Glenmorey' in March 1989.

Determination of *M. anisopliae* concentration in soil

After recording the numbers of insects, the soil sample unit was mixed before approximately 100 g of soil was taken for the determination of colony forming unit (CFU) concentrations. The five samples within each plot (treated and untreated by four blocks) were mixed together before 20 g of soil was diluted in 200 ml of sterile Ringer solution. After orbital shaking for 2 hours further dilutions were then made (10 ml/90 ml; followed by serial 1 ml/9 ml dilutions). At least three dilutions were plated in duplicate onto the medium of Doberski and Tribe (1980) using standard serial plating methods. After incubation for 14 days the numbers of *M. anisopliae* DAT F-001 colonies were recorded on all plates. Ecological studies undertaken by Yip and Rath (and much of which was later published, Yip, Rath and Koen, 1992) had shown that Doberski and Tribe's agar was highly selective for *M. anisopliae* strains 1, 2 and 3 (DAT F-001 is from strain 3). The results reported here truely reflect the reisolation of all of these strains, however, as none or very little *M. anisopliae* was ever recorded from the untreated plots, the recovered fungi is almost certainly DAT F-001. The dilution which gave between 10-100 colonies was used to determine the CFU's per gram soil. The figures were adjusted to give the equivalent of CFU's/g in the top 10 cm of soil. This method could only discern the presence of *M. anisopliae* once levels were above 10^2 spores/g soil. Consequently, *M. anisopliae* is recorded as not present when the soil levels are between 0 - 10^2 spores/g soil and present at any level above 10^2.

Field Mortality Model

The predictions of this model (Chapter 7) were recalculated using the actual soil temperature data recorded at each experimental site rather than the Midlands mean air temperature.

Statistical methods

At each sampling time, percentage survival data were calculated for each of the 20 strata by forming the ratio of the counts of each *A. couloni* life stage to those densities estimated from the same stratum immediately post-treatment application. A paired t-test (Snedecor and Cochran, 1980) was used to estimate the differences between the treatment effects. This pairing of plots within each stratum made possible a more accurate statistical comparison since the correlation between pairs effectively reduced the variance of the difference between treatments means. A full account of the statistics involved is given by Koen and Rath (1990). Unless otherwise stated all results are mean ± SE.

8.3 'GLENMOREY' SITE

8.3.1 Introduction

Pasture renovation is undertaken primarily in the autumn. Incorporation of *M. anisopliae* into the soil during renovation is the most economic way to introduce the fungus. These experiments examined the effect of three application times (early and late autumn and early winter) on the efficacy of *M. anisopliae*.

8.3.2 Materials and Methods

The site characteristics of 'Glenmorey' are listed in Table 8.1. The *A. couloni* infestation in 1987 had been heavy (Rath, 1989) and initial sampling in January and February of 1989, indicated that populations of L3 larvae could be over 400/m^2.

Table 8.1 General characteristics and principal profile forms of 'Glenmorey'

General characteristics*			Principal Profile Form		
land system code	mean rainfall (mm)	annual mean monthly air temperature range.	class†	description	pH
173121	375-500	5.3 - 15.1°C	GN1.81 suba	A Horizon: sandy clay loam	4.8
				B Horizon: sandy clay	6.0

* from Davies (1988)

† after Northcote (1979)

Table 8.2 **Application dates and rates of *M. anisopliae* DAT F-001 at 'Glenmorey' in 1989.**

	Application	
date	rate(kg/ha)*	soil concn (sp/g soil)†
20 March	146	$6.7 \pm 0.8 \times 10^4$
8 May	157	nr
26 June	161	$6.1 \pm 1.1 \times 10^4$

* kg/ha of grain/fungus mix

† spores/g average in the top 10 cm of the soil (mean ± SE, n=4)

nr = not recorded

Each of the four experimental blocks were placed on areas of large populations of L3 larvae with consistent densities, and compared three application times (Table 8.2). Each block consisted of three pairs of adjoining treated and untreated plots (as shown in Fig. 8.1), one pair for each application time. Paired plots for each application time were randomized within the block as was the relative position (left or fight) of the paired treatment and control plots. Because the soil was too hard for the seed drill to penetrate, the *M. anisopliae* treated and untreated plots (nil-dose) were ploughed and harrowed immediately before being drilled with the fungus. The plots were bare until natural regeneration occurred within 6 weeks.

After 1989, blocks 2, 3 and 4 were dismantled to allow the farmer to sow a crop in place of the damaged pasture. Block 1 was retained and sampled in May 1991 (mid autumn of a year in which large numbers of larvae were likely to be present). In each of the six plots (3 application times by 2 treatments) six ramdom samples (20 x 20 x 20 cm) were taken and the number of larvae counted. Within the site the three application times (March, May and June) were considered to be blocks and Analysis of Variance techniques were used to investigate the differences between treated and untreated plots.

8.3.3 Results

Survival of larvae in untreated plots

Ploughing of the existing pasture followed by drill application of the treatments resulted in a mechanically-induced larval mortality of between 53% and 63%. In the earliest application time (mid March; early autumn), this reduced the larval population from 462/m^2 to 218/m^2.

The decline in the number of larvae in the untreated plots, of the March experiment, between March and June (Fig. 8.5) was also shown by the pretreatment samples of the May (late Autumn) and June (early Winter) experiments which had declined by 35% and 70% respectively. By September, the survival in these two later experiments was less than 20 L3 larvae/m^2 and these experiments were abandoned.

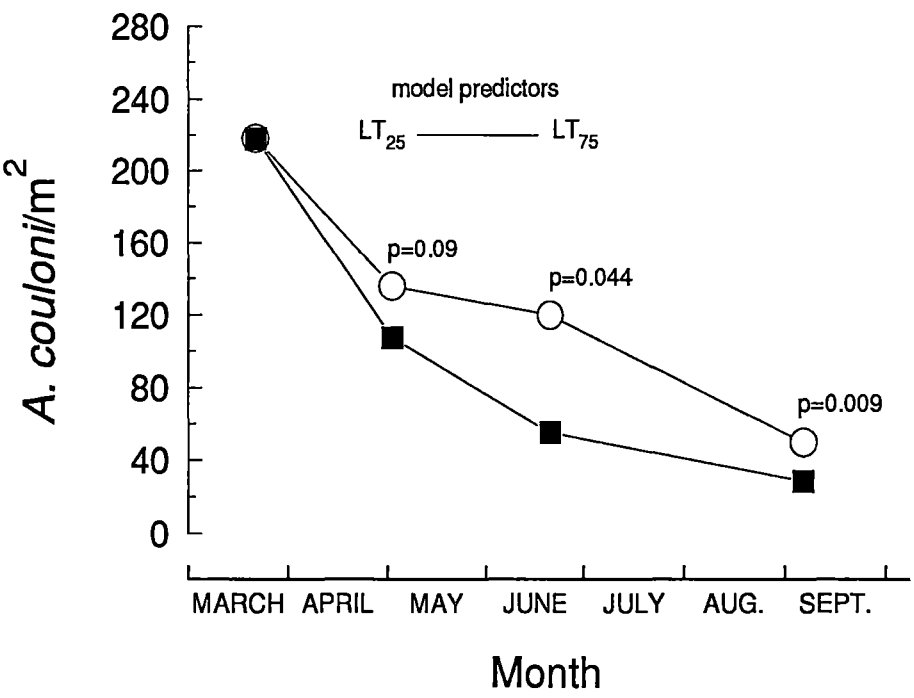

Figure 8.5 **Effect of early autumn (March) drill application of *M. anisopliae* DAT F-001 (6.7 x 10^4 sp/g soil) on the survival of (○) untreated and (■) treated L3 larvae of *A. couloni* at the 'Glenmorey' experimental site during 1989.**

Survival of larvae in *M. anisopliae* treated plots

The March experiment showed that *M. anisopliae* could enhance the speed of collapse of a *A. couloni* larval population (Fig 8.5). Six weeks after application of *M. anisopliae* there were 20% fewer (P=0.09) larvae in the treated plots and by 12 weeks this had risen (P=0.044) to 55%. At this time mummification of larvae averaged only 8/m^2.

In May 1991, there was a 47% difference (P=0.002) between the numbers of L3 larvae in treated (77 ± 15) and untreated plots (145 ± 19). There was no significant difference (P>0.05) between the application times (March, May, June 1989).

Field Mortality Model

The model predicted a 25% difference and a 75% difference between the treated and control populations, 7 and 12 weeks after application, respectively. There was a 20% difference (P=0.09) between the populations after six weeks, while the maximum difference of 55% was recorded after 12 weeks (Fig. 8.5).

Weather conditions

Mean winter soil temperatures were similar to the long-term air temperatures though summer-early autumn soil temperatures were 3°- 4°C higher than the air temperature (Fig. 8.6). For the first six weeks of this experiment the mean soil temperature was 15.2°C, while from 6 to 12 weeks the mean temperature had declined to 9.2°C. Rainfall was 35% below average over the early part (April to Sept.) of this experiment (Fig. 8.7) and continued to be in deficit while records were taken.

M. anisopliae concentration in the soil

The application of 146 kg/ha of the inoculant resulted in $6.66 \pm 0.82 \times 10^4$ CFU's/g in the top 10 cm of the soil. This declined initially ($P = 0.024$) and by 23 weeks (late Sept.) was $2.52 \pm 0.99 \times 10^4$ CFU/g. However, the *M. anisopliae* concentration remained at a relatively constant average CFU concentration (3×10^4 CFU/g soil) over the 30 months (Fig. 8.8). No *M.anisopliae* DAT F-001 was recovered from the untreated plots over the course of the experiment.

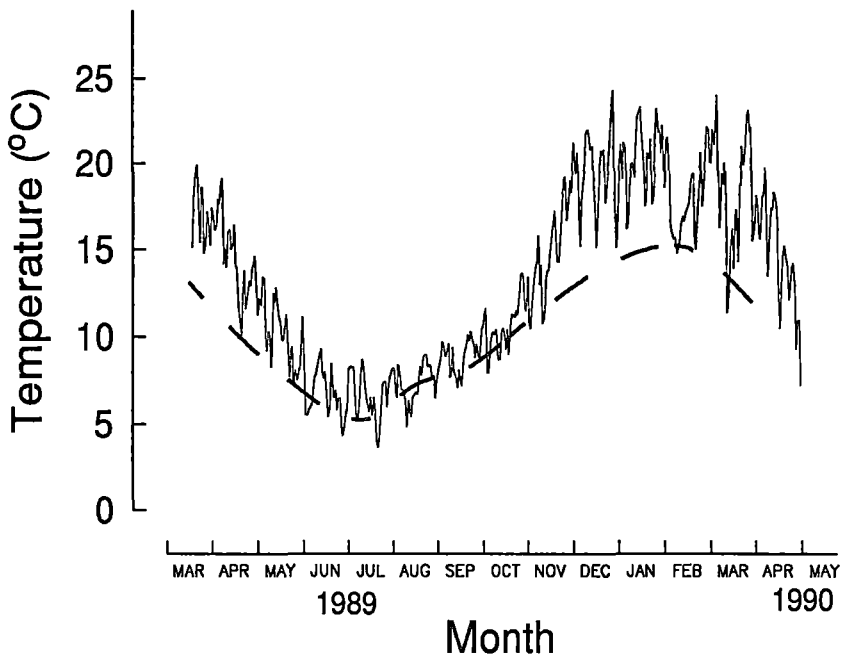

Figure 8.6 **Mean daily soil temperature (——) on 'Glenmorey' at a depth of 50 mm in comparison with the long-term (1884-1984) average air temperature (— —) for the Oatlands recording station (air data from Davies 1988).**

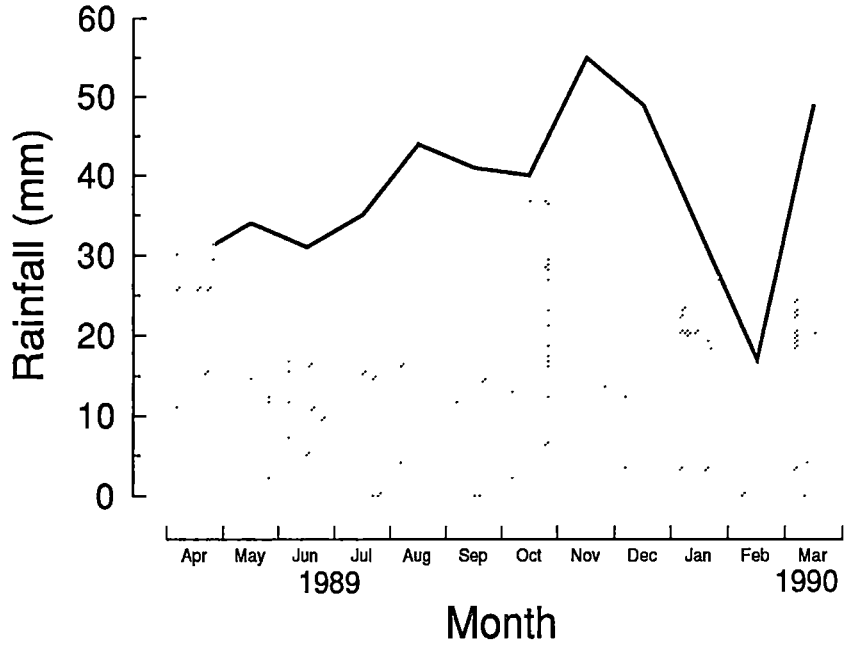

Figure 8.7 Total monthly rainfall (histograms) at 'Glenmorey' in comparison with the longer-term (1974-1985) average rainfall (——) at the Tunbridge recording station (Tunbridge data from Davies 1988).

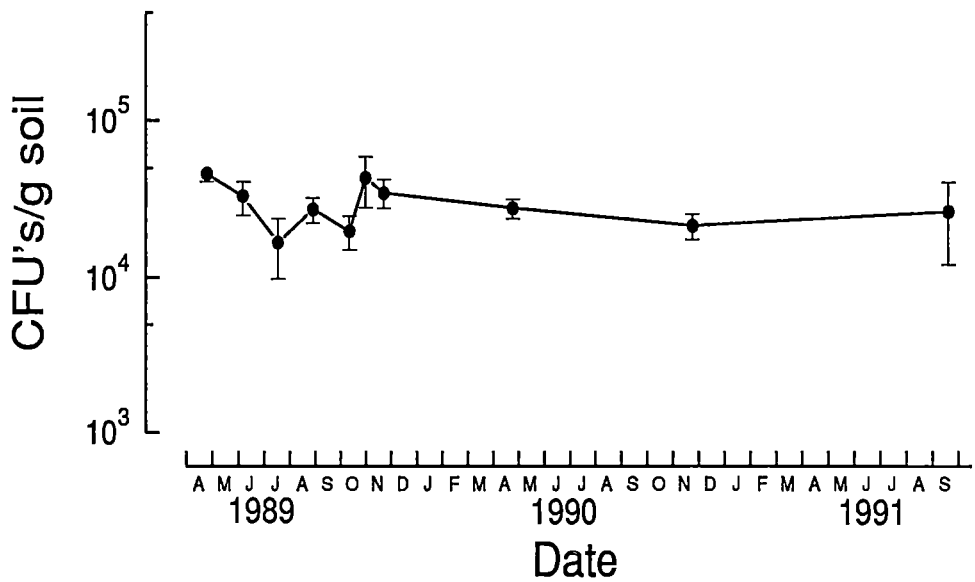

Figure 8.8 The concentration of *M. anisopliae* DAT F-001 in the soil over 30 months following ploughing and drill application into blocks at 'Glenmorey'.

8.3.4 Discussion

This experiment has shown that *M. anisopliae* DAT F-001 can reduce the survival of L3 larvae of *A. couloni* over the autumn and winter period. The soil temperatures over this period were low and this confirms the earlier laboratory studies (Chapter 6) which showed the effectiveness of the fungus at low temperatures. While rainfall was below average, this had little influence on the effectiveness of the fungus as the timing of *A. couloni* mortality was close to that predicted. The low level of mummification ($8/m^2$) may have been increased with greater rainfall. The survival of untreated larvae was poor in this experiment, perhaps aided by the harsh pretreatment (ploughing) of the blocks. While this treatment initially resulted in around 50% mortality of larvae, the ploughing also changed the soil structure which would have been detrimental to larval survival.

The 47% reduction in larval numbers in treated plots two years after application is more significant, in practical terms, than the initial result. While the larval population present in 1991 were the following generation to the one treated in 1989, it is likely that the control achieved is the result of not only continual reduction of the pest present in the blocks but also reduction of the pests migrating to the plots. The good survival (Fig. 8.8) of *M. anisopliae* was undoubtedly the reason why continued control of *A. couloni* was achieved. Presence of the fungus in the plots prior to egg laying (Oct.-Nov.) meant that all larval stages could encounter the fungus leading to adequate control of L3 larvae in autumn.

8.4 'WARRINGA' SITE

8.4.1 Introduction

Pretreatment of the 'Glenmorey' site resulted in 50-60% mortality of L3 larvae. While this physical control may be good from a practical farming perspective it changed the environment for *A. couloni* and probably aided the resultant population collapse at that site.

The 'Warringa' experiment was established to determine the efficacy of two application rates of *M. anisopliae* (209 kg/ha and 64 kg/ha) against a nil-dose control (untreated) introduced into the soil with minimal disturbance to the soil structure of the pasture.

8.4.2 Materials and Methods

The site characteristics of 'Warringa' are listed in Table 8.4. The experimental site was established in an area where L3 larval damage was already apparent

Table 8.4 General characteristics and principal profile forms of 'Warringa'.

General characteristics*			Principal Profile Form		
land system code	mean rainfall (mm)	annual mean monthly air temperature range.	class†	description	pH
172131	375-500	5.3 - 15.1°C	Db3.13 subb	A horizon: sandy clay loam	5.5
				B Horizon: brown duplex medium clay	8.6

* from Davies (1988)

† after Northcote (1979)

Table 8.5 Application dates and rates of *M. anisopliae* DAT F-001 at 'Warringa' in 1989.

	Application	
date	rate(kg/ha)*	soil concn (sp/g soil)†
24 April	64	$3.3 \pm 0.8 \times 10^4$
	209	$6.3 \pm 2.3 \times 10^4$

* kg/ha of grain/fungus mix

† spores/g average in the top 10 cm of the soil (mean ± SE, n=4)

nr = not recorded

because the density of larvae was both relatively consistent and high (~400/m^2).

Each of the four experimental blocks compared two treatment concentrations (Table 8.5), each of which was paired to the same untreated (nil-dose) plot. All plots were established by direct drilling with a Duncan triple disk drill into the uncultivated pasture at 155 mm spacings (Fig. 8.3). Space restrictions resulted in the plot widths being reduced to 7 m (2 m buffers, 3 m sampling zone).

8.4.3 Results

Survival of larvae in untreated plots

Subterranean application of the *M. anisopliae* and the nil-dose treatments into an established pasture resulted in a mechanically-induced mortality of 22.7%. This reduced the larval population from 385/m^2 to 298/m^2. The population declined by a further 37% for the first 12 weeks but then stabilized for the remaining 18 weeks (Fig 8.9).

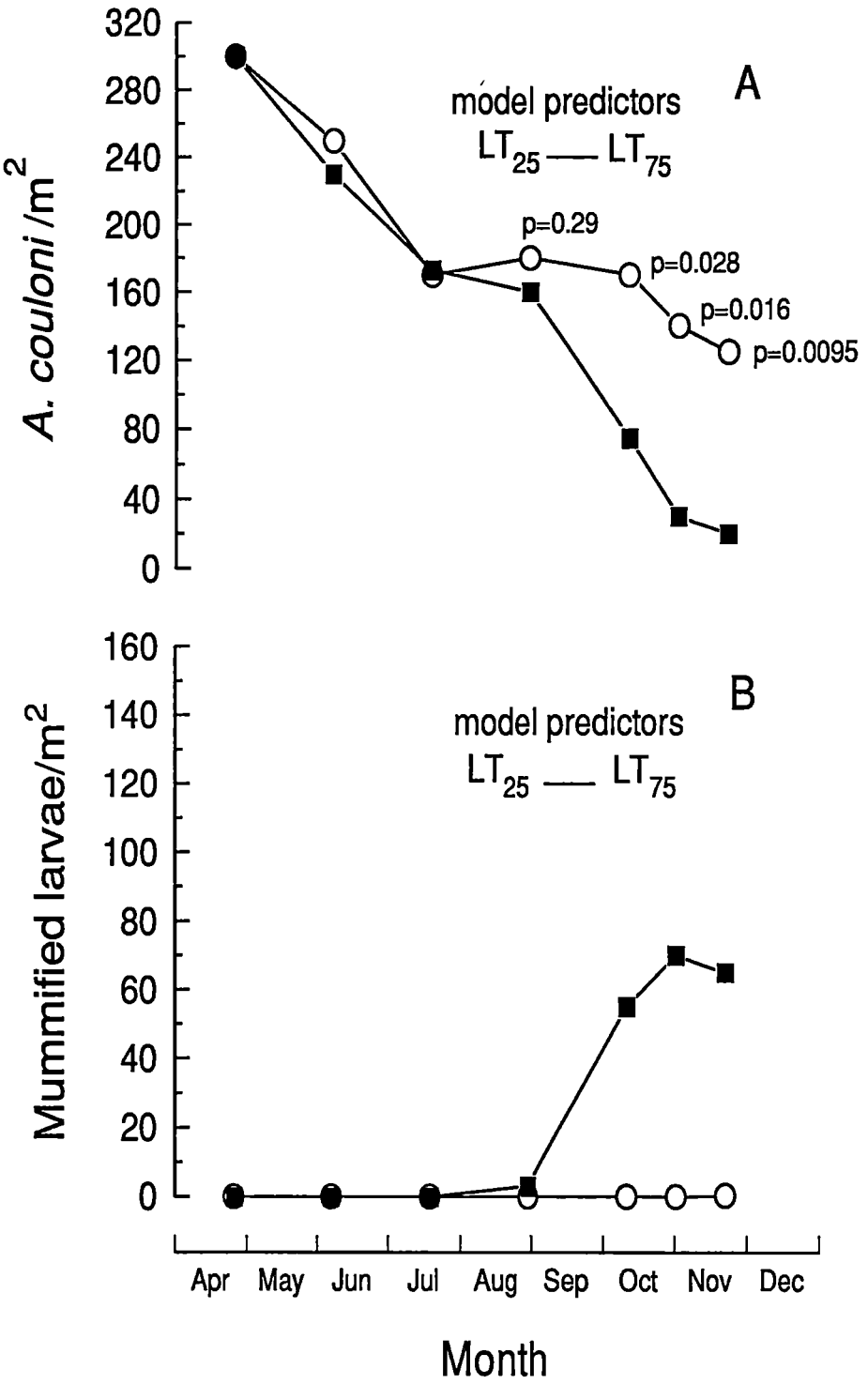

Figure 8.9 **Effect of autumn application (April 24) of *M. anisopliae* DAT F-001 (6.3 x 10^4 sp/g soil - high concentration) on (A) the survival and (B) the mummification of L3 larvae of *A. couloni* in the (○) untreated and the (■) treated plots at the 'Warringa' experimental site in 1989.**

Survival of larvae in *M. anisopliae* treated plots

The first diseased larvae were found in both the high and the low rate treatments 18 weeks (Sept.) after application. Six weeks later (Oct.) there was a 55% reduction (P=0.028) in surviving L3 larvae and 52 mummified L3 larvae/m^2 in the high rate treatment (Fig. 8.9).

L3 larval mortality in the high rate treated plots continued to increase with exposure time (Fig. 8.9) and reached 83% (P=0.0095) by late spring (Nov. - 30 weeks after application). In the low rate treatment 74% of L3 larvae had died by the same time, and this was not different (P>0.05) to the high rate treatment. The number of mummified larvae peaked at 70/m^2 in early November (Fig. 8.9).

The L3 larvae pupated in mid to late summer (Jan. - Feb.) and were all adults by the final (52 week) sample time. Of the control L3 population present at 30 weeks, 35% (44.3 adults/m^2) had developed into adults while only 11% (2.75 adults/m^2) of the *M. anisopliae* treated (high or low rate) L3 larvae developed into adults. This increased the *M. anisopliae*-induced mortality from 83% in L3 larvae to 94% in adults.

Overlapping Population

A small population of beetles from the overlapping population (Chapter 1) was present in the experiment from September 1989. These beetles would not have been exposed to the fungus prior to this time because they were 150 - 300 mm deep and the fungus had been drilled into the soil at a depth of 25 mm. Flying and mating occur between September and October, and beetles re-enter the soil to lay eggs. Egg laying occurs at depths of 0 - 80 mm in spring.

Mortality in these adults was related to both exposure time and concentration of *M. anisopliae* (Table 8.6), and by autumn of the following year (1990), there was an 80% difference (P=0.068) between the subsequent control and treated L3 populations (Table 8.6).

Field Mortality Model

The model predicted a 25% difference and a 75% difference between high rate and control populations after 19 and 22 weeks respectively. The first diseased

Table 8.6 **Effect of soil inoculated on 24 April 1989 with *M. anisopliae* DAT F-001 on the overlapping *A. couloni* population present in the 'Warringa' experimental site, six to twelve months after application.**

	Adults/m^2		Larvae/m^2
Treatment	10 Oct. '89	22 Nov. '89	24 April '90
nil dose (untreated)	$7.0 \pm 1.6^{a*}$	5.7 ± 1.4^{a}	14.3 ± 3.9^{a}
low concn ($3.3 \pm 0.8 \times 10^4$ sp/g soil)	9.0 ± 0.6^{a}	3.0 ± 1.1^{b}	6.0 ± 1.6^{b}
high concn ($6.3 \pm 2.3 \times 10^4$ sp/g soil)	9.5 ± 1.2^{a}	1.5 ± 1.2^{b}	2.8 ± 1.1^{a}

* At each sampling occassion, values followed by the same letter do not differ (P>0.05); mean ± SE, n=4

larvae and a 10% population difference (P>0.05) were found in the sample taken at 18 weeks. There was a 55% difference after 24 weeks and an 80% difference by 27 weeks (Fig. 8.9).

Weather conditions

Mean winter and spring soil temperatures were similar to the long-term mean air temperatures though the summer soil temperatures were 5°-7°C higher than the air temperature (Fig. 8.10). The mean soil temperature over the 24 week period required to reduce the larval population by 55%, was 7.3°C. Rainfall was close to average for the eight months that larvae were present in 1989 (Fig. 8.11).

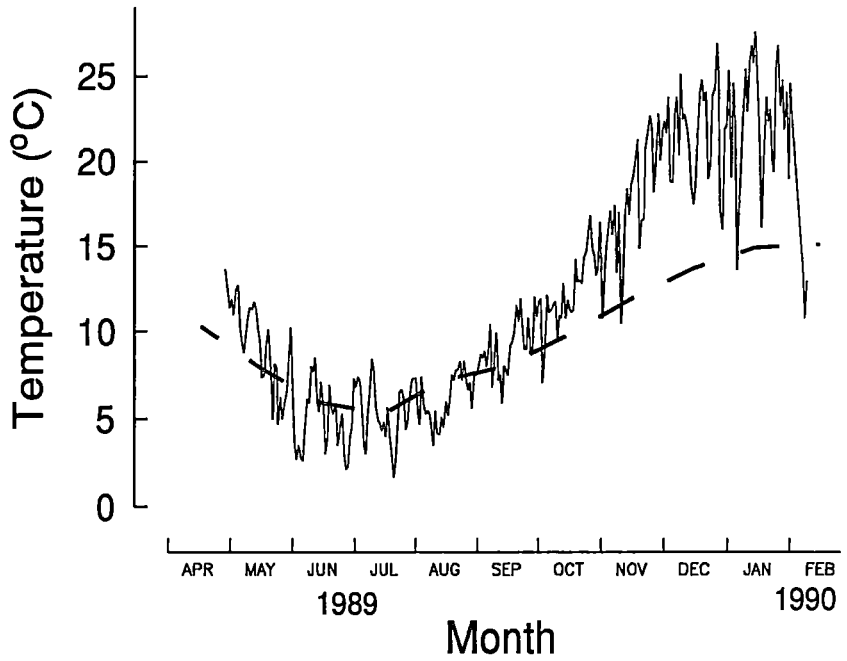

Figure 8.10 **Mean daily soil temperature (——) on 'Warringa' at a depth of 50 mm in comparison with the long-term (1884-1984) average air temperature (— —) for the Oatlands recording station (air data from Davies, 1988).**

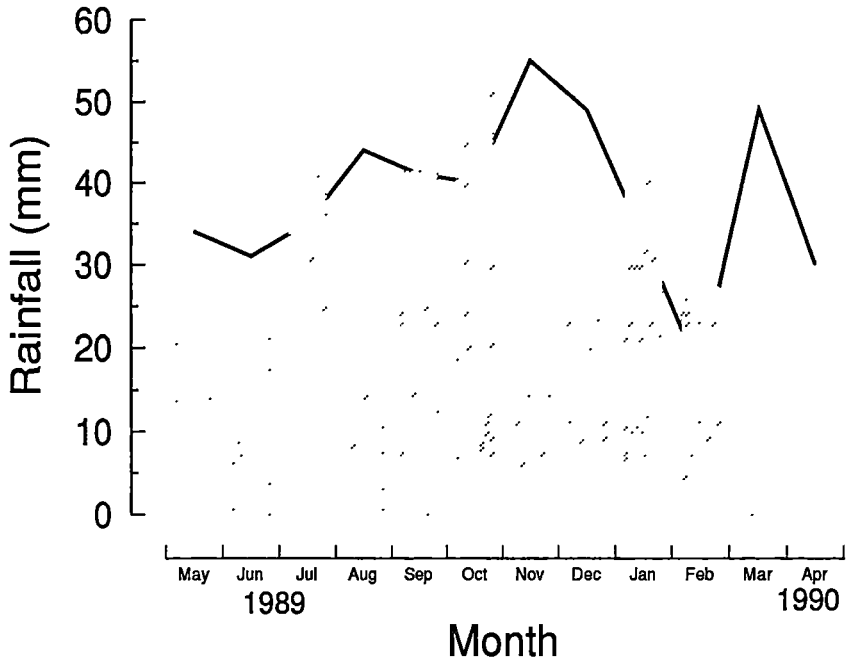

Figure 8.11 **Total monthly rainfall (shaded histograms) at 'Warringa' in comparison with the longer-term (1974-1985) average rainfall (——) at the Tunbridge recording station (Tunbridge data from Davies, 1988).**

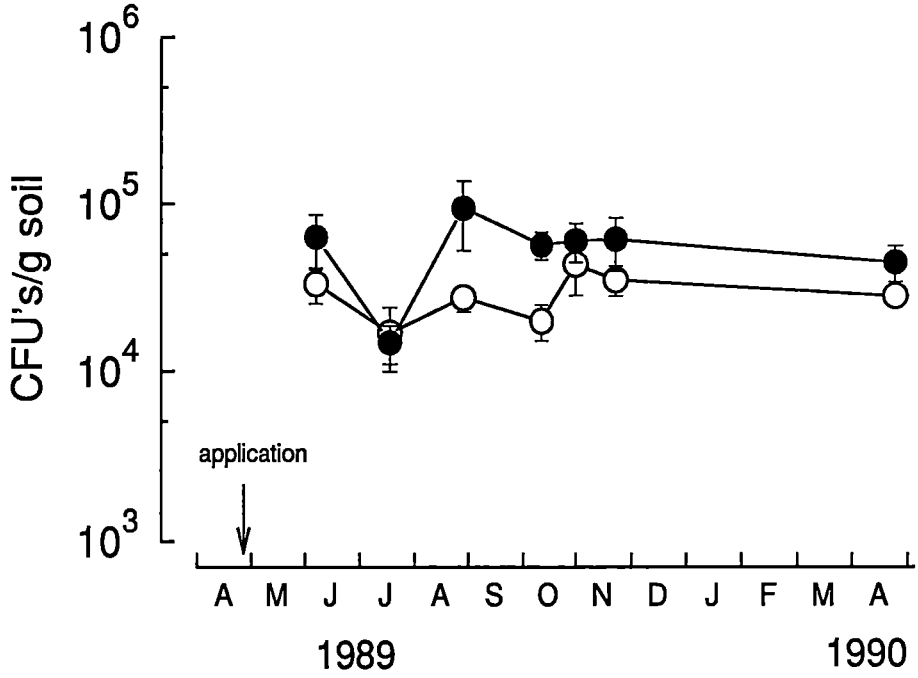

Figure 8.12 **The concentration of *M. anisopliae* DAT F-001 in the soil following direct-drill applications of (○) 3.3 x 10^4 and (●) 6.3 x 10^4 spores/g soil into blocks at 'Warringa'.**

M. anisopliae concentration in the soil

Application of 64.3 and 209 kg/ha of *M. anisopliae*-millet mix resulted in soil inoculum concentrations of 3.3 ± 0.8 x 10^4 and 6.3 ± 2.3 x 10^4 CFU's/g in the top 10 cm of the soil. These inoculum concentrations were maintained over 52 weeks (Fig. 8.12). No DAT F-001 was isolated from untreated plots.

8.4.4 Discussion

This experiment has shown that *M. anisopliae* DAT F-001 can reduce the *A. couloni* population by 94%. Application of either concentration caused similar larval and adult mortality. The mortality model was accurate in predicting the long lag time before larval mortality (caused by the fungus) was recorded, though the subsequent population decline was less swift than predicted.

Mummification and subsequent sporulation of cadavers was much higher at this site (70/m^2) compared to 'Glenmorey' (8/m^2). The warmer temperatures and higher rainfall present at 'Warringa' when the larvae were dying from the fungus

contrasts with the situation in June 1989 at 'Glenmorey' and probably accounts for the mummification differences. The decline in mummified larvae at 'Warringa' during November is more likely to be due to a lack of larvae (Fig. 8.9) than the sudden decline in rainfall (Fig. 8.11).

The enhanced mummification level did not increase the CFU concentrations in the soil (Fig. 8.12), even though mycelium was observed growing out of many L3 cadavers into the soil and producing additional spores, in much the same manner as described for *B. bassiana* by Gottwald and Tedders (1984). While this suggests that there was considerable loss of CFU's at this site it would depend upon the spore production per mummified cadaver. If an average of 10^7 spores/cadaver were produced, the increase in soil CFU concentration would be negligible (at 70 cadavers/m^2), while at 10^8 spores/cadaver a doubling of the CFU concentration could be expected (provided there was no attrition of the applied concentration). Under ideal laboratory conditions Rath (unpublished data) found that L3 cadavers could produce 10^7 spores/cadaver but additionally the mycelium grew out from the cadaver into a sand-peat mix producing more spores (up to 7 x 10^7 spores/larval cadaver). Weiser and Matha (1986) have shown that a similar insect, *Melolontha melolontha*, when infected with *M. anisopliae* typically produces 10^5 spores/cadaver. Consequently it would seem unlikely that L3 larvae of *A. couloni* would significantly increase the CFU concentration in pasture soils. It is more likely that the spores from the cadavers would only serve to aid the maintenance of applied CFU concentrations.

8.5 'INVERELL' SITE

8.5.1 Introduction

The previous two experiments have shown that application of *M. anisopliae* to pastures in autumn does not result in reduction of *A. couloni* numbers until winter or spring (depending on application timing). Consequently, there was no saving of autumn-winter pastures by application of the fungus in the autumn of an infestation. In subsequent years the 'Glenmorey' site showed that larval control could be achieved in autumn probably saving some pasture productivity. Consequently, the fungus could probably be introduced into pastures at any applicable time (autumn to spring) and achieve similar long-term control.

The 'Inverell' experiment was established to determine both the short-term and long-term efficacy of *M. anisopliae* following application in mid-winter and early spring.

Table 8.7 General characteristics and principal profile forms of experimental sites.

General characteristics*			Principal Profile Form		
land system code	mean rainfall (mm)	annual mean monthly air temperature range.	class†	description	pH
273231	500-625	5.3 - 15.1°C	Dy5.12 suba	A horizon: silt loam B horizon: light olive grey mottled yellowish brown light clay	5.3 7.4

* from Davies (1988)

† after Northcote (1979)

8.5.2 Materials and Methods

The site characteristics of 'Inverell' are listed in Table 8.7. This property had a major infestation for the first time in the autumn of 1989 and the pastures were extensively damaged when the experiments began.

Each of the four experimental blocks compared two application times (Table 8.8). The treatment and nil-dose control plots were established by direct-drilling into the uncultivated pasture. The plots were sampled over a 29 month period and the sampling times for the July application are shown in Table 8.8a.

8.5.3 Results

Survival of larvae in untreated plots

Subterranean application of both the *M. anisopliae* treated and the nil-dose controls (untreated) to an established pasture resulted in a mechanically-induced L3 mortality of 7% ($P>0.05$) in the mid-winter (July) experiment (Table 8.8a) and 18% ($P=0.009$) in the spring (September) experiment. After this the numbers of larvae in the untreated plots (of both the July and the September treatments) remained relatively constant (~350/m^2) until early summer (December) when a further decline left only 60% of larvae remaining (~225/m^2) (Fig. 8.13; Table 8.8a). This time coincided with the development of L3 larvae into prepupae (Fig. 8.13). This density of insects then remained constant

Table 8.8 Application dates and rates of *M. anisopliae* DAT F-001 to experimental sites.

	Application		
Field site	date	rate(kg/ha)*	soil concn (sp/g soil)†
'Inverell'	10 July	84	$5.1 \pm 0.7 \times 10^4$
	13 Sept	122	$7.0 \pm 2.5 \times 10^4$

* kg/ha of grain/fungus mix

† spores/g average in the top 10 cm of the soil (mean ± SE, n=4)

nr = not recorded

through 1990 as *A. couloni* developed into pupae and then adults. In October 1990 adult flights occurred, after which there were 40 ± 5 females/m^2 in the untreated plots of the July application (Table 8.8a), and 57 ± 5 females/m^2 in the September application. A large population of ~ 900 L2/m^2 was present in early March, 1991 and these declined to ~ 500 L3/m^2 by October 1991 (Fig. 8.13; Table 8.8a).

Survival of larvae in *M. anisopliae* treated plots

July application: The first diseased larvae of the primary population were found 15 weeks (23 Oct., 1989) after application. The numbers of larvae in the treated and untreated plots were not different until December 1989 (21 weeks after application) when there were 30% less larvae in the treated plots (Fig. 8.13; Table 8.8a). There was 60% less pupae in the treated plots after 27 weeks (Jan.,1990), and by 44 weeks (May., 1990) there was 69% less adults (Fig. 8.13; Table 8.8a).

The stabilization of insect survival after 27 weeks, coincided with the movement of the population (pupal and adult populations during 1990) deeper into the soil (20 - 30 cm) where exposure to *M. anisopliae* would be minimal. The difference in survival of adults between treated and untreated plots remained basically unchanged throughout the life span of the adults (Feb. - Sept. 1990) until November (Fig. 8.13). The November sample was taken after the mating flights had occured in October 1990; there were no differences in the numbers of adult females in the treated (40/m^2) and untreated (35/m^2) plots in November.

In early autumn (March) of 1991, there were 63% less L2 larvae in the treated plots (than in the untreated plots and this increased to 79% less in the L3 population by spring (November; Fig. 8.13; Table 8.8a). This reduction in larval numbers brought the population below the economic threshold in autumn (May, 1991; Table 8.8a), and while lack of rain limited pasture productivity, there was visibly less damage in the treated plots compared with the untreated plots (Fig. 8.14).

September application: These treatment and control plots were established 9 weeks after the July application but the warmer spring temperatures meant that the first diseased larvae were found only three weeks later (Nov. 1989) than those in the July plots (9 weeks after application). There were 38% less pupae ($P = 0.011$) in the treated plots (151/m^2) after 18 weeks (Jan. 1990) than in the untreated plots (243/m^2) and after 35 weeks (May 1990) there were 46% less adults ($P = 0.05$) in the treated plots (104/m^2) than in the untreated plots (193/m^2). As with the July application the difference between treated and control populations remained constant as the population moved deeper into the soil and away from the high concentrations of *M. anisopliae* spores.

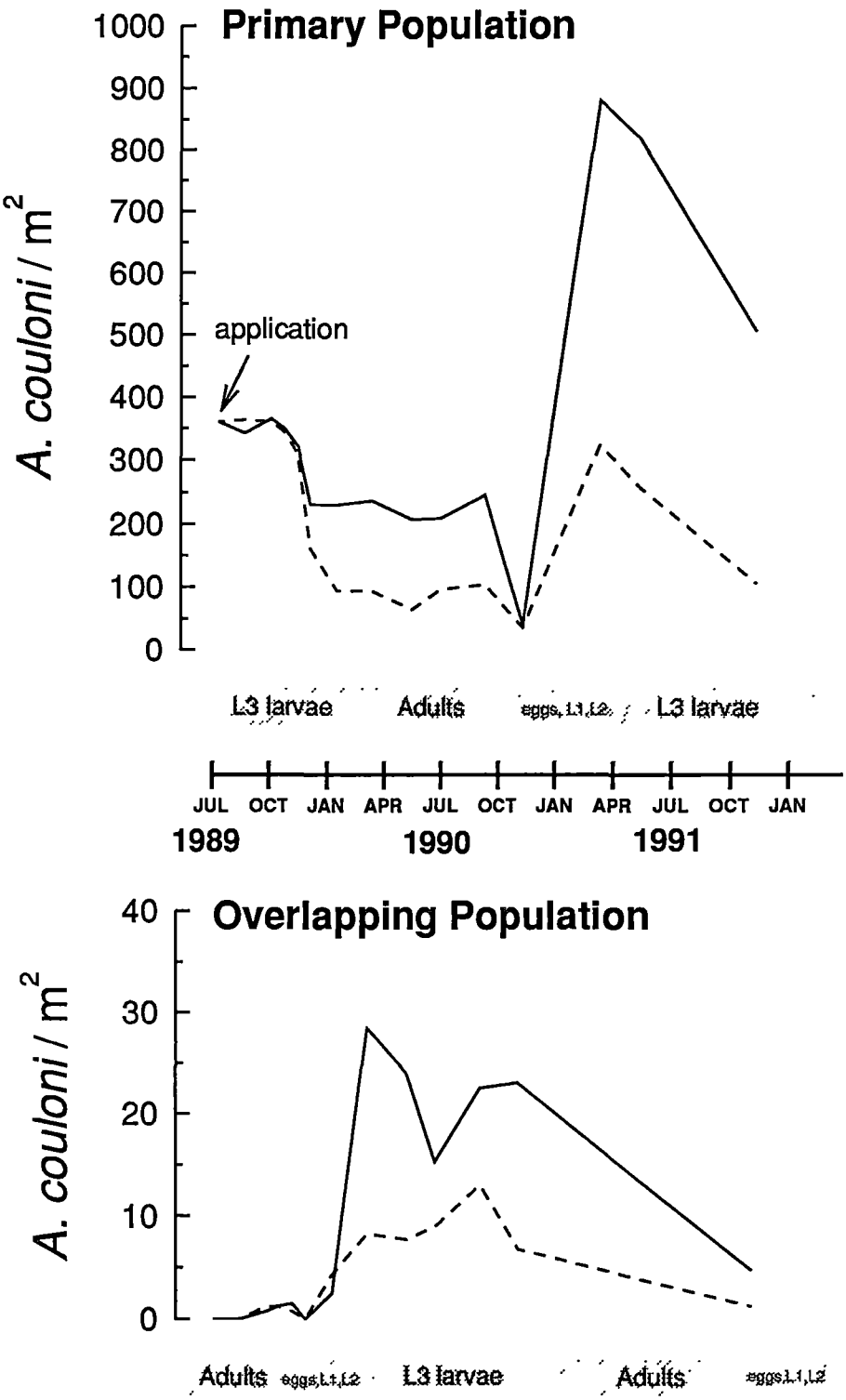

Figure 8.13 Effect of *M. anisopliae* DAT F-001 applied in July 1989 on the (——) untreated and the (— —) treated primary and overlapping populations of *A. couloni* at 'Inverell'.

Table 8.8a **Effect of *M. anisopliae* DAT F-001 applied in July 1989 on the untreated and treated primary population of *A. couloni* at 'Inverell'.**

sampling date	insect stage	larvae/m^{2*} untreated	larvae/m^{2*} *M. anisopliae* treated	%mortality	P value
preliminary sample					
03/07/89	L3	387 ± 44	387 ± 52	0.0	0.98
immediate post application sample					
10/07/89	L3	373 ± 46	346 ± 43	7.2	0.26
routine samples					
21/08/89	L3	342 ± 24	364 ± 28	-6.4	0.70
02/10/89	L3	367 ± 27	360 ± 592	1.9	0.46
23/10/89	L3	349 ± 19	345 ± 33	1.1	0.46
13/11/89	L3	321 ± 33	309 ± 37	3.7	0.28
04/12/89	L3/prepupae	231 ± 28	161 ± 13	30.3	0.049
15/01/90	pupae	223 ± 26	94 ± 12	57.8	0.0019
12/03/90	adults	236 ± 27	94 ± 23	60.1	0.0046
15/05/90	adults	207 ± 52	65 ± 12	68.6	0.022
09/07/90	adults	217 ± 21	97 ± 37	55.2	0.016
19/09/90	adults	245 ± 13	104 ± 21	57.7	0.0013
19/11/90†	adults	40 ± 5	35 ± 6	12.5	0.26
20/03/91	L2	884 ± 84	325 ± 33	63.2	0.0034
22/05/91	L2/L3	819 ± 38	258 ± 59	68.5	0.0029
18/11/91	L3	506 ± 58	106 ± 15	79.2	0.0015

* mean ± SE, n=4

† after mating flights; females/m^2 only

Figure 8.14 Damage to pasture due to the presence of L3 larvae of *A. couloni* in the pasture surrounds this *M. anisopliae* DAT F-001 treated plot at 'Inverell' in early June 1991.

There were more (p = 0.014) female adults in the untreated plots of the 'September' experiment (57/m^2) than in the treated plots (37/m^2) after mating flights in 1990 and as a consequence less samplilng effort was expended on these plots than on the July applied plots (the July plots were not significantly different at this time whereas the differences in these September plots may have automatically led to increased numbers of larvae in 1991). During 1991 these plots were only sampled in May and showed a 70% reduction (P=0.0018) in the numbers of L3 larvae in the treated plots (287/m^2) over the untreated plots (948/m^2); a very similar result to that shown in the July applied plots (Table 8.8a).

Overlapping Population

In September 1989, a small population of immigrant adults laid eggs in the paddock in which this experiment was established. By autumn (May 1990), the larval population in the treated plots (8/m^2) of the July applicatiaon was 68% lower (P=0.02) than in the untreated plots (24/m^2) and the difference was maintained as the larvae pupated and became adults in 1991 (Fig. 8.13). The September applied plots showed a similar situation with 51% less (P=0.0075) larvae in the treated plots (18/m^2) than in the untreated plots (36/m^2) in May 1990.

Field Mortality Model

July application: The model predicted the 25-75% difference to occur from 16 to 18 weeks. The first diseased larvae were found after 15 weeks though there was no significant mortality in the treated plots until 21 weeks when 30% mortality was recorded (Fig. 8.13).

September application: The model predicted the 25-75% difference after 12 to 14 weeks. The first diseased larvae were found after nine weeks though there was no significant mortality in the treated plots until 17-18 weeks when 38% mortality was recorded (Fig. 8.13).

Environmental conditions

Mean soil temperatures were similar to the long-term average air temperatures except that during the spring of 1990 and 1991 the soil temperatures were around 2°C below average while both summers were 2° - 3°C higher than average (Fig. 8.15). This site maintained a rainfall deficit over the course of the experiment and this most noticeable in the autumn (March-May) of 1991 (Fig. 8.15).

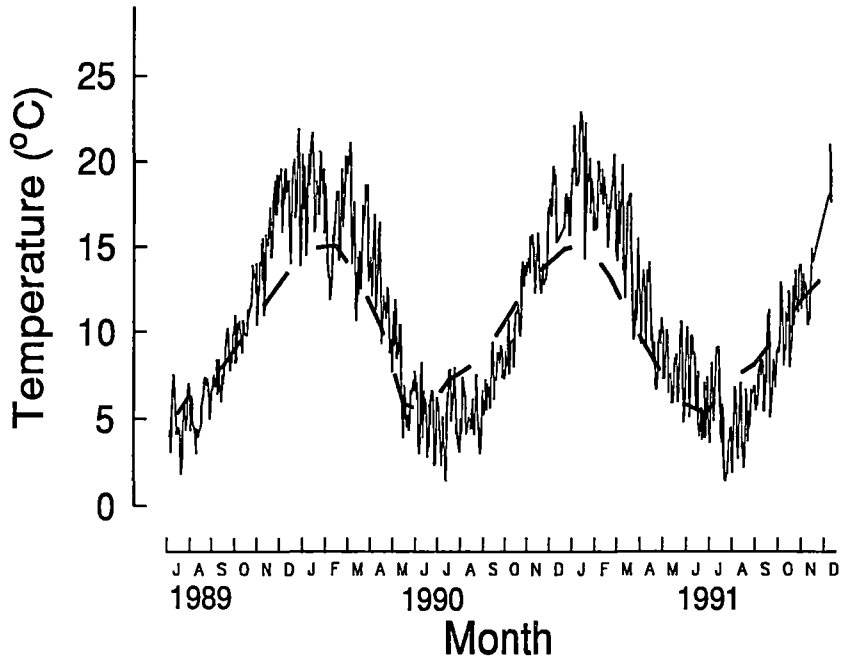

Figure 8.15 Mean daily soil temperature (——) on 'Inverell' at a depth of 50 mm in comparison with the long-term (1884-1984) average air temperature (— —) for the Oatlands recording station (air data from Davies 1988).

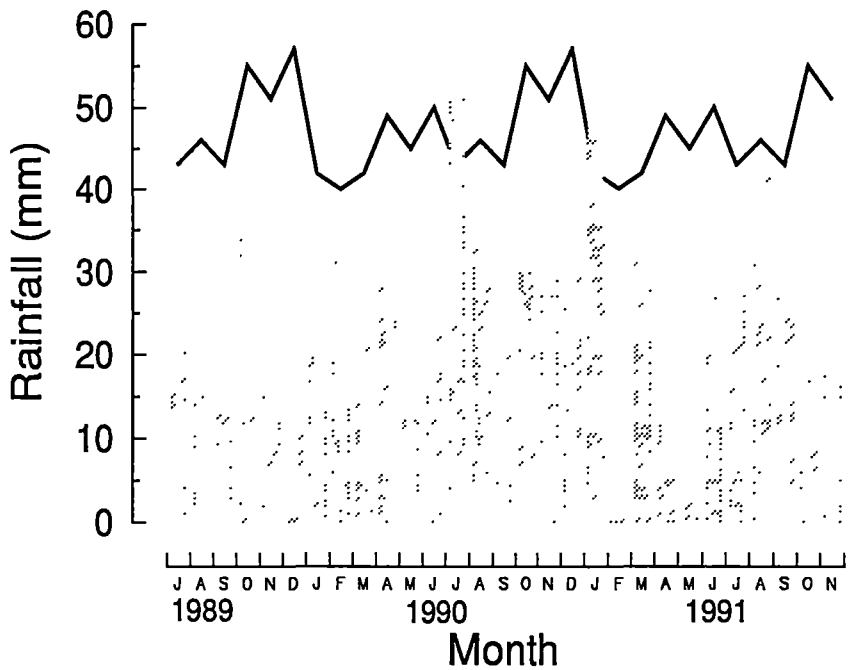

Figure 8.16 Total monthly rainfall (shaded histograms) at 'Inverell' in comparison with the longer-term (1884-1984) average rainfall (——) at the Oatlands recording station (Oatlands data from Davies 1988).

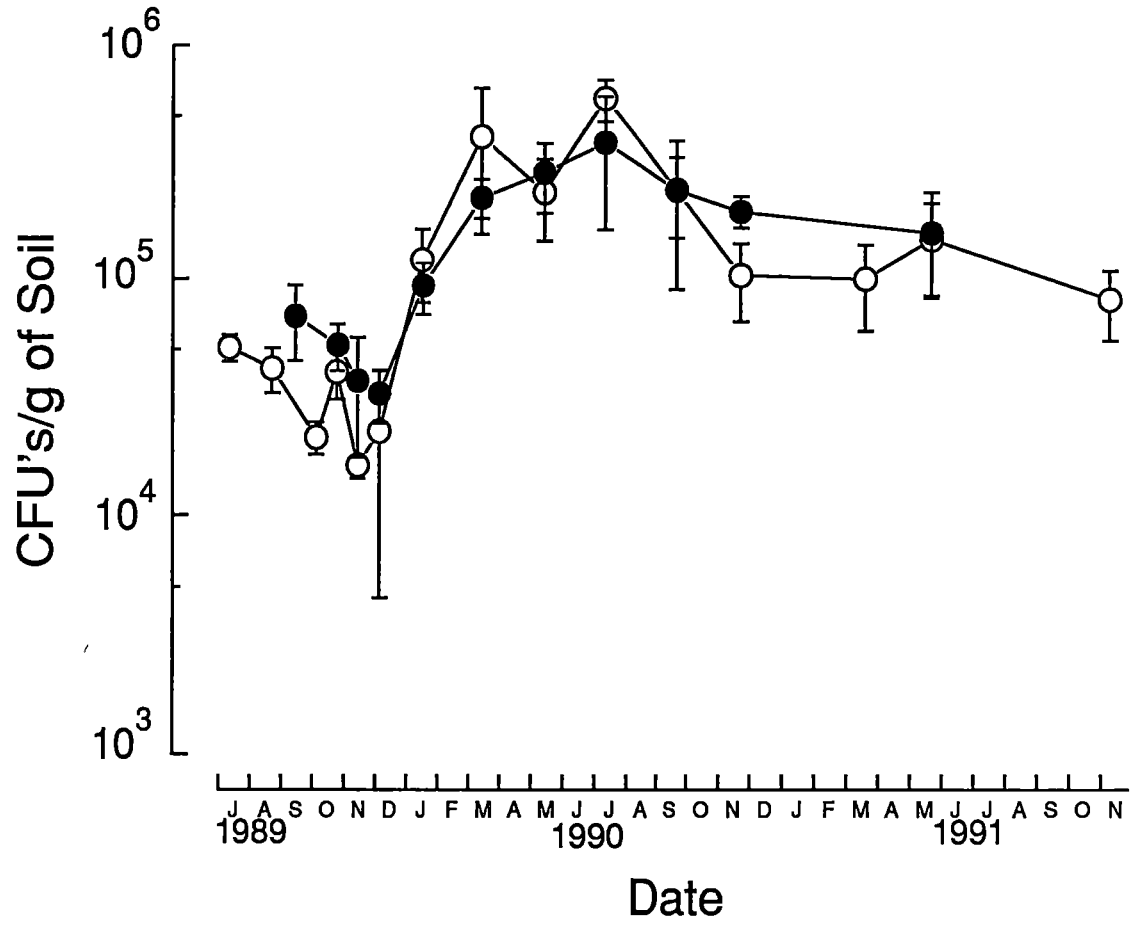

Figure 8.17 **The concentration of *M. anisopliae* DAT F-001 in the soil over 29 months, following direct-drill applications of (○) 5.1 x 10^4 spores/g soil in July and (●) 7.0 x 10^4 spores/g soil in September to the blocks at 'Inverell' in 1989.**

M. anisopliae concentration in the soil

The concentration of *M. anisopliae* DAT F-001 in the top 10 cm of pasture soil was maintained at concentrations close to the applied concentration (5.1 - 7.0 x 10^4 CFU/g soil) until the mummified cadavers began to sporulate. Sporulation increased the soil CFU concentration 5 - 10 fold to 4.0 - 6.0 x 10^5 CFU/g soil (Fig. 8.17) but this concentration declined slowly over the next 20 months to 0.8 - 1.0 x 10^5 CFU's/g soil. No *M. anisopliae* was recorded from the untreated plots.

Effect on non-target organisms

During 1989, there were no differences ($p>0.05$) in the numbers of blackheaded cockchafers (Coleoptera: Scarabaeidae: *Aphodius tasmaniae*) (Table 8.9), yellowheaded cockchafers (Col.: Scar.: *Sericesthis* spp.) (Table 8.10) or corbies (Lepidoptera: Hepialidae: *Oncoperae* spp.) (Table 8.11). There were more ($p = 0.0001$) blackheaded cockchafers in the treated plots 29 months after application than in the control plots (Table 8.12).

Table 8.9 **Blackheaded cockchafer (*Aphodius* spp.) numbers* in the *M. anisopliae* DAT F-001 treated and the untreated plots at 'Inverell' in 1989.**

Month	weeks after application	larvae/m^2 untreated	treated
July	0	31	28
August	6	39	41
October	12	12	12
November	15	6	8
December	18	2	2

* all results not significantly different ($p>0.05$)

Table 8.10 **Yellowheaded cockchafer (*Sericesthis* sp.) numbers* in the *M. anisopliae* DAT F-001 treated and the untreated plots at 'Inverell' in 1989.**

Month	weeks after application	larvae/m^2 untreated	treated
July	0	19	23
August	6	19	11
October	12	6	3
November	15	2	5
December	18	0	1

* all results not significantly different ($p>0.05$)

Table 8.11 **Corbie grub (*Oncoperae intricata*) numbers* in the *M. anisopliae* DAT F-001 treated and the untreated plots at 'Inverell' in 1989.**

Month	weeks after application	larvae/m^2 untreated	treated
July	0	17	8
August	6	17	9
October	12	1	1
November	15	0	1
December	18	0	0

* all results not significantly different (p>0.05)

Table 8.12 **Effect of DAT F-001 on non-target organisms at 'Inverell' on 18/11/91, 29 months after application of *Metarhizium anisopliae*.**

Organism	Density/m^2 untreated	treated
Diptera		
larva A	1.3	1.0
larva B	0.5	1.0
Lepidoptera		
Oncopera	0.0	0.3
Mythimna	0.5	0.5
Coleoptera		
Aphodius	5.3	26.3*
Sericesthis	2.8	3.3
dung beetles	0.0	0.0
Elaterids	3.0	15.0
other	0.5	2.0
Annelids		
earthworm A	0.3	0.3
earthworm B	0.0	0.0

* P=0.0001; all other results p>0.05

8.5.4 Discussion

Application of *M. anisopliae* to an L3 *A. couloni* population in winter or spring had little impact on the larval numbers but significantly reduced the pupal and adult populations the subsequent year. In this experiment the subsequent generation was reduced by 60 - 70% before and during early autumn. This result is similar to that shown at 'Glenmorey' in 1991. The 70% reduction in the overlapping population at 'Inverell' in 1990 was identical to that shown at both 'Glenmorey' and 'Warringa'.

The concentration of *M. anisopliae* DAT F-001 increased 5 - 10 fold as the cadavers began sporulating (Jan.-March, 1990). The concentration was still twice that of the applied concentration 21 months later. While the fungus was responsible for a 70% reduction L2 larvae by March 1991, few cadavers were found (probably because these smaller larvae - L1/L2 - would disintegrate more quickly than L3 larvae). There must have been some input to the CFU concentrations in the soil from these cadavers but there is no rise in CFU concentrations at that time in 1991. As at 'Warringa', rainfall may be responsible for this difference between years. The late summer/autumn (Feb-March) of 1990 at 'Inverell' was considerably wetter than the same period in 1991. It is also likely that the difference is due to the life stage of the cadavers. In 1990 the rise in CFU concentrations occurred as mummified L3 and pupae sporulated while in 1991, mortality due to the fungus occurred prior to the L2 stage, suggesting eggs and/or L1 larvae as the CFU source. Earlier life stages produce fewer spores than later stages (Weiser and Matha, 1986). Weiser and Matha (1986) also show that *Melolontha* pupae can produce 10^{10} spores/cadaver, if the spore production from *A. couloni* is similar it would explain why their was a rise in CFU concentration at 'Inverell' and not at 'Warringa'.

M. anisopliae DAT F-001 had no adverse effect on the range of invertebrates present in this experiment and this was consistent with the studies of Goettel *et al.* (1990) and Nuutinen *et al.* (1991). However, 29 months after application, there were significantly higher numbers of another pest, the blackheaded cockchafer, in the *M. anisopliae* treated plots than in the untreated plots, but they were still well below the economic threshold of 125/m^2 (McQuillan and Ireson, 1987). This pest produces vertical tunnels in the pasture and emerges at night to feed on the aerial parts of ryegrass and other pasture grasses

(McQuillan and Ireson, 1987). While blackheaded cockchafer is probably the most serious pest in Tasmanian pastures it is easily controlled by chemical insecticides.

The reason for the increase in pest numbers in *M. anisopliae* treated plots may be quite simple: Large infestations of *A. couloni* move back and forth horizontally in the soil and continually cover the burrows of tunnelling insects such as blackheaded cockchafers. Large infestations of *A. couloni* have very few other insects present. At 'Inverell' in 1991 the dramatic differences in the *A. couloni* populations between control (900/m^2) and treatment (300/m^2) plots enabled a greater survival of blackheaded cockchafer in the treated plots.

This should not be seen as the resurgence or replacement of one pest with another because the blackheaded cockchafer is by far the more widely distributed and damaging of the two pests. The farmer would have had a blackheaded cockchafer problem except for the presence of *A. couloni*. In the absence of *A. couloni* there is a restoration of the blackheaded cockchafer population.

Coles and Pinnock (1982, 1984) were developing another isolate of *M. anisopliae* for blackheaded cockchafer control, however, commercial development has ceased (R. deGroot, personal communication, Sept. 1991).

8.6 DAT F-001 SURVIVAL IN THE ABSENCE OF HOST

8.6.1 Introduction

The experiments previously discussed in this chapter are specifically designed to test the efficacy of DAT F-001 against *A. couloni*. The survival of *M. anisopliae* DAT F-001 was an important but secondary aim of those experiments. In the 'Inverell' experiment the mummification of *A. couloni* significantly increased the CFU concentration in the soil. At the other two sites, the effect of spore production on cadavers was not obvious.

This field experiment examined the variation in *M. anisopliae* DAT F-001 concentration in the soil in the absence of *A. couloni*.

8.6.2 Materials and Methods

'Bendeveron' is a property situated at Bothwell which is 42 km south-west of the other sites. Infestations of *A. couloni* were not present on 'Bendeveron' over the study period. The site characteristics of this property are listed in Table 8.13.

Table 8.13 General characteristics and principal profile forms of 'Bendeveron'.

General characteristics*			Principal Profile Form		
land system code	mean rainfall (mm)	annual mean monthly air temperature range.	class†	description	pH
272251	500-625	5.3-15.5°C	DB4.12 suba	A Horizon: loam B Horizon: mottled heavy clay	5.5 6.5

* from Davies (1988)

† after Northcote (1979)

The datalogger at this site was faulty and intermittent data was recorded during 1989/90. This data was deleted.

The experimental design consisted of four adjacent plots (20 m x 20 m). Inoculant was applied to the soil following disk ploughing. The soil was then ploughed (to a depth of 15 cm) to cover the fungus, followed by harrowing and sowing of pasture seeds. At each sampling occasion, four soil samples (50 x 50 x 100 mm; l x w x d) were taken from each plot. Within a plot the soil samples were mixed together prior to determination of CFU concentrations as described in section 8.2.

8.6.3 Results and Discussion

The CFU concentrations declined rapidly for the first 18 months, followed by a period where the concentration averaged 10^4 CFU/g soil (Fig. 8.18). Soil temperatures were similar to those described on the other three sites (Fig. 8.19). An apparent rise in CFU concentrations from Jan. 1990 to Aug./Oct.

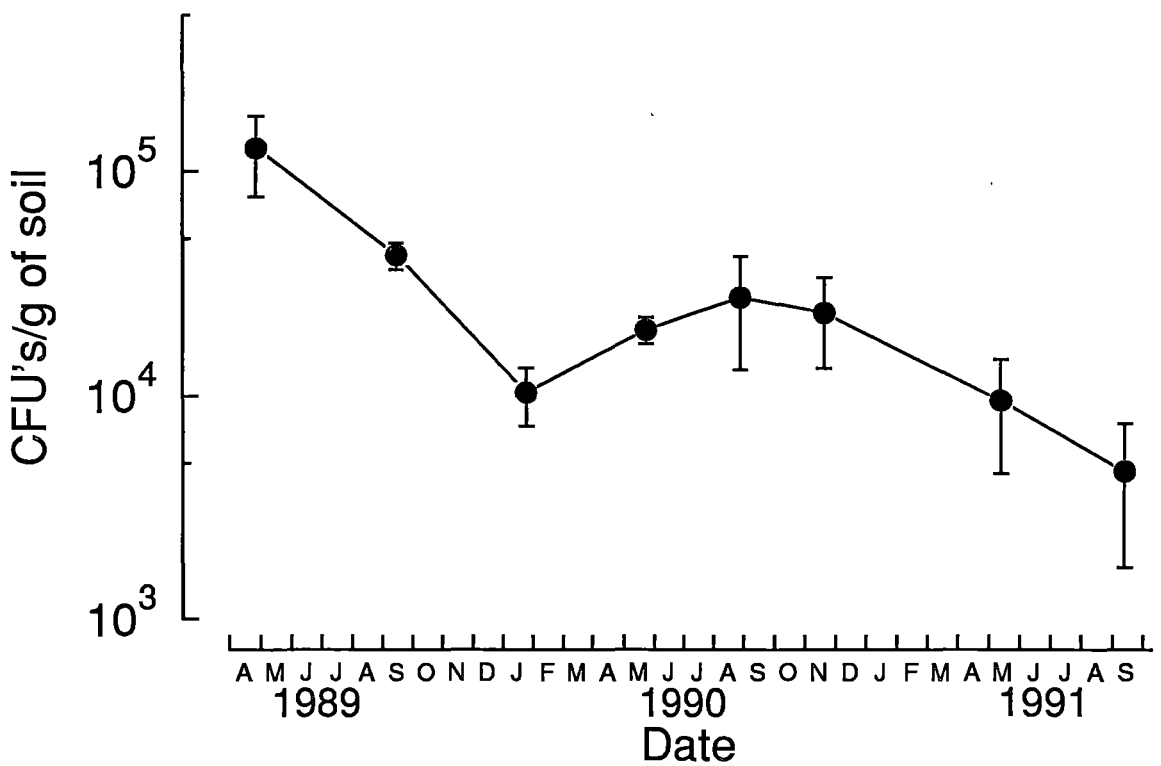

Figure 8.18 **The concentration of *M. anisopliae* DAT F-001 in the soil over 29 months, following ploughing of surface-applied fungus into the blocks at 'Bendeveron' in 1989.**

1990 may be in part due to the rainfall averages at this time. During 1990 the rainfall was similar to the long-term average (Bothwell; Davies, 1988), while in 1991 the rainfall was in deficit (Fig. 8.20). During this deficit period the CFU concentrations declined from their late 1990 high point (Fig. 8.18). While the 'Glenmorey', 'Warringa' and 'Inverell' sites showed either a constant CFU concentration or a rise in CFU concentration, the concentration declined in this experiment. However, Latch and Falloon (1976) found that rainfall and temperature did not affect the survival of *M. anisopliae* var. *major* in the breeding sites of *Oryctes rhinoceros* over a 24 months period. In contrast to the laboratory data, Milner and Lutton (1976) found that field survival of *M. anisopliae* remained unchanged after 12 months.

It has already been discussed that the rise in CFU concentrations at 'Inverell' was associated with sporulating cadavers while at 'Warringa', sporulating cadavers did not increase the CFU concentration. The 'Bendeveron' data, in comparison with these other sites, suggests that the presence of the host is a the major factor in maintaining CFU concentrations. Muller-Kogler and Zimmerman (1986) reported a decline in *B. bassiana* in field sites in both summer and winter in the absence of the host.

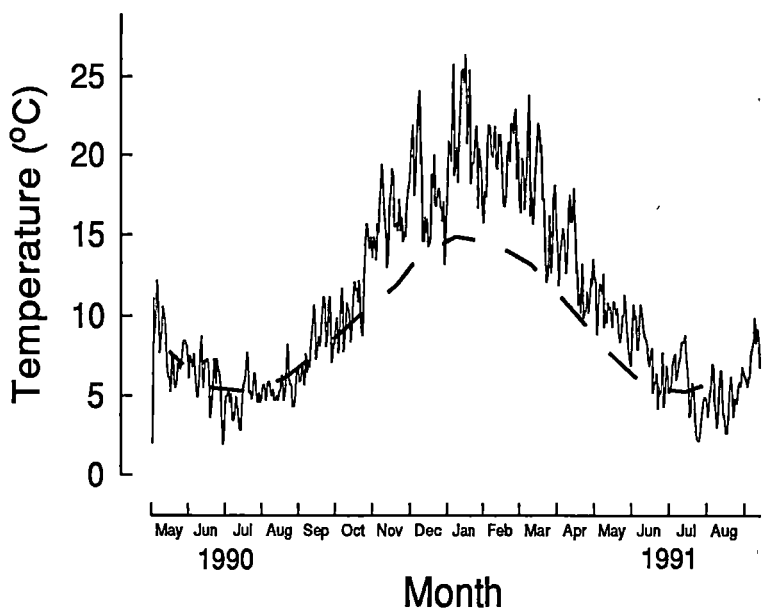

Figure 8.19 **Mean daily soil temperature (——) on 'Bendeveron' at a depth of 50 mm in comparison with the long-term (1884-1984) average air temperature (— —) for the Bothwell recording station (air data from Davies 1988).**

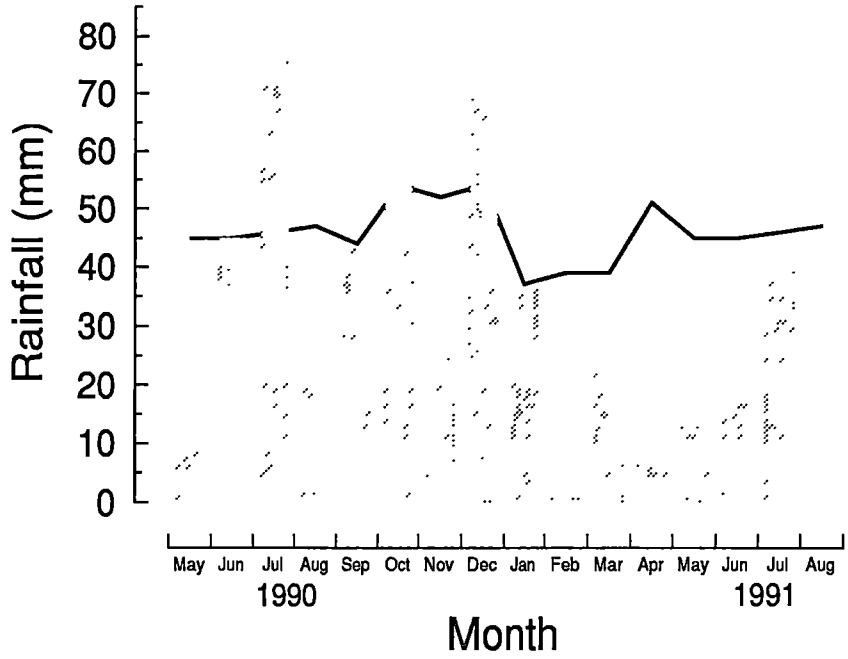

Figure 8.20 **Total monthly rainfall (shaded histograms) at 'Bendeveron' in comparison with the longer-term (1974-1985) average rainfall (——) at the Bothwell recording station (Bothwell data from Davies, 1988).**

8.7 GENERAL DISCUSSION

These field experiments have shown that *M. anisopliae* DAT F-001 can reduce L3 larval survival by up to 83% (Fig. 8.9), and the timing of the population reduction is consistent with a model (Chapter 7) developed from laboratory data. Control of both the overlapping larval population the year following application, and the second generation of the primary population two years after application, reached 60 - 70% before the autumn period when pasture damage would normally occur. These control rates should lead to increased pasture productivity for several years after a single application of *M. anisopliae*.

Efficacy

All three field experiments were established when *A. couloni* were in the final instar (L3) stage. As a result, a comparison of the experiments can be regarded as an investigation of the application timing. Experiments established before winter ('Glenmorey' and 'Warringa', Figs. 8.5 and 8.9) showed that DAT F-001 was a major contributing factor in the decline of larval numbers. The experiments established in mid-winter and early spring ('Inverell', Fig. 8.13) had minimal impact on the larval population but significantly reduced the numbers of pupae (40 - 60% reduction).

The effect of field temperatures can be easily be seen in these experiments. In the 'Glenmorey' experiment (Fig. 8.5), DAT F-001 was able to reduce the larval population by 50% in 12 weeks during the warm temperatures of early autumn. Application of DAT F-001 six weeks later (mid autumn) could not reduce the larval population by 50% until approximately 24 weeks ('Warringa', Fig. 8.9). The slow growth of the fungus, combined with reduced larval activity at winter temperatures (Chapter 6), significantly reduced the fungal virulence.

The 'Inverell' experiment further highlights the influence of temperature, as this experiment included a mid-winter and an early-spring application time. The first diseased larvae in the spring experiment were found only three weeks after similar findings in the mid-winter experiment even though the experiments were established nine weeks apart. Latch and Kain (1983) found that the field mortality rates of *Wiseana* sp. exposed to *M. anisopliae* were increased with increasing average temperature.

While adults and L3 larvae were equally susceptible to DAT F-001 (Chapter 5), in the 'Inverell' experiment the maximum reduction in *A. couloni* survival was reached as the L3 larvae pupate, and it was apparent that after this time there was no additional DAT F-001 induced mortality (Fig. 8.13). This was because the larvae moved deeper into the soil as they prepared to pupate. Consequently, they removed themselves from the soil zone (50 mm deep) where they were likely to encounter DAT F-001. The data suggest that the only pupae that died were those infected or contaminated by DAT F-001 as larvae before migration down the soil profile.

These experiments have shown a greater reduction in the larval numbers of *A. couloni* in the year of application than have other studies of scarab pest control which use similar application methods. Ferron (1981) found that application of *Beauveria brongniartii* to a population of the scarab *Melolontha melolontha* did not cause an epizootic until the end of the larval stage one year later when 69% mortality was recorded (Ferron as cited by Keller 1986). These delayed epizootics in *M. melolontha* have also been reported by Ferron (1983), Keller (1986) and Keller *et al.* (1986). Jackson (1989) found that application of *Serratia entomophila* to pastures caused mortality in grass grub (*Costelytra zealandica*) populations just prior to pupation, which then resulted in 55% differences between treated and untreated populations.

Application rates were only compared in the 'Warringa' experiment. Application of 64 kg/ha ($3.3 \pm 0.8 \times 10^4$ sp/g soil) was as effective as 209 kg/ha (6.3 ± 2.3 10^4 sp/g soil) in the reduction of both L3 larvae and the subsequent adult population. This result was supported by the laboratory dosage data (Chapter 6) and the data of many other authors (including Muller-Kogler and Stein, 1976; Marchal, 1976; Quintella *et al.*, 1990) who have shown that 10-fold reductions in concentration are required to make significant differences in larval survival. This study suggests that much lower concentrations of DAT F-001 (10^3 - 10^4 sp/g soil) may be effective in reducing L3 larval populations given the length of time of the larval stage. However, the concentrations used are much lower than that used to effectively control *M. melolontha*. Keller *et al.* (1986) used a concentration of ~ 2.6×10^{14} blastospores of *B. bassiana*/ha, while the concentrations cited above (64 and 209 kg/ha) equate to ~ 5.0×10^{13} spores/ha.

These experiments have shown that in the years following DAT F-001 application, the overlapping larval population and the next generation of the primary population were reduced by 70% over the summer period (Table 8.6 and Fig. 8.13) before the damaging L3 stage is reached. This indicates that DAT F-001 will have a longer-term effect by suppressing the populations over a number of generations. The data in Chapter 6, which showed that DAT F-001 in soil was pathogenic for eggs and L1 larvae, support this argument. Most importantly, eggs are laid in the top 80 mm of the soil and this places them and L1 larvae in the zone of high DAT F-001 spore (CFU) concentrations. In this respect, this work has shown similar results to those of Ferron (1981) and Keller (1986).

Model

The field mortality model (Chapter 7) was accurate at predicting the onset (as measured by initial mummification) of mortality in L3 larvae. However, prediction of the LT_{25} and the LT_{75} was not accurate. This is not surprising when three assumptions of the model are considered:

(a) The assumed concentration of 1×10^5 sp/g soil was never reached, while,

(b) The reduction of larval movement at temperatures of 5°-10°C (Chapter 6) would suggest that application of spores in mid-winter would not result in exposure of the larvae within a few days, and

(c) The assumption of the efficacy of DAT F-001 at 5°C was shown to be optimistic (Chapter 6).

As a consequence, the predicted LT_{25}'s should have been at later times. Minor modification of this model to include the concentration data from Chapter 6 and the temperature data from Chapter 7 would significantly enhance the predictability of a model that promises to be useful under field conditions. A modified model is described in Chapter 9.

DAT F-001 CFU concentration in soil

The concentration of DAT F-001 CFU's in the soil over the course of these experiments shows that there is potential for long-term suppression of *A. couloni*. In the 'Glenmorey' experiment the fungus concentration declined only slightly over six months but was then maintained for over 30 months. The concentration was also maintained for 12 months in the 'Warringa' experiment even though at this site the mummification of larvae was high (Fig. 8.9) and an increase in CFU concentrations could have been expected. This increase was seen in the 'Inverell' experiment where mummification coincided with a 5 - 10 fold increase in soil CFU concentrations.

Lewis and Papavizas (1984) have shown that the CFU concentrations of the plant pathogen antagonist *Trichoderma* sp. increased when applied to the soil as a mycelial preparation with bran. In the experiments reported here the use of the grain substrate, on which *M. anisopliae* was grown, as the inoculant carrier did not lead to a rise in CFU concentrations shortly after application.

The population dynamics of insect pathogenic fungi is regulated by many complex biotic and abiotic factors which have not been fully characterized (McCoy *et al.*, 1988). The decline in CFU concentrations at 'Bendeveron' suggest that the presence or absence of the *A. couloni* host is a major factor in the maintenance of CFU concentrations. This maintenance of CFU concentrations could have been aided by low soil temperatures (Milner and Lutton, 1976), a lack of U.V. light and enhanced spore survival due to lessened desiccation in the soil. The results described here were probably not unexpected as Milner and Lutton (1976) predicted that *M. anisopliae* spores could survive many years in soil at 16°C. However, Tyni-Juslin and Vanninen (1990) found *M. anisopliae* counts in a range of field soil types were only 34% of the original applied concentration after 12 months.

Agricultural Implications

The mortality model and the efficacy data presented here both indicate that there is almost no practical application time which would reduce L3 larval numbers sufficiently (below 100-200/m) to save pasture productivity in the first year. Major damage to pastures occurs between April and June, and while application in February and March would probably result in pasture savings due

to reductions in larval numbers, application at this time is inappropriate for two reasons:

(1) Farmers will not see any effect of the pest before late March. Reactive application of DAT F-001 at this time could be completely wasted (and hence costly), as natural population collapses are common as larvae develop from L2 to L3 larvae in March-April; and

(2) Subterranean application of DAT F-001 at the end of summer is generally impossible because of the dry, hard soil.

Even if DAT F-001 could act fast enough in autumn to save pasture production, this pest is often distributed over 500-1,000 ha on each affected farm. In general, farmers do not have the resources to apply DAT F-001 to more that 100-200 ha each autumn. As a result, only a small saving in pasture production would be expected.

M. anisopliae DAT F-001 control of *A. couloni* will only succeed if the fungus can maintain reduced pest populations over several years. The results show that the use of DAT F-001 can lower adult numbers by up to 96%. Additionally, the L1 and L2 larvae can be reduced, in subsequent years, by 70% over summer before the autumn when the L3 larvae damage pasture.

The maintenance of *M. anisopliae* in the soil is the key component of the control strategy. Unlike short-lived insects with multi-generations per year (Hall, 1982), it is unlikely that the 'epizootic potential' of fungus will be important in *A. couloni* control. The epizootic potential of infected immobile eggs or L1/L2 larvae that move only small distances, is probably nil. Adequate control will only be achieved with an 'even' distribution of an 'acceptable' concentration. This will guarantee maximum probability of eggs and L1/l2 larvae being exposed to, and subsequently succumbing to the fungus before the damaging L3 stage. All the experiments reported here used a concentration of ~ 10^5 spores/g soil and gave very good control over several years. Consequently, an 'acceptable' concentration may be much lower than those studied.

Long-term control would not only make a final biocontrol product effective but would help to lessen the high initial cost of approximately US$40/ha (Rath *et al.*, 1990; appendix 7). This cost is based upon an application rate of 25 kg/ha of the 10% a.i. product. These concentrations used in these experiments ranged from 66 kg/ha (US$106/ha) to 209 kg/ha (US$334/ha). As these costs are

untenable in pasture, both a reduction in application rate (to at least 25 kg/ha) as well as the long-term control is still needed. Field experiments to examine the efficacy of concentrations as low as 1 kg/ha are reported in Chapter 10.

CHAPTER 9

9.0 MODIFICATION OF THE MODEL WHICH PREDICTS THE TIMING OF FIELD MORTALITY.

9.1 INTRODUCTION

The model developed in Chapter 7 was accurate in its prediction of the onset of fungal induced mortality in L3 larval populations. The model became less accurate when the *M. anisopliae* application time progressed from winter into spring. This was due to the movement of the L3 larvae down the soil profile (down to 300 mm) in readiness for pupation.

There were several inherent flaws with the early model. Most importantly, the model was not designed to include variation in application rates and consequently the model was limited in its exploratory capability. Further, the model utilized an exponential equation, whereas later data (Chapters 5 and 6) had shown that logistic curves were more appropriate for explaining the variations in both temperature and spores concentration data. Logistic models were used by Pinnock and Brand (1981) to explore the interactions between the dose of *Bacillus thuringiensis* and the time required to kill the red-humped caterpillars, *Schizura concinna*.

This chapter describes the revision of the initial model by the use of a single logistic equation incorporating both the effects of spore concentration and temperature on fungal virulence. The model was verified using the independent field data (Chapter 8) before being used to examine application time/rate strategies for the control of *A. couloni*. The conclusions of the model were used to design subsequent field experiments.

9.2 MODEL DEVELOPMENT

This model used the same procedures as described for the development of the earlier model (section 7.2) except that the total laboratory data sets for L3 larvae from Chapter 5 (spore concentration at 20°C; 10^1 to 10^7 spores/g sand-peat) and Chapter 6 (change in virulence due to temperature decline; 15° to 5°C at 4 x 10^6 spores/g sand-peat) were combined and analyzed using the GLIM® statistical package.

The data were only part of the total 'temperature by concentration' matrix and consequently the resultant model had to predict the missing parts of the matrix. The accuracy of these predictions could not be assessed except by intuition.

The same logistic function as used in Chapters 5 and 6 was used here:

$$P = \frac{e^x}{1 + e^x} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \textcircled{1}$$

where P = proportion of larvae surviving,
and x = a function of concentration, temperature and time

GLIM® enabled the x function to include the main factors as well as any combination of factor interactions. Initially, the full model was fitted (ie. concentration, temperature, time and all possible combinations), however, this caused three major problems:

1. The biological explanation of some of the interactions was not intuitively obvious,

2. The inclusion of several 'time' factors made the resultant equations virtually unsolvable for 'time' alone, and

3. The model had a tendency to give parabolic predictions (ie. the model tended to overestimate LT_{50}'s at mid concentrations and mid temperatures while often the LT_5's were underestimated at the extremes of concentration and temperature - often to the extent of giving negative predictions).

These problems were solved by using only the two parameters of concentration and temperature and incorporating time as a three way interaction,

$$x = a + b(concn) + c(temp) + d(concn \times temp \times time) \ldots . \quad ②$$

However, this model was still limited by the parabolic effect mentioned above (3.). The incorporation of a $(dose)^2$ factor in equation ② reversed the parabolic effect and was perhaps easier to accept 'biologically' than a $(time)^2$ factor. The new equation became:

$$P = \frac{e^x}{1 + e^x}$$

where $x = a + b(concn) + c(concn)^2 + d(temp) + e(concn \times temp \times time) \ldots ③$

The major interest in this model was to solve the equation for 'time', as concentration, temperature and the proportion of surviving larvae were the 'known' variables. The equation became,

$$\text{Time required to achieve P survival} = \frac{\ln[P/(1-P)] - [8.679 + 2.00 \times concn - 0.2287 \times (concn)^2 - 0.3721 \times temp.]}{-0.001551 \times (temp \times dose)}$$

This equation was then incorporated into the computer programme SuperCalc IV® and a matrix of the mean monthly soil temperatures of each of the three field experiments ('Glenmorey', 'Warringa' and 'Inverell') conducted in 1989 was used to validate the model.

The model was then used to predict (using the three year average mean monthly soil temperature at 'Inverell'),

1. The application rates required to achieve 50% larval mortality by April 30 if applied at times from January to April, and

2. The effect of application of 10^{12}, 10^{13} and 10^{14} spores/ha at varying application times over the length of the L2/L3 life stage.

9.3 RESULTS

Model validation

This model accurately predicted the initiation of disease (LT_5) and the LT_{50} in the L3 larval population at the 'Glenmorey' and 'Warringa' field sites (Figs. 9.1 and 9.2). The predicted time were 14 to 28 days earlier than the actual times (Fig. 9.3), though at the predicted LT_5 the first mummified larvae were recorded (Chapter 8, p. 8 - 28).

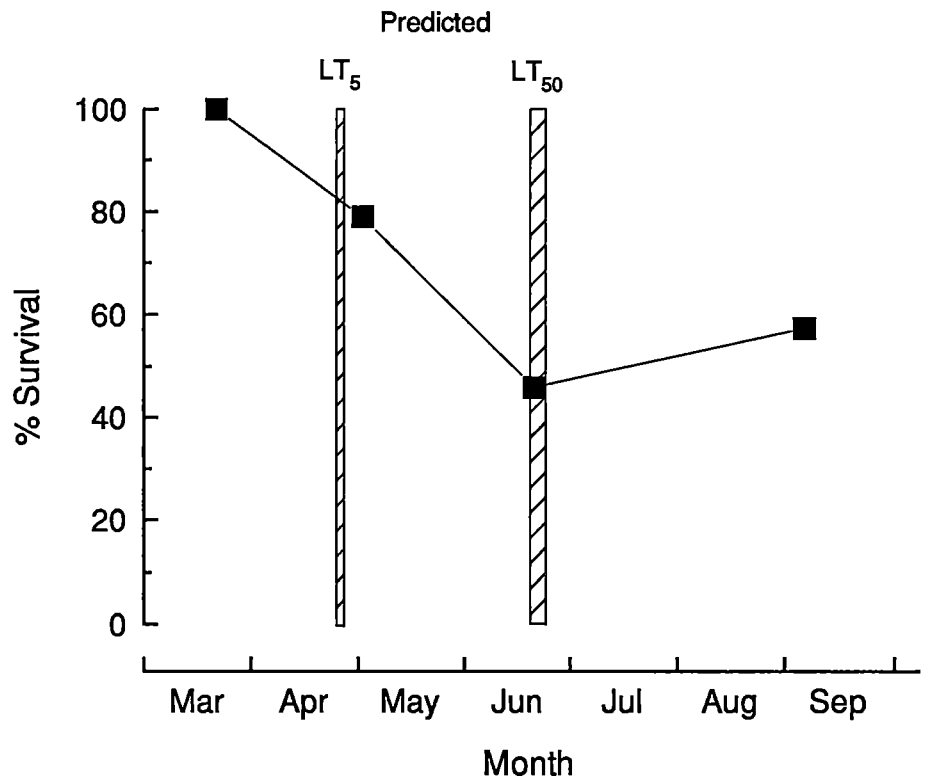

Figure 9.1 **The model predicted LT_5 and LT_{50} of the 'Glenmorey' field experiment plotted with the data from Fig. 8.5 converted to percentage survival. The width of the LT_5 and LT_{50} bars represent the difference between using long-term average regional air temperature (left-hand side of bar) and the actual mean monthly soil temperature recorded during the experiment (right-hand side of bar).**

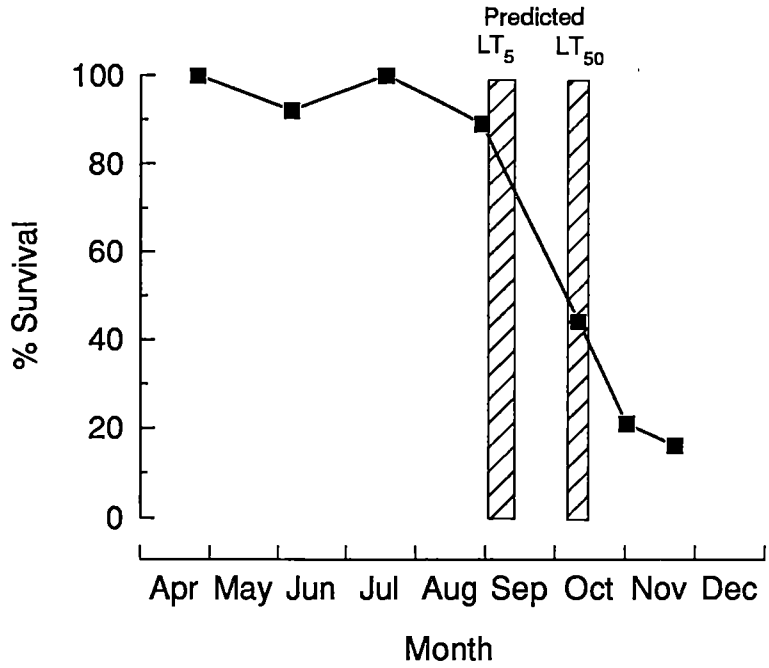

Figure 9.2 **The model predicted LT_5 and LT_{50} of the 'Warringa' field experiment plotted with the data from Fig. 8.9 converted to percentage survival. The width of the LT_5 and LT_{50} bars represent the difference between using long-term average regional air temperature (left-hand side of bar) and the actual mean monthly soil temperature recorded during the experiment (right-hand side of bar).**

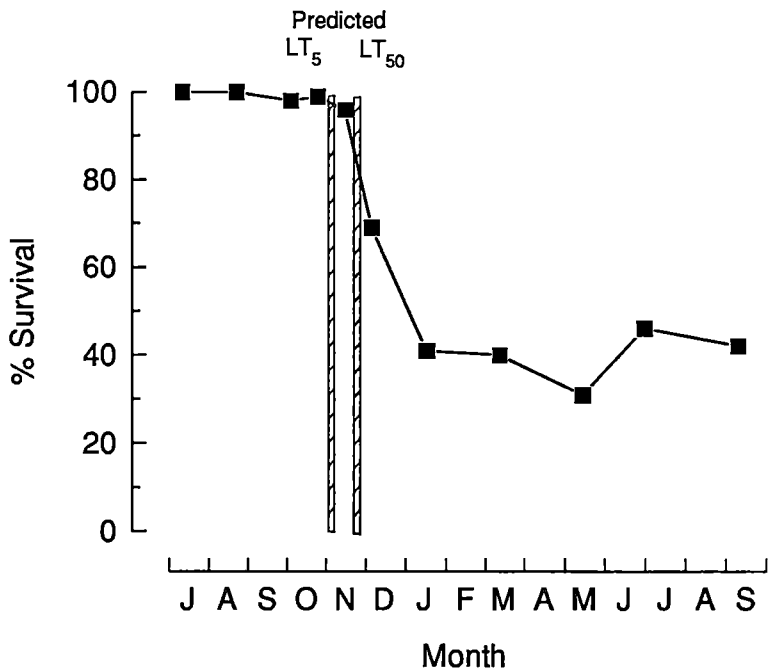

Figure 9.3 **The model predicted LT_5 and LT_{50} of the 'Inverell' field experiment plotted with the data from Fig. 8.13 converted to percentage survival. The width of the LT_5 and LT_{50} bars represent the difference between using long-term average regional air temperature (left-hand side of bar) and the actual mean monthly soil temperature recorded during the experiment (right-hand side of bar).**

Predictions of short-term application rates

The combined effect of decreasing temperatures (from January to March) and decreasing time (to 30 April) resulted in an exponential increase in the quantity of *M. anisopliae* required to achieve a 50% reduction in larval numbers by 30 April (Fig. 9.4). Application rates greater than 100 kg/ha were required from late summer (February) onward while this rate could be reduced to only 100 g if applied in early January (Fig. 9.4).

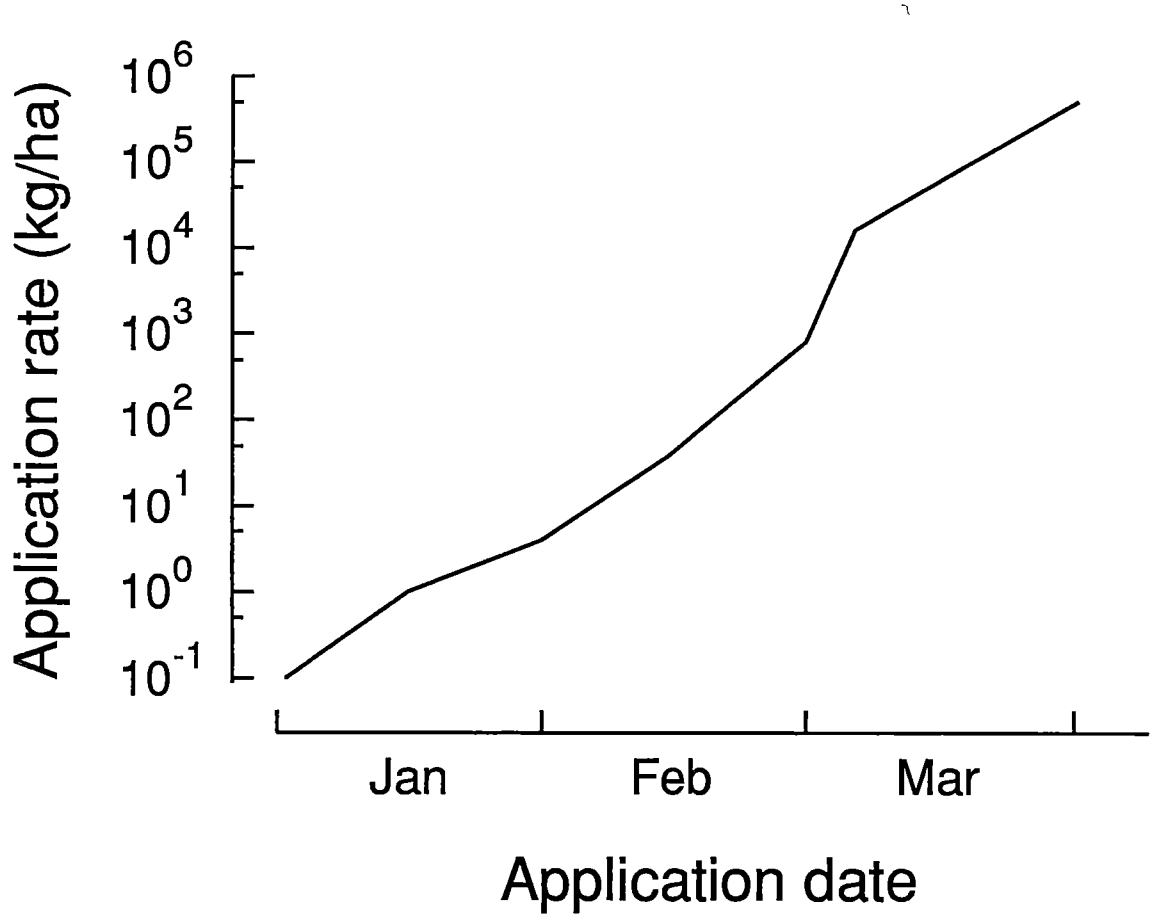

Figure 9.4 **Model prediction of the *M. anisopliae* DAT F-001 application rates (10% a.i.) and dates required to achieve 50% larval mortality by mid autumn (30 April).**

Predictions of effective autumn application rates

Application of *M. anisopliae* at rates of 10^{12} to 10^{14} spores/ha during autumn (March to May) was predicted to reduce the larval population by 50% after the autumn/early winter damage had occurred (Fig. 9.5). However, these rates would reduce the population over the year. Application of the fungus from mid-winter (July) to spring (Sept. - Nov.) would have minimal impact on the larval population at any of the rates.

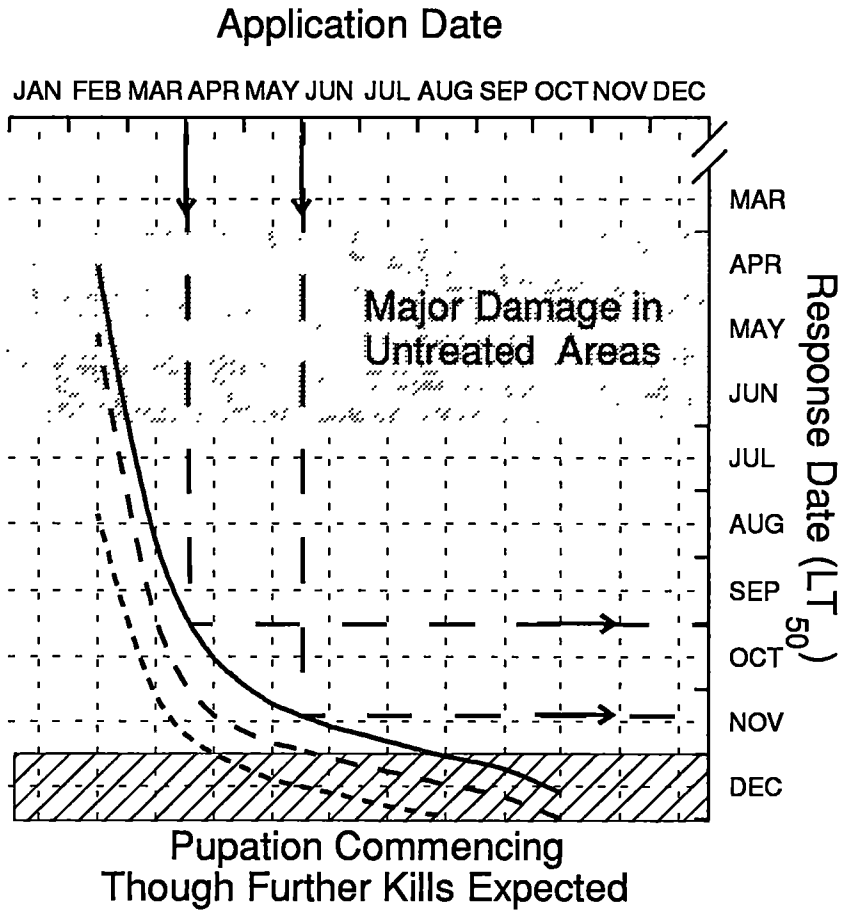

Figure 9.5 **Model predictions of the LT_{50} following application of *M. anisopliae* at (———) 10^{14} spores/ha, (— — —) 10^{13} spores/ha and (– – –) 10^{12} spores/ha.**

9.4 DISCUSSION

This model was very accurate in the prediction of the LT_5 and the LT_{50} in the field experiments where *M. anisopliae* had been applied in autumn, but when the fungus was applied in mid-winter (Fig. 9.3) the predictions preceded the actual mortality dates. This inaccuracy was probably due to the movements of the larvae which were not incorporated into the model. Larval mobility was reduced at the low temperatures (5° - 8°C) which prevailed when *M. anisopliae* was applied at the 'Inverell' field site (the effect of temperature was previously discussed in Chapter 6). As a consequence of reduced larval movement, the larvae would have taken longer to encounter a lethal dose of the fungus. This 'encounter' factor could be incorporated into the model as a multiplier which varied depending on the temperature which prevailed at application.

Prediction of the application rate required to kill 50% of larvae by April 30, indicated that very low rates (0.1 - 10 kg/ha) could be used in mid-summer. However, the pasture soil in mid-summer would be too dry to effectively drill *M. anisopliae* to a depth of 20 - 25 mm and the larvae would generally be below the driest topsoil (below ~ 80 mm). Consequently application at that time would not be as effective as the model predicted (unless the fungus was ploughed into the soil, though ploughing would kill the larvae anyway). However, these predictions suggested that the fungus, if applied in late spring when the soil was moist and adults were ovipositing, could reduce the population before April 30.

The modelling indicated that autumn application of *M. anisopliae* at rates as low as 1 kg/ha (10^{12} spores/ha) would have reduced the larval population by 50% by December (Fig. 9.5). Consequently, application rates 100 x lower than those used previously (Chapter 8) could be effective in the medium to longer-term control of *A. couloni.*

The predictions of this model are examined in the next chapter.

Chapter 10

10.0 FIELD EFFICACY (2)

10.1 GENERAL INTRODUCTION

The efficacy of *M. anisopliae* DAT F-001 at application rates ranging from 64 to 209 kg/ha (~ 10% *M. anisopliae* spores; 3.3 - 7.0 x 10^4 spores/g soil) was demonstrated in field experiments established in 1989 (Chapter 8). The timing of application was the major factor in determining the population reduction by the end of the L3 larval life stage. Application rate was less important than timing in these experiments. *M. anisopliae* DAT F-001 killed 55-84% of L3 larvae when the fungus was applied in autumn, while fewer larvae were killed when the fungus was applied in winter (30%) or spring (0%). In these later experiments, many larvae were infected and subsequently succumbed to the fungus during the pupal stage resulting in a cumulative population mortality of 69% and 46% respectively. These two later experiments continued to be studied in 1990 and 1991 (section 8.5) and in both experiments the overlapping population of larvae and the next generation of the primary population were reduced by 60-70%. The reduction in subsequent populations was also shown in the earlier experiments (section 8.3 and 8.4), and consequently, the longer-term control of *A. couloni* looks feasible.

Two major concerns still remain which effect the commercial viability of *M. anisopliae* as a control agent for *A. couloni*: (1) The high cost of a *M. anisopliae* product for use in pastures; and (2) The lack of short-term (2 - 6 wk) effectiveness of the fungus in the year of application.

The high cost of the product (A$46/ha) was briefly discussed by Rath, Pearn and Worladge (1990 - Appendix 7) and more fully outlined in Chapter 2. Their costing was based on an application rate of 25 kg/ha of a material containing approximately 10% *M. anisopliae* spores on a grain substrate (material similar to that used in Chapter 8). This application rate had not been field tested but was considered likely to be effective based on both the efficacy modelling (Chapter 9) and the field data (Chapter 8). The field data had shown that an application rate of 64 kg/ha was as effective as 209 kg/ha in the total reduction of the population (section 8.4). In 1989, the yield of spores in production was only 5 x 10^8 spores/g grain, whereas Rath *et al.* (1990) reported yields of 1 x 10^9 spores/g grain. Consequently, the 64 kg/ha rate used in 1989 would be reduced to 32 kg/ha at this higher yield; a rate very similar to 25 kg/ha.

An increase in the efficiency of spore production or a reduction in application rate could reduce product price. The efficacy modelling suggested that a concentration of 1 x 10^{12} spores/ha (~ 1 kg/ha of fungus on grain) could reduce 50% of an L3 larval population by December if applied in April. This concentration was 100 times lower than that generally used in Chapter 8, and 25 times lower than that costed at A$46/ha by Rath *et al.*(1990).

The second concern was the lack of short-term (2 - 6 weeks) effectiveness of the fungus in the first year. Both the field experiments (Chapter 8) and the efficacy modelling (Chapter 9) had shown that there was no application time when the lower rates discussed above, would be expected to reduce the L3 larval population by 50% while damage was occurring (autumn). *M. anisopliae* product (10% a.i.) rates of 10 to 100 tonnes/ha were predicted (Chapter 9) to be required if 50% reduction in larvae was to be achieved in four to six weeks after autumn application. The use of chemical insecticides as an adjunct to the fungus was examined by Rath and Headlam (Appendix 8) but was found not to increase the mortality rate of L3 larvae.

Early autumn control of L3 larvae of both the primary and overlapping populations (~ 70% reduction in population numbers) was achieved in both the second and third years after application of *M. anisopliae* (Chapter 8). This implied that the fungus was reducing the early instar populations over the summer period. If *M. anisopliae* was applied in November (spring), after adult mating flights and four to five months before damage would be expected, effective population reductions may be achieved.

The experiments described here were established to determine both the effectiveness of both low *M. anisopliae* application rates, and fungal application in November, prior to a major infestation.

10.2 CONTROL OF EARLY INSTAR *ADORYPHORUS COULONI* FOLLOWING LATE SPRING APPLICATION OF *METARHIZIUM ANISOPLIAE*

10.2.1 Introduction

Metarhizium anisopliae DAT F-001 reduced visible pasture damage (Fig. 8.14) caused by *Adoryphorus couloni* because the number of insects reaching the L3 stage in autumn was reduced by 70% (section 8.5.3). This control was only achieved in the second and third years following application to pastures infested with L3 larvae (section 8.5). While the L3 larval population could be reduced by 84% in the year of application, no short-term (2 - 6 weeks) control was achieved, and consequently pasture damage still occurred in that year (Chapter 8).

These experiments test the hypothesis that application of *M. anisopliae* to pastures after the mating flights can reduce the L3 larval population by early autumn.

10.2.2 Materials and Methods

Five experimental paddocks were chosen on three adjoining farms following preliminary sampling of pastures for adult females in September and October 1990. The farms were in the York Plains region of Tasmania and centered around the property 'Inverell' (discussed in section 8.5). Each paddock was approximately 10 ha in size. The site characteristics for 'Inverell' are reported in Table 8.7. An Environdata® computerized weather station was located at 'Inverell' and the daily rainfall and the mean soil temperature (5 cm below the soil surface) was recorded.

Each paddock was divided into 10 m wide strips which ran the length of the paddock. One of three treatments were randomly assigned to each strip: no treatment (equipment check), a drilled non-dosed treatment (untreated) and a drilled *M. anisopliae* treatment (treated) (Table 10.1). Four experimental blocks were established on areas of each paddock where the numbers of female beetles were both high and consistent in density (Table 10.1). Each block consisted of paired *M. anisopliae* treated and untreated plots (25 m x 10 m) identical to those reported in Chapter 8 and was analysed as described previously (page 8-3). Each paddock was established within the first two weeks

Table 10.1 **Site, application rate of *M. anisopliae* and density of adult females (after mating flights) at each of five experimental field sites at establishment in November 1990.**

experiment	site	application rate* (per ha)	Adult females/m^2		P value
			untreated	*M. anisopliae* treated	
1	Inverell	100 kg	49.8	47.3	0.32
2	Inverell	25 kg	40.5	42.8	0.38
3	Inverell	10 kg	38.0	42.0	0.28
4	Rockville	25 kg	39.3	44.8	0.13
5	Headlam	25 kg	31.3	33.8	0.31

* 1 kg contains approximately 10^{12} *M. anisopliae* spores

of November 1990 using one of three *M. anisopliae* rates (10, 25 or 100 kg/ha; Table 10.1).

M. anisopliae DAT F-001 was produced on a grain substrate as described in section 8.2, except that 2 kg kibbled barley (*Hordeum vulgare*) (rather than Japanese millet) was mixed with 1.2 L tap water in a plastic autoclave bag (50 x 100 cm) (rather than a steel drum). Each bag was sealed except for a 50 mm diameter polypropylene pipe (75 mm long) which contained a steristopper® (Fig. 10.1). Unlike the drums described previously (Chapter 8), the bags were laid horizontally and left to develop a thick mycelial mat for 10-11 days prior to breaking-up the mat (Fig. 10.2). The steristopper® was removed after that to allow additional air flow which enhanced sporulation. Incubation continued for an additional 10 days. Over a four month period 1500 kg of *M. anisopliae* inoculant was produced in this manner. All bags were stored at 10°C prior to use and no bag was over three months old when used.

The variation over time, of the concentration of *M. anisopliae* DAT F-001 in the soil, was assessed (using the methods described in Chapter 8) at all three 25 kg/ha experiments (expt. 2, 4 and 5).

The numbers of non-target invertebrates in each 1.0 x 0.2 x 0.3 m sample was recorded at the last sampling occasion at the 'Inverell' 25 kg (expt. 2) and 'Rockville' 25 kg (expt. 4) experiments (October, 1991).

All other materials and methods were as described in Chapter 8 (section 8.2).

Figure 10.1 ***Metarhizium anisopliae*** **DAT F-001 growing in plastic bags at 20°C using the methods described in the text.**

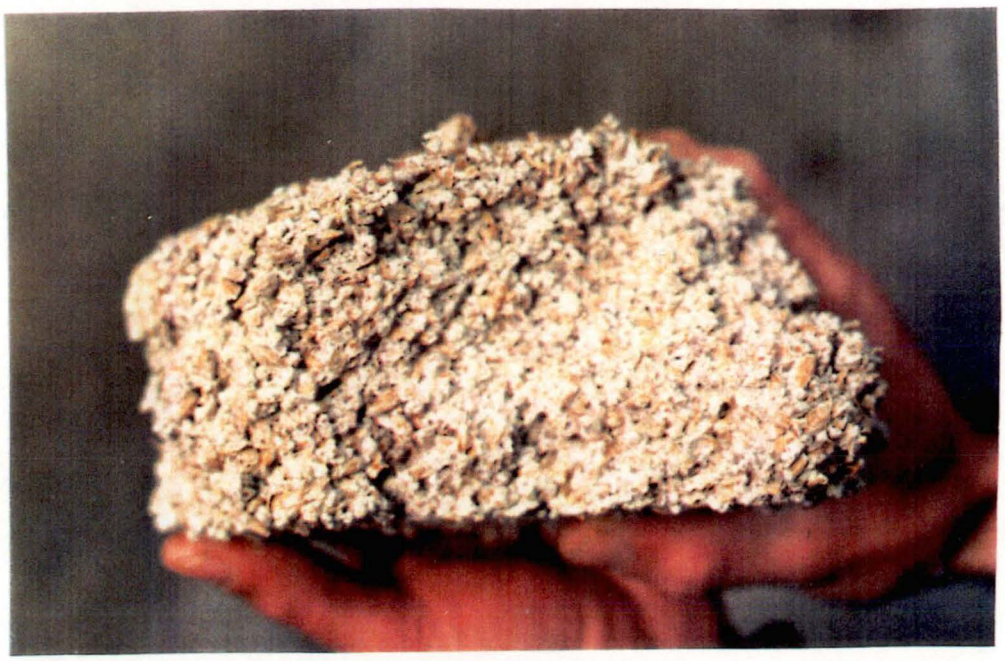

Figure 10.2 ***Metarhizium*** **'cake', a thick mycelial matting of kibbled barley. This mat is the result of 10 days incubation at 20°C, after which it is broken-up (still in the autoclave bag) and left an additional 10 days to sporulate.**

10.2.3 Results

Experiment 1: 'Inverell' 100 kg

There were 20% fewer larvae in the *M. anisopliae* treated plots than in the untreated plots in autumn (April) (Table 10.2). The treated population declined more rapidly than the untreated population for the rest of the year, and by December there was a 52% difference between populations. The larval population was slow to develop: There were only 22% L3 larvae by April 24, and this had increased to only 67% by December 1 (compare with Fig. 1.1).

Experiment 2: 'Inverell' 25 kg

There were no differences in the numbers of larvae in the treated or untreated plots until winter (June) when there were 18% less larvae in the *M. anisopliae* treated plots (Table 10.3). The numbers of larvae declined markedly in all plots from June to October but the percentage difference between the treated and untreated plots remained at 15 - 18%. The larval population was slow to develop as there were 61% L3 larvae in the plots by May and only 77% by October (compare with Fig. 1.1).

Experiment 3: 'Inverell' 10 kg

This site was sampled in both June when there were 13% more (P=0.24) larvae in the *M. anisopliae* treated plots (750 ± 47/m^2) than in the untreated plots (666 ± 46/m^2), and in November when there were 35% fewer (P=0.016) larvae in these treated plots (321 ± 37) than in the untreated plots (493 ± 61/m^2). Larval development was 'normal' as there were 97% L3 larvae in the plots in June (compare with Fig. 1.1).

Experiment 4: 'Rockville' 25 kg

There was no difference between the *M. anisopliae* treated and the untreated plots in either April or May. The numbers of larvae in the untreated plots remained the same at each sample date and by October, there were 16% fewer larvae in the *M. anisopliae* treated plots (Table 10.4).

Table 10.2 **Effect of *M. anisopliae* DAT F-001 applied at a rate of 100 kg/ha in November 1990 on L2 and L3 larvae of *A. couloni* at 'Inverell' during 1991.**

sampling date (1991)	larvae/m^2*		% mortality	P value	instar ratio (L1:L2:L3)
	untreated	*M. anisopliae* treated			
March 25	512 ± 63	410 ± 91	20	0.14	2:93:5
April 29	479 ± 58	377 ± 48	21	0.0085	0:76:22
June 3	521 ± 43	391 ± 41	25	0.0031	0:52:48
August 9	447 ± 49	273 ± 44	39	0.0391	0:52:48
December 1	235 ± 30	114 ± 23	52	0.0055	0:33:67

* mean ± SE, n=4

Table 10.3 **Effect of *M. anisopliae* DAT F-001 applied at a rate of 25 kg/ha in November 1990 on L2 and L3 larvae of *A. couloni* at 'Inverell' during 1991.**

sampling date (1991)	larvae/m^2*		% mortality	P value	instar ratio (L1:L2:L3)
	untreated	*M. anisopliae* treated			
March 27	1257 ± 104	1217 ± 146	3	0.39	1:80:19
May 3	1157 ± 39	1070 ± 86	8	0.16	0:39:61
June 5	1007 ± 30	828 ± 23	18	0.0029	0:47:53
August 1	713 ± 42	609 ± 33	15	0.051	0:23:77
October 14	433 ± 79	364 ± 73	16	0.022	0:23:77

* mean ± SE, n=4

Table 10.4 **Effect of *M. anisopliae* DAT F-001 applied at a rate of 25 kg/ha in November 1990 on L2 and L3 larvae of *A. couloni* at 'Rockville' during 1991.**

sampling date (1991)	larvae/m^{2*} untreated	*M. anisopliae* treated	% mortality	P value	instar ratio (L1:L2:L3)
April 3	768 ± 31	677 ± 70	12	0.068	0:84:16
May 8	773 ± 97	710 ± 77	8	0.26	0:30:70
October 28	663 ± 48	553 ± 58	16	0.039	0:18:82

* mean ± SE, n=4

Experiment 5 'Headlam' 25 kg

This site was sampled in both July, when there were 20% more (P=0.041) larvae in the *M. anisopliae* treated plots (672 ± 100/m^2) than in the untreated plots (559 ± 119/m^2), and November, when there were 3% fewer (P>0.05) larvae in the *M. anisopliae* treated plots (492 ± 32/m^2) than in the untreated plots (506 ± 46/m^2). Only 40% of the larval population were at the L3 stage at each sample date.

Field Mortality Model (2) (Chapter 9)

While the model was only designed to predict the mortality of L3 larval caused by *M. anisopliae*, the high summer temperatures should have allowed the fungus to kill most larvae over January and February. This did not happen, and even when the populations were modelled from March onwards, the model proved inaccurate.

Environmental conditions

The period from application (November 1990) to April (1991) was characterized by warm temperatures (Fig. 10.3) with below average rainfall (Fig. 10.4). The period from February to November was well below the expected rainfall except

in late-winter (August). Previous data (Fig. 8.16) indicated that this site had below average rainfall for 18 months prior to the establishment of these experiments.

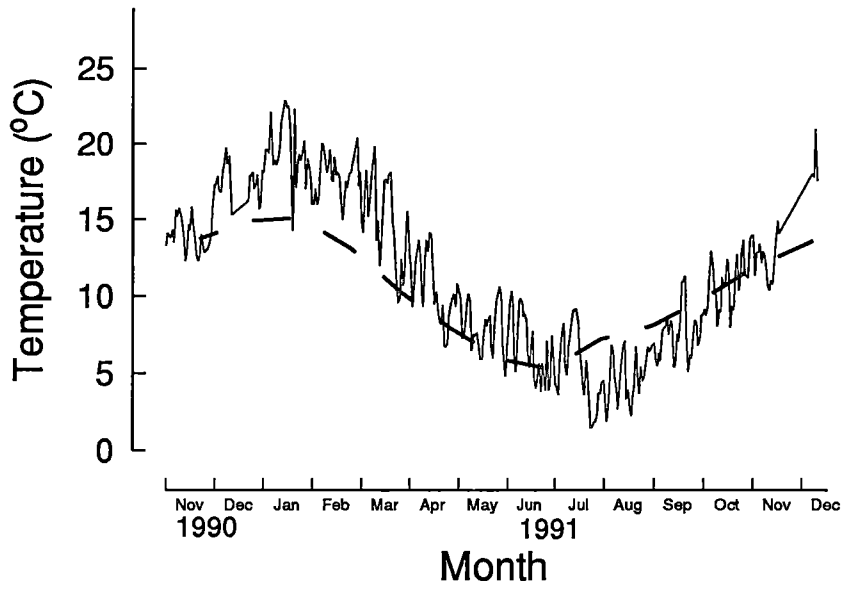

Figure 10.3 **Mean daily soil temperature (——) on 'Inverell' at a depth of 50 mm in comparison with the long-term (1884-1984) average air temperature (— —) for the Oatlands recording station (air temperature data from Davies 1988).**

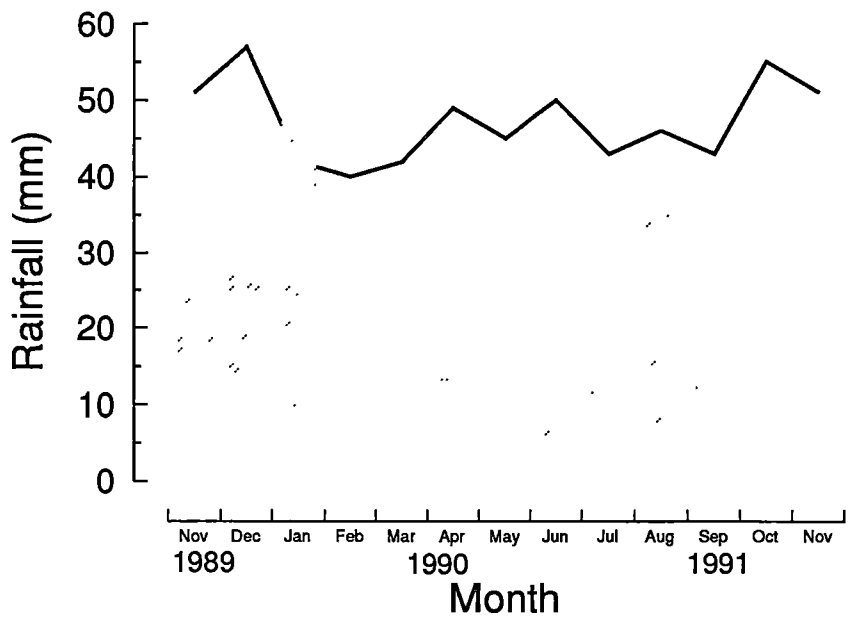

Figure 10.4 **Total monthly rainfall (shaded) at 'Inverell' in comparison with the longer-term (1884-1994) average rainfall (——) at the Oatlands recording station (Oatlands data from Davies 1988).**

M. anisopliae concentration in the soil

There was considerable fluctuation in the concentrations of *M. anisopliae* in the soils, probably as a result of the low application rate. The concentrations shown here (Fig. 10.5) equate to only a few colonies on the selective agar plates and a few extra colonies on the agar result in a large change in the CFU count. In general, the concentration of *M. anisopliae* remains close to the applied rate, though there may be a decline at 'Headlam' (expt 5) (Fig. 10.5). *M. anisopliae* was not recorded in any control plots.

Effect on non-target organisms

There were no differences (P>0.05) in the densities of a range of non-target invertebrates at either of the two sites ('Inverell' 25 kg, expt 2, Table 10.5; 'Rockville' 25 kg, expt 4, Table 10.6) examined at the last sampling occasion in October 1991.

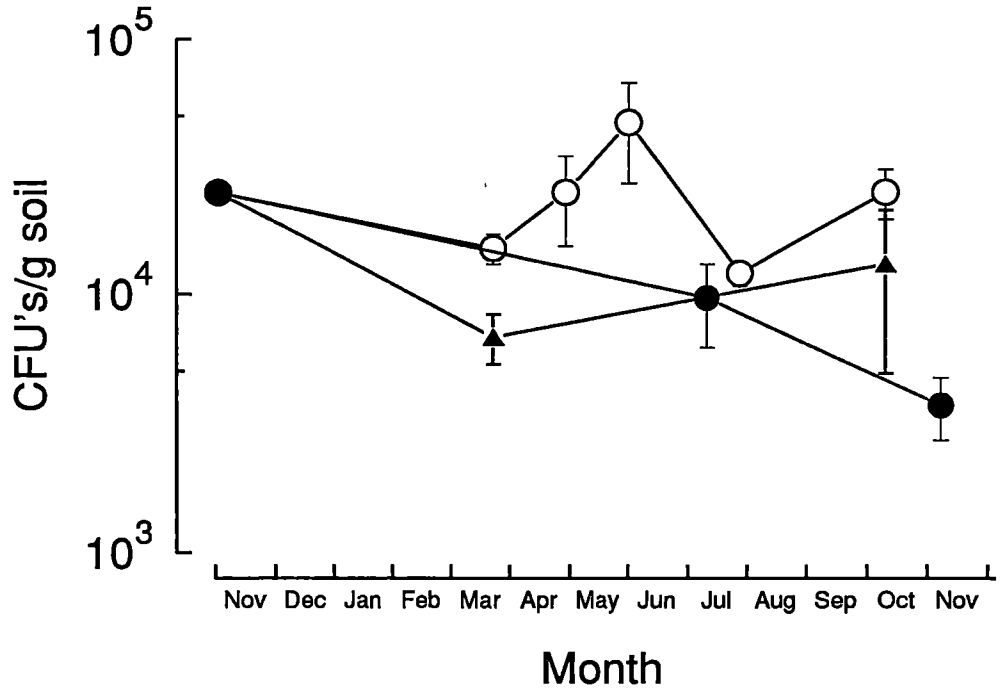

Figure 10.5 **The concentration of *M. anisopliae* DAT F-001 in soil over 12 months, following drill application of 2.5 x 10^{13} spores/ha (25 kg/ha) into the treatment plots at (○) 'Inverell' (expt 2), (▲) 'Rockville' (expt 4) and (●) 'Headlam' (expt 5) in November 1990.**

Table 10.5 **Effect of *M. anisopliae* DAT F-001 on the density of non-target organisms in the plots at 'Inverell' 25 kg (expt 2) on 15/10/91, 11 months after application.**

Organism	Density/m^{2*}	
	untreated	*M. anisopliae* treatment
Diptera		
larva A	1.3	2.5
larva B	2.5	2.3
Lepidoptera		
Oncopera	0.3	0.3
Mythimna	0.0	1.0
Coleoptera		
Aphodius	9.3	4.0
Sericesthis	11.5	5.0
Onthophagus	0.0	0.3
Elateridae	5.8	6.5
other spp.	0.8	1.0
Annelids		
earthworm A	4.3	1.0
earthworm B	2.8	0.5

* all results not significantly different (P>0.05)

Table 10.6 **Effect of *M. anisopliae* DAT F-001 on the density of non-target organisms in the plots at 'Rockville' 25 kg (expt 4) on 28/10/91, 11 months after application.**

Organism	Density/m^2 untreated	*M. anisopliae* treatment
Diptera		
larva A	3.3	2.8
larva B	2.8	2.5
Lepidoptera		
Oncopera	5.0	3.8
Mythimna	1.8	1.3
Coleoptera		
Aphodius	0.8	1.5
Sericesthis	7.3	5.5
Onthophagus	0.0	0.0
Elateridae	4.0	5.8
other	1.3	1.3
Annelids		
earthworm A	0.0	0.0
earthworm B	0.8	2.3

* all results not significantly different (P>0.05)

10.2.4 Discussion

These experiments have shown that *M. anisopliae* DAT F-001 could not reduce populations of early instar larvae over the summer (1990-1991) period. Only experiment 1, which used an application rate of *M. anisopliae* of 100 kg/ha had lower levels of larvae in the treated plots in early autumn. Even at this high rate, the reduction in larvae was only 20% and consequently, there were large

populations of larvae (>300/m^2) which could damage pastures throughout the autumn and winter of 1991.

The effectiveness of the 100 kg/ha rate (~ 10^{14} spores/ha) in one year was shown by the 52% reduction in larval numbers by December 1991, though this level of mortality was lower than shown in earlier experiments where *M. anisopliae* was applied in autumn (Chapter 8).

By mid to late spring, the 25 kg/ha experiments had only reduced the larval populations by 15 - 20% while by late spring the 10 kg/ha experiment had reduced the population by at least 26% . These differences were perhaps due to larval activity. High levels of larval activity would have increased the probability of larvae encountering a dose of *M. anisopliae* in the soil which was likely to cause death. The larvae at the 10 kg site were all L3 larvae by June while at the 25 kg sites there were large numbers of L2 larvae still present in October-November, suggesting greater activity in the 10 kg experiment.

The relationship between larval activity and mortality in relation to temperature was discussed in Chapter 6. However, differences in instar development rates in these experiments would not be a result of the temperature effect on larval activity as the temperature at all sites would have been very similar to that recorded (Fig. 10.3). Instead, the effect of limited moisture creating hard impenetrable soils in the 25 kg/ha experiments was perhaps the cause. The 10 kg/ha site was a lighter textured soil and even when very dry, would have enabled greater larval movement than the other sites which had heavier textured soils.

The lack of larval mortality over the period from application (late spring) to autumn was probably due to a combination of moisture and fungus placement. It is well established that scarab larvae move vertically in response to soil moisture (references cited by McQuillan, 1990). In preliminary sampling during summer (January and February) it was noted that the L1 larvae were below the hardened top soil (0 ~ 6 cm). The fungus was drilled into the ground at a depth of 2 - 2.5 cm at all sites and consequently the larvae would encounter little *M. anisopliae* until the autumn rains moistened the top soil. The differences in vertical positioning of the fungus and the larvae suggest that reductions in L1 larvae in summer is only likely to occur in years with above average rainfall. The movement of larvae away from the upper layers of soil which contain the

pathogen and towards deeper soil layers, as a response to soil moisture, has also been found to limit control of the European chafer, *Rhizotrogus majalis*, by the bacterium *Bacillus popilliae* in the U.S.A. (Klein, 1981).

The results reported here contrast markedly with the situation in an on-going field experiment being conducted in an adjoining paddock at 'Inverell'. That experiment (section 8.5) had been established in July 1989, and the numbers of female beetles laying in the *M. anisopliae* treated and untreated plots over October and November of 1990 were identical at 37/m^2. However, by March 20 (1991), there were 881 larvae/m^2 in the untreated plots and 325 larvae/m^2 in the treated plots, a difference of 63%.

Several factors were probably responsible for the apparent differences in efficacy. The concentration of *M. anisopliae* in the soil was one factor as the concentration of *M. anisopliae* CFU's, in November 1990, in the early 'Inverell' (1989) experiment was nearly 10 times the concentration in the 25 kg experiment (expt 2). Additionally, in the 100 kg experiment (expt 1) the population had been reduced by 20% by March 25 whereas the 25 kg experiment (expt 2) had only reduced the population by 3%. Both these later experiments were conducted in paddocks next to the 'Inverell' (1989) experiment.

The fungus distribution, both horizontally and vertically, could have been another factor. In the earlier experiment ('Inverell', 1989), the fungus distribution could be more uniform than that applied initially by the drill (20 - 25 mm deep, 155 mm apart) due to both the vertical movement of *M. anisopliae* spores through leaching (this has been studied with *Nomuraea rileyi* by Ignoffo *et al.* (1977) and with *Beauveria bassiana* by Storey and Gardner, 1986), and the horizontal and vertical transportation by insects. Transportation would be achieved by the spores adhering to, and dislodging from, the cuticle of many invertebrates including the host insect. The transportation of *M. anisopliae* by non-target invertebrates has been shown by Schabel (1982), Zimmermann and Brode (1983) and Balazy *et al.* (1987). The densities of insects recorded as non-target invertebrates in Tables 10.5 and 10.6 were not affected by *M. anisopliae* DAT F-001 and could easily have aided the distribution of the fungus.

Larvae of *A. couloni* transported *M. anisopliae* vertically. The L3 larvae in the early 'Inverell' (1989) experiment migrated to a depth of 200 - 300 mm before

pupation (section 8.5). The maximum mummification and the major reduction in the treated population occurred as pupae at those depths.

A third factor could have been the exposure of adults to *M. anisopliae*. Ovipositing females had been exposed to the fungus in the earlier experiment (section 8.5) from the moment they entered the treatment plots (and perhaps before if treatment adults re-entered the soil where they left it), whereas in these experiments (expt 1 to 5) the fungus was only applied late in the egg laying period. This would limit the exposure of these adults to the fungus. This finding was supported by laboratory studies (Chapters 4 and 5) which have shown that egg production could be reduced if adults became infected with *M. anisopliae*. Consequently, there could have been significant differences in the density of eggs between treatment and control plots in November 1990 in the earlier experiment ('Inverell', 1989) but not in these experiments (expt 1 to 5).

Even after the summer period, these experiments failed to show that *M. anisopliae* could reduce the population over the larval life stages. Only the 100 kg experiment (expt 1) resulted in greater than 50% reduction in larval numbers. All other application rates resulted in 15 - 16% population reductions. The failure of the 25 kg (expt 2, 4 and 5) and the 10 kg (expt 3) experiments could be solely due to these low application rates being ineffective. However, the rainfall over the autumn-spring period was in deficit at these sites and the resultant lack of moisture probably inhibited larval activity (as discussed above) resulting in reduced exposure to the fungus, or may have had a direct effect on fungal infectivity.

These experiments pose the question as to what will happen to the population in subsequent years. The earlier 'Inverell' experiment ('Inverell' 1989, section 8.5) had only reduced the L3 population by 30% by December 1989 (when applied in July) and there was no reduction by that time when the fungus was applied in September. In both cases, however, there was a major reduction in adult survival the next year (69% and 46% respectively) and a 70% reduction in larval survival the year after. This suggests that *M. anisopliae* is likely to further reduce the 1991/92 *A. couloni* population present in these experiments through 1992, perhaps leading to the longer-term control seen in the earlier experiment. As a result, the extent of control given by application rates of 25 kg/ha and 10 kg/ha is not likely to be known until April or May 1992 when all the larvae have pupated into adults. The long-term effectiveness (3 - 6 years) of these rates may be similar, suggesting that even lower rates could be used. These experiments will continue to be monitored in 1992 and 1993.

10.3 FIELD EFFICACY OF *METARHIZIUM ANISOPLIAE* DAT F-001 AT 1, 10 AND 25 KG/HA

10.3.1 Introduction

M. anisopliae DAT F-001 used at 100 kg/ha (10% a.i.; ~10^{14} spores/ha) reduced larval populations of *A. couloni* both in the year of application and for the next two years. However, there was no reduction in visible pasture damage in the autumn of the year of application because the larval numbers were not reduced quickly enough (Chapter 8). Application of *M. anisopliae* in late spring, prior to the development of large populations of L3 larvae, only reduced the numbers by 20% in the autumn (section 10.2).

Consequently, there is no prospect of adequate larval control (giving pasture savings in autumn) in that first year and as a result, the control of *A. couloni* with *M. anisopliae* relies on the longer-term reduction of *A. couloni* (Chapter 8). However, at an application rate of 100 kg/ha, *M. anisopliae* would be too expensive (at least A$184, Appendix 7) for use in a low value crop such as pasture.

Reduction in application rates would reduce the cost of a *M. anisopliae* product and would possibly be as efficacious as the 100 kg/ha rate if 30 - 50% of larvae were killed in the first year. The 'Lester' experiment (described in Chapter 8) showed that late applications of the fungus only killed 0 - 30% of larvae but reduced the adult population by 46 - 69% the following year. Further, the larval population in both the second and third years were reduced before autumn damage occurred. Lower rates, applied earlier, may be as effective as the 100 kg/ha rate.

Efficacy modelling (Chapter 9) predicted that rates as low as 1 kg/ha, applied in autumn, would reduce the larval population by 50%. This experiment examines the field efficacy of *M. anisopliae* DAT F-001 applied at 1, 10 and 25 kg/ha in autumn, over the larval life-stage.

10.3.2 Materials and Methods

This experiment was conducted at 'Inverell'. The plots were located 400 m from the experiment discussed in section 8.5 and the site characteristics are similar to those described in Table 8.7 (section 8.5).

The plots were established using similar equipment and experimental designs as described for previous field experiments (Chapters 8 and 10 - section 10.2). Each of the four experimental blocks compared three *M. anisopliae* DAT F-001 application rates with a zero concentration (untreated) control (Table 10.7). Treatments were randomized within each block though the untreated plot was confined to either of the two inner plots to allow for more efficient pairing of treatment and control samples. There were no significant differences (P=0.53) in the numbers of larvae between plots or blocks at establishment.

All plots were established on 15 April 1991 by direct-drilling mixtures of ryegrass and *M. anisopliae* into uncultivated pasture (Table 10.7). The efficacy model (Chapter 9), predicted that no mortality due to the fungus would occur until October. Samples were taken in October, November and December.

Table 10.7 Quantity of ryegrass and *M. anisopliae* DAT F-001 in mixtures drilled into the pasture covered experimental plots on April 1991

	M. anisopliae concentration		Application rates (kg/ha)		
treatment	spores/ha	(spores/g soil) theoretical	*M. anisopliae**	ryegrass	Total
1	0	0	0	18.0	18.0
2	1.0×10^{12}	1.0×10^{3}	1.4	21.2	22.6
3	1.0×10^{13}	1.0×10^{4}	9.8	14.6	25.4
4	2.5×10^{13}	2.5×10^{4}	24.4	0	24.4

* *M. anisopliae* on kibbled barley. These application rates include the grain substrate. The quantity of *M. anisopliae* is approx. 10% of the figure quoted.

10.3.3 Results

Larval mortality

Immediately post-application there were 1249 ± 68 larvae/m^2 in both the treated and untreated plots (P>0.05). Of these, 32% were third instar. The number of larvae in the untreated plots declined to 559 larvae/m^2 in October and 457 in December (Table 10.8). At the highest concentration (2.5 x 10^{13} spores/ha; 25 kg/ha) the treated population declined more rapidly than the untreated population, resulting in a 35% difference (P=0.0025) by December 1 (Table 10.8). At 10 kg/ha (1.0 x 10^{13} spores/ha) larval survival was reduced by 16% The larval survival at the lowest concentration was not different from the survival in the untreated plots (Table 10.8). The level of mummified larval cadavers increased with both *M. anisopliae* application rate and sample time, reaching a maximum of 46/m^2 (Table 10.8). In December, 18% of the population (treated and untreated) were still L2 larvae and there were no prepupae (compare with Fig. 1.1).

Field Mortality Model (2)

The model accurately predicted an 8% difference in the high concentration treatment in October but in all other cases the actual field mortality was much lower than that predicted (Table 10.8).

Environmental Conditions

The soil temperatures from this site were little different from the long-term average air temperatures (Fig. 10.4). The higher soil temperature over summer was typical of that found in other experiments (Figs. 8.6, 8.10 and 8.19).

This site had a serious rainfall deficiency over the autumn period (Fig. 10.5). Monthly rainfall did not reach the long-term average rainfall over the course of the experiment. By December, there had been only 208 mm of rainfall over the previous nine months while the 100-year average (1884 - 1984) for the same period was 424 mm. This site had below average rainfall for the two previous years (Fig 8.14).

M. anisopliae concentration in the soil

The concentration of *M. anisopliae* DAT F-001 in the top 10 cm of pasture soil after seven months (1 Dec) was close to the applied concentration (15 April) (Table 10.9). The initial recovery of DAT F-001 from the 10^4 treatment was only 38% of the concentration that was theoretically applied. This increased to 76% at seven months (Table 10.9). No *M. anisopliae* was recovered from the untreated plots.

Table 10.8 Effect of three concentrations of *M. anisopliae* DAT F-001 applied in April 1991 on L2 and L3 larvae of *A. couloni* at 'Inverell'.

sampling date (1991)	application rate (kg/ha)	larvae/m^2*	% mortality: predicted†	% mortality: actual	mummified cadavers/m^2
Oct 2	1	n.r.	1		
	10	n.r.	4		
	25	507 ± 50^a	8	9.5	17
	(untreated)	$(559 \pm 42)^a$			(0)
Nov. 5	1	568 ± 45^a	9	0	1.3
	10	484 ± 37^a	42	13.4	18
	25	466 ± 21^a	65	16.6	17
	(untreated)	$(558 \pm 50)^a$			(0)
Dec. 1	1	527 ± 35^a	39	0	6
	10	381 ± 15^b	88	16.6	27
	25	295 ± 39^c	95	35.4	46
	(untreated)	$(457 \pm 40)^a$			(1.3)

* mean ± SE, n=4. Within a sample date, values followed by the same letter do not differ significantly (P>0.05).

† Model developed in Chapter 9.

n.r. = not recorded

Table 10.9 The concentration of *M. anisopliae* DAT F-001 in soil over 7 months at 'Inverell' in 1991.

application rate (kg/ha)	applied cfu/g soil	recovered *M. anisopliae* (cfu/g soil)*	
		15 April	1 December
0	0	n.r.	n.r.
1	1.0×10^3	n.r.	$2.5 \pm 0.9 \times 10^3$
10	1.0×10^4	$3.8 \pm 1.3 \times 10^3$	$7.6 \pm 1.0 \times 10^3$
25	2.5×10^4	$2.6 \pm 1.0 \times 10^4$	$1.9 \pm 0.6 \times 10^4$

* mean ± SE, n=4.

n.r. = not recoverable

10.3.4 Discussion

This experiment has shown that an *M. anisopliae* concentration of 2.5×10^{13} spores/ha could reduce an L2/L3 population of *A. couloni* by 35% in the year of application. Lower concentrations were less effective with a concentration of 1×10^{12} spores/ha producing few diseased larvae and no reduction in larval numbers. The revised field mortality model (Chapter 9) was inaccurate in its prediction of mortality events but like the initial model (Chapter 7), was reasonably accurate in predicting the long lag time before the onset of fungal-induced mortality.

The 'Warringa' experiment established in 1989 (section 8.4) included a concentration of $3.3 \pm 0.8 \times 10^4$ spores/g soil, which was similar to the highest concentration studied here of $2.6 \pm 1.0 \times 10^4$ spores/g soil (25 kg/ha; 2.5×10^{13} spores/ha). Additionally, the 'Warringa' experiment was established at a similar time (24 April 1989, compared with 15 April, 1991). However, the mortality recorded in this experiment (35% by early December) was much lower than that recorded at 'Warringa' (74% by late November).

Weather conditions may have been important in limiting the efficacy of *M. anisopliae*. At 'Warringa' in 1989, there was 318 mm of rainfall from May to November, with an average temperature of 9.3°C. In this current experiment there was only 161 mm of rainfall at an average temperature of 7.9°C. This lack of rainfall at 'Inverell' had prevailed for a number of years (Fig 8.14; section 8.5). The 100-year average at the Oatlands recording station is 560 mm/year (Davies, 1988), yet over the 29 months which experiments were conducted at 'Inverell' the rainfall totalled only 736 mm (approximately 54% of the expected rainfall).

The low rainfall had little impact on fungal survival (Table 10.8), instead it appeared to have a negative effect on larval development. At 'Warringa' in April 1989, 25% of larvae were L2 while in this experiment, 68% were L2. By early June 1989, only 6.5% of larvae were still L2 at 'Warringa' while there were still 18% L2 larvae at 'Inverell' in December, 1991. The developmental rates of larvae from L2 to L3 at 'Warringa' was typical of that described by McQuillan and Ireson (1987)(Fig. 1.1) while at 'Inverell' larval development was retarded. The retardation of larval development was probably due to the dry hard soils limiting larval movement and consequently, larval feeding (McQuillan, 1990). This decline in larval development rate was also seen in the late-spring experiments discussed earlier (section 10.2). Lessened larval activity could mean reduced exposure to the fungus and subsequently less infection. However, lessened activity probably also meant reduced pasture damage leading to the hypothesis that in 'normal' environmental conditions the fungus would control the pest (as shown in Chapter 8) while in drought conditions the weather would determine the extent of the pest damage.

The lower temperatures prevailing at 'Inverell' in 1991 should not have had any major impact on efficacy as the reduced temperatures were incorporated into the field efficacy model (Chapter 9). From this experiment it was clear that soil moisture may need to be incorporated into the model. Recent laboratory data of Pung and Rath (unpublished) has shown that as sand-peat moisture was reduced from 25% to 8% the LT_{50} increased from 46 to 63 days (4.5×10^4 spores/g sand-peat; 20°C). When this data was examined using the model developed in Chapter 9, the LT_{50} at the low moisture was equivalent to a concentration 100 times less than at the higher moisture (ie. 4.5×10^2 spores/g sand-peat).

10.4 GENERAL DISCUSSION

These experiments show that broad-acre application of *M. anisopliae* can be practically achieved using conventional farm machinery. Both investigations reported here (sections 10.2 and 10.3) failed to adequately address the question of the efficacy of rates lower than 100 kg/ha. The 10 kg experiment at 'Inverell' (section 10.2) was more effective in reducing the larval population than any of the three 25 kg experiments, however, comparison of these experiments is confounded by many factors including location and soil texture. Even so, the suggestion remains that the performance of *M. anisopliae* in 1991 was sub-optimal and perhaps the worst that can be expected.

This data highlights two concerns with the analysis of the effect of *M. anisopliae* on *A. couloni* populations. The first is that some experiments, notably 'Inverell' 25 kg (Table 10.3) and 'Rockville' 25 kg (Table 10.4), were last recorded in October 1991 while some other sites were recorded 4-6 weeks later. High *A. couloni* mortality recorded from the latter sites may have been due to sampling time rather than application rate. This is supported by data from the 'Inverell' 25 kg experiment which at the last sampling (16 October) recorded only a 16% reduction in larval numbers in the treated plots. Block 4 from this site was sampled again on December 11 and recorded a 50% difference between treatment and control whereas at the October sample the difference was only 6%. This suggests that the experiment may have been more effective than reported here.

The second concern relates to the interpretation of the mortality data. The %mortality has been quoted as the difference between the treated and control populations at each sample time. However, the control populations also decline through the year and as reported in these experiments this reduction may be as high as 60 -70% (Table 10.3). Consequently, some researchers may wish to reinterpret this data to determine the impact of *M. anisopliae* as one of a series of mortality factors. In this case, instead of a 52% difference between treated and control populations at the 'Inverell' 100 kg experiment, *M. anisopliae* would only account for 24% of the total population decline in the treated plots since application (Table 10.2). The field data in both this chapter and Chapter 8 has been presented in such a way to enable either interpretation.

Both concerns mentioned above are probably minor as data presented in this thesis shows repeatedly that there is little prospect for control of the pest in the year of application, while control in subsequent years reduces the larval populations before April/May when the major damage would normally occur. At this time it is total numbers that are most important, not percentage reduction. Populations below 100/m^2 cause no damage under any situation, while populations above 300/m^2 are generally required before severe pasture damage occurs (Allen, 1986). Populations of 400 - 500 L2/L3 larvae/m^2 are commonly found in damaged pastures. In such cases the fungus would only be required to reduce the population by 40% for 'control' to be achieved. Earlier experiments (Chapter 8) have shown that populations as high as 900/m^2 or as low as 30/m^2 can be reduced by 60 - 70% before the autumn period when damage is likely.

As previously discussed (sections 10.2.4 and 10.3.4) the effect of the soil environment on the development of *A. couloni* larvae in 1991 was probably the major factor limiting *M. anisopliae* efficacy. In earlier experiments (Chapter 8) the maximum efficacy was not achieved until the second and third year after application and this may be the case in these experiments. However, it appears (P.B. McQuillan, pers. comm, February 1992) that the stressed L3 larval populations failed to pupate over the January - February period and consequently may either die or continue to develop and combine with the smaller overlapping population to pupate in January 1993. Events such as this are probably responsible for the development of equal sized primary and overlapping populations which occur in north-west Tasmania and most of Victoria.

While development of the *A. couloni* populations and the effect of *M. anisopliae* on them will be further monitored in 1992 and 1993, the potential of the fungus as a control agent is still chiefly shown by the longer-term study initiated at 'Inverell' in 1989 (section 8.5). It was suggested, in the light of this data, that the commercial partner market *M. anisopliae* DAT F-001 at a rate of 10^{13} spores/ha. This was a calculated risk deemed necessary because of the projected product cost and the low value of sheep pastures. Registration of the fungus as a control agent is currently proceeding using the data contained in this thesis and it remains to be seen whether the registration authorities accept the efficacy of the 10 kg rate (10^{13} spores/ha). Further trials will need to be undertaken by the commercial partner.

Whatever concentration is used commercially the importance of this control agent is its ability to reduce *A. couloni* populations for many (5 to 10?) years. The 1989 'Inverell' experiment (section 8.5) showed that the timing of population reductions (due to *M. anisopliae*) was appropriate and could result in the lessening of visual pasture damage (Fig. 8.14). The concentration of *M. anisopliae* in the top 10 cm of the soil is the major factor which will determine long-term efficacy. Further experiments to examine the effect of soil type, pH, moisture, temperature, chemicals and other factors relevant to the concentration of *M. anisopliae* recovered from soils have been initiated and should highlight potential problem areas. However, the study on the distribution and abundance of *M. anisopliae* in Tasmanian pastures reported in Chapter 11 suggests that the environmental variables should have little impact on the level of *M. anisopliae* CFU's in the soil, as that study concludes that the host will have the major impact of CFU numbers.

Chapter 11

11.0 THE INFLUENCE OF ABIOTIC FACTORS ON THE DISTRIBUTION AND ABUNDANCE OF *METARHIZIUM ANISOPLIAE* IN TASMANIAN PASTURE SOILS.

11.1 INTRODUCTION

Ecological studies on insect fungal pathogens usually focus on one of two aspects. The first is the environmental and biological factors which affect the insect-host interaction. The second aspect concerns the persistence and stability of pathogens following introduction into an ecosystem. Both types of studies have involved laboratory and field experiments which examine the influence of soil-type, moisture, temperature, light, competition, chemicals, etc (McCoy, Samson and Boucias, 1988; Milner, 1989) and have led to an increased understanding of the factors which govern pathogen efficacy.

The study of insect fungal pathogens has an ultimate aim of releasing large quantities of specific strains into an ecosystem to control an insect pest. In the case of soil-borne insects, a secondary aim has been to enhance the long-term persistence of the fungal agent (McCoy *et al,* 1988; Rath and Yip, 1989, appendix 2; This thesis Chapter 2 and 8). With the exception of two studies (Hokkanen and Zimmermann, 1986; Weiser and Matha, 1986) there has been little attempt to investigate the existing insect-pathogenic or non-pathogenic flora into which these biocontrol agents are being released.

A better understanding of soil ecology is essential to the development of insect pathogens of soil-inhabiting insects (Klein, 1988). Studies of the natural insect pathogenic flora can be used to help assess which isolates are best suited to a particular environment, determine which factors in that environment may be detrimental to the persistence of the introduced pathogen, and provide environmental data required for registration of the pathogen.

As part of the study on *Metarhizium anisopliae* control of the subterranean pasture scarab *Adoryphorus couloni*, the natural *M. anisopliae* flora present in the pasture soils of the Australian island state of Tasmania was investigated. Part of this study resulted in the classification and description of 16 *M. anisopliae* strains (Yip, Rath and Koen, 1992). This chapter examines the distribution and abundance of these strains in pasture soils in relation to the

abiotic factors of soil-type, pH, rainfall/moisture, conductivity, temperature and altitude.

11.2 MATERIALS AND METHODS

Collection of soil samples

Soil samples were taken from all major pastoral localities in Tasmania. Within each locality single samples were taken randomly from pastures. The distribution of soil samples reflects the distribution of the state's 900, 000 ha of sown pasture (Appendix 6). The samples were collected by Dr. H.Y. Yip on five separate occasions from June to November (winter to spring) of 1988. Each of the 419 samples measured 50 x 50 x 100 mm (L x W x D) and weighed approximately 150-200 g (wet weight). Samples were stored at 5°C for a maximum of seven days before plating.

Soil Analysis

Soil samples were pretreated by sieving (2 mm sieve) except in the case of clay soils, which could not be sieved because of the high moisture content. In these clay soils, coarse material such as roots and pebbles were removed by hand.

Abiotic factors

Soil type was classified as sandy loam, loam, clay loam or clay according to the behaviour of the moist bolus as described by McDonald *et al.* (1984).

The pH (1:5 soil:0.01M $CaCl_2$) and conductivity (millisiemens/cm) (1:5 soil:deionised water) of the soil samples were determined. The mean moisture content of each soil sample was calculated after drying three separate 20-25 g quantities of the soil in an oven at 105°C for 12 hours.

Average annual rainfall and altitude were determined for each sample site by comparing its grid reference (1: 25,000 map) with a land system code (Davies, 1988). Tasmania has been divided into seven regions, and each region has been extensively studied and classified into land types (or land systems). This provides broadscale information on soils, vegetation, geology, rainfall and land use (Davies, 1988). Three land system regions were used in this study, Region

3 (Richley, 1978), Region 4 (Pinkard, 1980) and Region 6 (Davies, 1988). Land system numbers encode rainfall (1 - 7 : 375 - 2000 mm) and altitude (1 - 5 : 0 - 1500 m).

Temperature characteristics were studied using the mean summer maximum isotherms of Jackson (1988) and by calculation of mean winter average isotherms from the mean winter minimum isotherms (Jackson, 1988) combined with long term average temperature data from 35 weather recording stations within the study area.

M. anisopliae determination

After moisture determination, moist soil from each sample equivalent to 20 g oven-dried weight was placed in a 500 ml Erlenmyer flask containing 200 ml sterile Ringers solution (Oxoid, BR52). Each flask was shaken on an orbital shaker (150 rpm) for 30 minutes before two serial (1: 9) dilutions in Ringers solution were made. Five replicates of each dilution were spread-plated (0.1 ml) onto selective agar (Milner and Lutton, 1976) and incubated at 20°C for 15 days before examination. The number of colonies of *M. anisopliae* in each plate was recorded and live cultures of the fungi were classified to strains by Dr. H.Y. Yip using the methods of Yip *et al* (1992).

Statistical Analysis

The distribution of *M. anisopliae* was analyzed on a presence/absence basis from the 419 soil samples in conjunction with each of the associated abiotic factors using the Chi-square test of independence procedure in Minitab (Ryan, Joiner and Ryan, 1985). The soil type, rainfall and altitude results were grouped according to the land system codes discussed earlier while pH's were grouped at 0.25 unit intervals and conductivities at 0.025 unit intervals. Initial comparisons of *M. anisopliae* strains used standard regression and ANOVA methods, and where appropriate, LSD (5%) was used to test for specific strain differences.

Complete data sets were collected for 108 *M. anisopliae* isolates representing 14 strains. Each of these isolates was considered as a separate point in 5 dimensional space, the coordinates of which was defined by the characteristics of the "sample site", namely soil type, pH, conductivity, rainfall and altitude.

Canonical variate analysis (CVA) (Krzanowski, 1990), a multivariate statistical technique which aims at summarizing, and yet highlighting, the structure within such high dimensional data sets in fewer dimensions (ideally one or two) was employed to more easily display and comprehend interrelationships between the strains.

CVA condensed the salient variation within these 5 dimensions into fewer orthogonal dimensions (axes) so that the relative spatial segregation of 14 strains present could be more readily depicted. CVA involves the creation of a new coordinate system which are linear combinations of the original 5 measured variables. The first canonical variate represents an axis which is chosen to give greatest separation or discrimination between strain groups by maximizing the ratio of the between groups variance to the within groups variance in a manner analogous to a one way analysis of variance. The second axis is then formed perpendicular to the first and provides maximum separation of the groups utilizing information not previously utilized by the first canonical variate. A further three axes are similarly formed, thereby accommodating the 5 dimensional data set under study. Inspection of the weighting coefficients (or loadings) used in forming the new coordinate system provided an insight into those original variables which played a dominant role in the formation of the canonical axes. A good account of the actual mathematics involved is given by Seal (1964).

M. anisopliae density

The CFU (colony forming units) counts from the spread plates were averaged for each isolate of *M. anisopliae*. These single means were then tested for correlation with the other measured variables. Standard regression and ANOVA techniques were used initially, with the more powerful Best Subsets Regression then being used to correlate CFU counts with six abiotic variables (soil type, pH, conductivity, rainfall, moisture and altitude). Best subsets Regression (Minitab Reference Manual, 1989) uses the r^2 criterion to select the smallest subset of explanatory variables which explain a maximum proportion of the variation in CFU counts.

11.3 RESULTS

11.3.1 *Metarhizium* distribution

Of the 419 soil samples collected from the pastoral zone in Tasmania, 28% (119) contained *M. anisopliae.* Of these, 107 were single strain isolations, 11 contained two strains and one soil sample yielded three strains. The most commonly isolated strains (3, 4, 14, 15; Table 11.1) showed no clear geographical delimitation (Appendix 6).

Soil Type. *M. anisopliae* was more often ($P<0.05$) isolated from a loam soil relative to other soil types (Fig. 11.1A). The greater isolation of *M. anisopliae* from loam was not correlated with pH, rainfall, altitude or conductivity. However, analysis of the soil type preference of the four most commonly isolated strains (3,4,14,15) showed that only strain 4 was specific, with a greater isolation rate ($P<0.05$) from loam soils.

pH. The isolation of *M. anisopliae* from soils was not affected by the pH of the soil (Fig. 11.1B). While there were differences ($P<0.001$) in the mean pH over the four soil types, this was not related to the presence or absence of *M. anisopliae.* Strains 3, 4, 14, or 15 could not be separated on the basis of preferred soil pH.

Conductivity. The presence or absence of *M. anisopliae* was not related to soil conductivity (Fig. 11.1C). Conductivity was not related to soil type. The isolation of strains 3, 4, 14 or 15 was also not related to soil conductivity.

Rainfall. The number of soil samples taken from each of the rainfall zones reflected the area of the pastoral zones subject to these rainfall averages. The isolation of *M. anisopliae* from soils was not influenced by average annual rainfall, though from 450-1250 mm rainfall, the proportion of soils yielding *M. anisopliae* increased (Fig. 11.1D). Of the soil types, only clay loam soils showed a greater isolation ($P<0.05$) of *M. anisopliae* with increasing rainfall. Strain 4 was more commonly isolated ($P<0.001$) from low rainfall zones than were the other three main strains (3, 14, 15).

Table 11.1 **The number of isolates of *M. anisopliae* strains and the group average of each of the sample site characteristics.**

Strain No.*	No. Isolates	SOIL Type†	SOIL pH	SOIL Conductivity (mS/cm)	SOIL Rainfall†	SOIL Altitude†
1	4	2.50	4.85	0.025	4.25	1.25
2	2	3.50	4.60	0.025	5.50	1.50
3	14	2.43	4.84	0.048	4.00	1.21
4	16	1.75	5.12	0.035	2.94	1.06
5	2	2.00	5.65	0.040	2.00	1.00
6	1	2.00	5.20	0.030	2.00	1.00
7	1	3.00	4.80	0.020	5.00	2.00
8	3	1.67	5.07	0.050	2.67	1.00
9	2	2.50	4.75	0.035	3.00	1.00
10	1	1.00	4.10	0.030	3.00	1.00
11	8	2.38	4.70	0.043	4.13	1.13
12	1	4.00	5.10	0.010	3.00	1.00
14	28	2.64	4.86	0.035	4.43	1.14
15	25	2.56	5.14	0.040	4.12	1.20

* Strains classified using the key of Yip, Rath and Koen (1992).

† Soil type, rainfall and altitude are encoded variables (Davies, 1988):

soil type: 1=sandy loam, 2=loam, 3=clay loam, 4=clay

rainfall: 1=375 - 500 mm, 2=500 - 625 mm, 3=625 - 750 mm, 4=750 - 1000 mm, 5=1000 - 1250 mm, 6=1250 - 1500 mm, 7=1500 - 2000 mm.

altitude: 1=0 - 300 m, 2=300 - 600 m, 3=600 - 900 m.

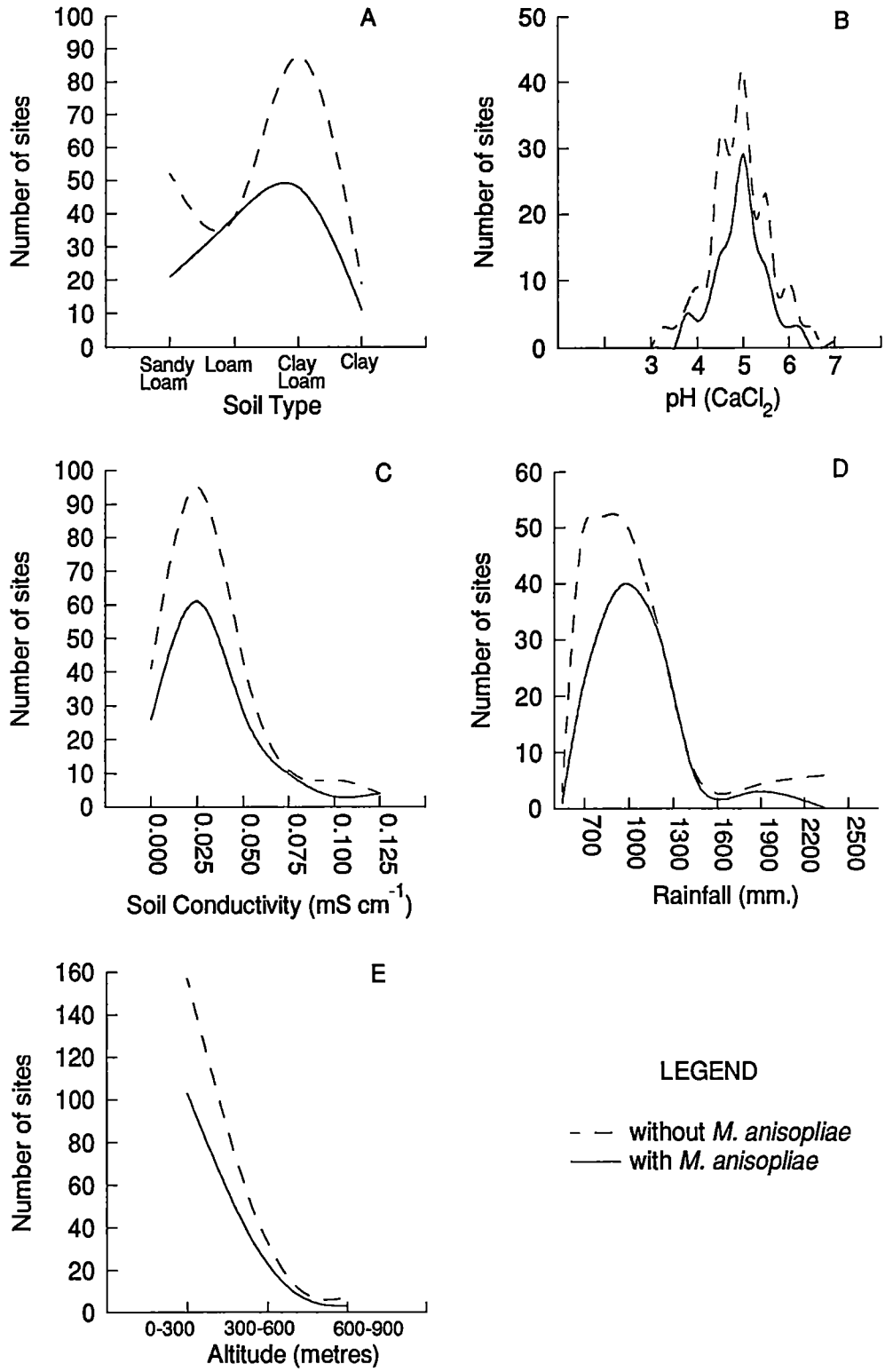

Figure 11.1 **Numbers of soil samples (sites) in which *M. anisopliae* was present or absent plotted against the sample characteristics of A. Soil type, B. pH, C. Conductivity, D. Rainfall and E. Altitude.**

Percentage soil moisture was positively correlated ($P<0.0001$, $r^2=16.8\%$) with rainfall. Amongst the four common strains, the mean %soil moisture was lower ($P<0.01$) for isolations of strain 4 than for strain 16. Strains 3 and 15 did not differ from one another or the other two strains.

Altitude. The isolation of *M. anisopliae* from soil was not related to altitude (Fig. 11.1E) and even though rainfall was positively correlated ($P<0.001$) with altitude, strain 4 (which preferred drier soils) and the other three strains were evenly distributed across altitude.

Temperature. The mean average mid-winter temperatures ranged from 4°C in the highlands to 10°C along the coast, while the mean maximum summer temperatures ranged from 20°-24°C. There was no obvious relationship between the temperature isotherms and the distribution of these *M. anisopliae* strains.

Canonical Variate Analysis. The weights (equivalent to regression coefficients) for the 5 variables of the first 2 new axes are given in Table 11.2. Collectively, these 2 new axes account for 81.8% of the variance in the original data cloud, which gave an indication of the reasonable effectiveness of the variables in discriminating between strains.

Examination of the actual site characteristics (Table 11.1), in addition to the variable weightings (Table 11.2), indicated that the separation of strains along axis 1 (Fig. 11.2) was due to variation in average annual rainfall. Separation along axis 2 appeared to be due to differences in both average pH and soil-type. The stability of this separation pattern was tested by re-performing the CVA on the sub-set of data comprising strains 3,4,14 and 15, that is, those strains representing 83 of the 108 isolates. The relative positioning of these 4 most abundant strains remained unaltered, thereby giving added confidence to the interpretation of the causes of strain separations displayed in Fig. 11.2.

Ellipses were drawn around the four most common strains to include any other strain isolated from the same soil sample (Table 11.3). These multi-isolate samples clearly indicated that the distribution of *M. anisopliae* could be divided up into sets and subsets of strains which occupy similar niches, and that isolates from opposite environmental niches were not found together (Fig. 11.2).

Table 11.2 **Weighting coefficients for the first two new axes derived from Canonical Variate Analysis (CVA). CVA has reduced the 5 variables into 2-dimensions (axes 1 and 2). The coefficients indicates the weight given to each variable in forming each of the axes.**

Variable	Axis I	Axis 2
Soil type	0.23	- 0.41
Soil pH	- 0.29	- 0.92
Conductivity	- 0.23	- 0.05
Rainfall	1.02	- 0.18
Altitude	0.08	0.12
% Variance Accounted for	68.70	13.10

11.3.2 *Metarhizium* Abundance

Of the 14 *M. anisopliae* strains examined only four (strains 3,4,14,15) are common and they account for 77% of the isolations (Table 11.1). Strain 11 is less common (7% of isolations), while all other strains can be regarded as rare (1-4% of isolations).

The density of *M. anisopliae, per se*, in soils was low with 85% of isolations being recorded at 5 x 10^4 CFU's/g soil or less (Fig. 11.3). The modal density was 1 x 10^3 CFU/g soil while the average density was 1.97 ± 0.7 x 10^3 (mean ± SE, n = 132). Counts around 10^2-10^3 CFU's/g soil equated to the minimum concentration discernable with the method used here, equalling an average of 0.1 - 1 colonies (respectively) on the five agar plates (10^{-1} dilution).

The density (log_{10} CFU's/g soil) of isolates was not singularly related to the soil moisture, soil type, pH, conductivity, rainfall or altitude. There were no significant differences in the densities of the 14 *M. anisopliae* strains. The moisture content of the soil samples at collection ranged from 0.6-51.1% (median = 8.3%; n=132).

Best Subsets Regression of the CFU counts against the six factors indicated that none of the factors, singularly or combined, was able to adequately explain the data. The r^2 values of the best models were less than 1.5%.

Table 11.3 Soil samples yielding more than one isolation of a *M. anisopliae* strain.

Soil sample	*M. anisopliae* strains present
D096	3,4
D196	3,7,14
D235	3,15
D096	4,3
D271	4,6
D079	4,8
D196	14,3,7
D195	14,9
D187	14,11
D184	14,11
D167	14,15
D133	14,15
D340	15,1
D235	15,3
D179	15,11
D167	15,14
D133	15,14

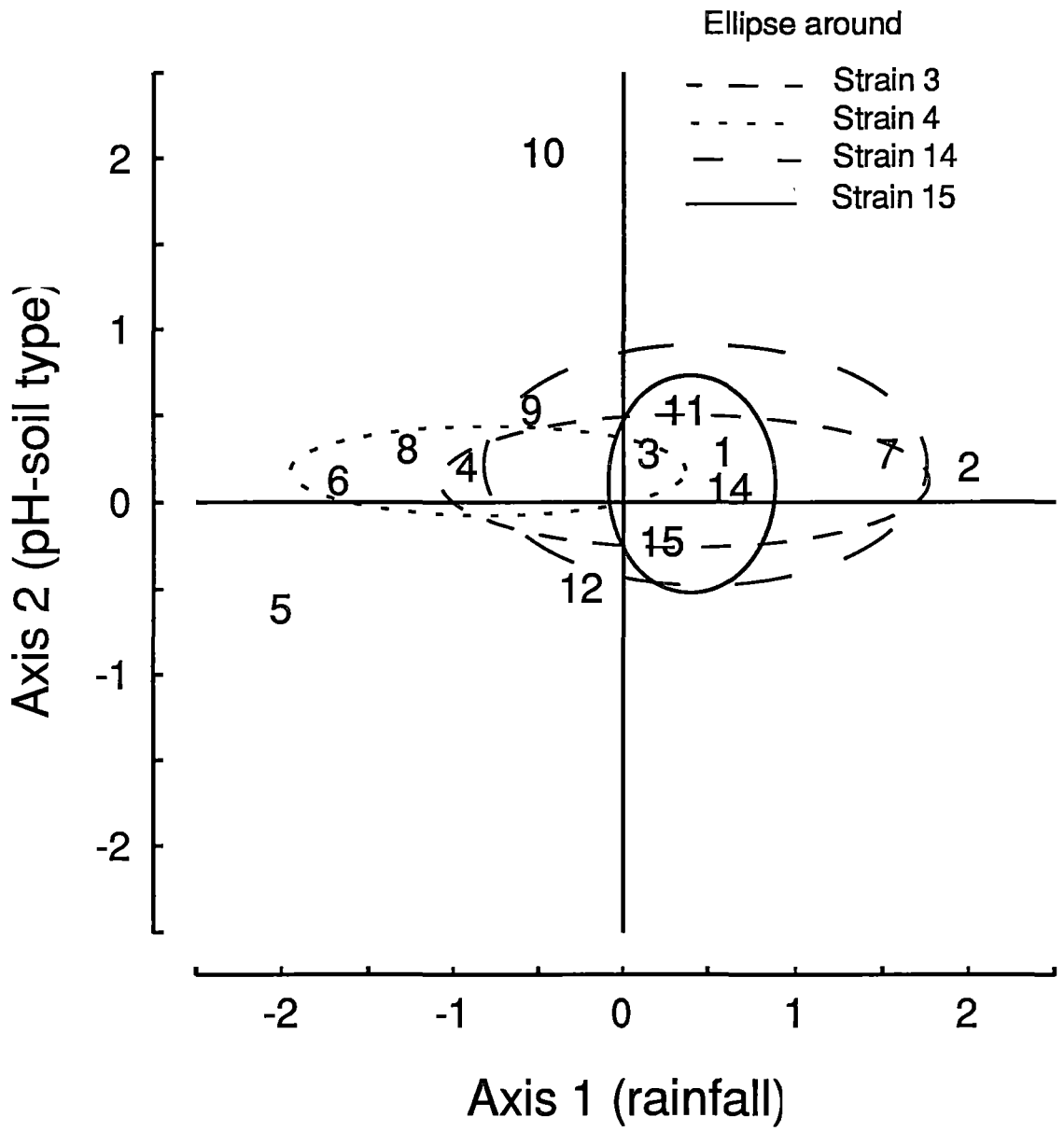

Figure 11.2 **Separation of the 14 *M. anisopliae* strains using axes 1 and 2 derived from the five dimensional variates of soil type, pH, conductivity, rainfall and altitude (examination of the weighting coefficients in Table 11.2 allowed the determination of the predominant variable or variables for each axis). Integers represent strain numbers as per Table 11.1. Ellipses for specified strains (3,4,14 and 15) encompass other strains isolated from identical soil samples (Table 11.3) (see text for further description).**

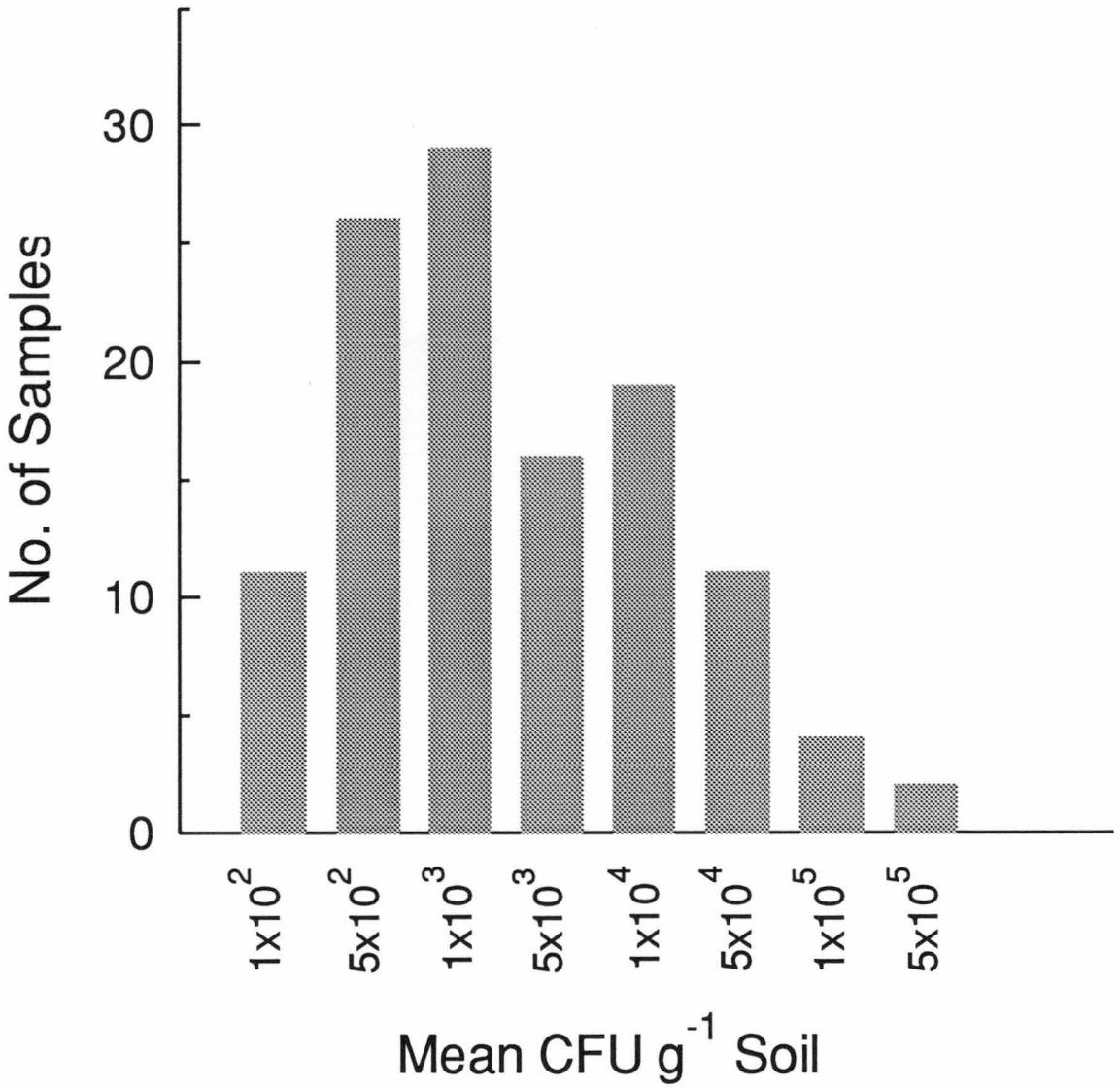

Figure 11.3 **Mean densities (CFU/g soil) of *M. anisopliae* plotted against the numbers of soil samples.**

11.4 DISCUSSION

This study has shown that *M. anisopliae* var. *anisopliae* is a commonly encountered soil fungus which is distributed across all the soil environments examined and that the isolation rate and density is little affected by differences in the soil environment. However, the results (Fig. 11.2) clearly showed distributional differences, based on rainfall, pH and soil type, in the 14 strains of *M. anisopliae* identified.

M. anisopliae var. *anisopliae per se*

The soil samples studied here were small (only 200 g) and yet nearly 1/3 (119/419) of these had *M. anisopliae* present. By contrast, Hokkanen and Zimmermann (1986) who used a modified "*Galleria* bait method" (Bedding and Akhurst, 1975) only isolated *M. anisopliae* from 5 out of 30 soil samples taken from a range of agro-forestry soils.

The pasture soils examined ranged widely in relation to the studied abiotic factors of soil type, pH, conductivity, moisture, rainfall, altitude and temperature yet only soil type affected the distribution and abundance of *M. anisopliae* as a species. In this case there was a greater than expected isolation rate of the fungus from loam soils. None of these factors was correlated individually or in combinations with the density (CFU's/g soil) at which the fungus was found.

Tyni-Juslin and Vanninen (1990) showed that after one year, the persistence of a released strain of *M. anisopliae* was the same for all four soil types tested (clay, loam, humus and peat). However, they found that in loam soils spores were found in the top 0-5 cm while in the others, vertical migration of the spores led to recovery of *M. anisopliae* at deeper soil levels (5 - 15 cm and 15 - 20 cm). Ignoffo *et al* (1977) also found a greater retention of *Nomuraea rileyi* spores in loam soils when compared with sandy soils. Storey and Gardner (1986) found that in laboratory mixes of clay and sand the retention of *Beauveria bassiana* in the upper 5 cm was positively correlated with sand content and negatively correlated with clay content. This study only examined the top 10 cm of the soil and this may have some bearing on the greater isolation rates of naturally occurring *M. anisopliae* from loam soils (Fig. 11.1A).

However, it is probably the 0-10 cm depth that is most relevant to soil pest control. Placement of biocontrol strains of *M. anisopliae* at a depth of 2-3 cm was ideal for the control of eggs and larvae of the subterranean scarab pest, *A. couloni* (Chapter 2). Leaching of the fungus would lead to reduced pest control.

It is not surprising that pH has little effect on the distribution and abundance of *M. anisopliae* as the fungus is able to germinate over a wide pH range. Milner (1989) stated that germination was inhibited below pH 5.0 and above pH 8.5, but Rath and Pearn (unpublished data) have shown that one isolate of *M. anisopliae* (DAT F-001) was able to grow across all pH's from 4.0 to 7.8. In the mass production of entomogenous fungi the pH is often reduced to 3.5 to limit bacterial contamination (Barlett and Jaronski, 1988). The $CaCl_2$ method used to determine pH in this study generally gave results 0.3-0.5 pH units lower than results attained without $CaCl_2$. This must be taken into account when comparing pH values.

The average annual rainfall was a more important determinant of moisture than either %soil moisture or water potential (A_w) because these short-term results were unlikely to influence the distribution of *M. anisopliae.* This study did show that %soil moisture taken at the time of sampling could be regressed against average rainfall but the r^2 value was low (16.8%) indicating considerable variation in the data. The ratio of the number of *M. anisopliae* isolations to soils sampled increased from 25% at 450 mm to 50% at 1250 mm average rainfall (over 4 rainfall zones). However, further direct comparison at higher rainfalls was not warranted because the numbers of pasture soils at higher rainfall declined dramatically (Fig. 11.1C). Additionally, the long-term average rainfall may not have been as important as the rainfall in the previous six months. Moisture retention is further complicated by effects of temperature and evaporation.

High humidity or free water is generally required by entomogenous fungi for germination (McCoy *et al*, 1988). In the soil, water is probably not limiting as many fungi can still utilize the available soil moisture at water potentials far lower (-20 MPa) than permanent wilting point (-1.5 MPa) (Yanagita, 1990). Further, insects surviving in soil probably give fungi the high humidity required for germination through their respiration and the closeness of their contact with the soil.

While density would probably be more meaningful if the population was measured over time and over different seasons, the density at which *M. anisopliae* was found was not influenced by any of the abiotic factors studied here. Other abiotic factors may be important: Milner (1989) believes that increased organic matter in soils aids infection. However, most authors have found soil to have a fungistatic effect which acts to maintain viable conidia for several years (McCoy *et al*, 1988). Fungistasis is commonly found to be related to the presence of other micro-organisms, particularly Actinomycetes (Milner, 1989), rather than any abiotic factor.

Consequently, it appears likely that the major factor influencing density is biotic rather than abiotic. Milner (1989) considers that fungi that are insensitive to fungistasis may not persist well in the soil. On the other hand, conidial production on the host can dramatically influence spore density. Weiser and Matha (1986) have shown that 1×10^{10} *M. anisopliae* conidia can be produced by one infected *Melolontha melolontha* prepupa.

Correlation of the data with host is impossible as the natural hosts of the 119 isolates are unknown. However, Yip *et al* (1992) tested 192 cultures of *M. anisopliae* var *anisopliae* isolated from Tasmanian soils, including the 119 examined here, for pathogenicity to *A. couloni*. They found that 50% of the isolates of strain 1 and 3 were pathogenic. Strains 1 and 3 had a statewide distribution and yet the pathogenic isolates were all found in the northern part of Tasmania. This matches the current distribution of *A. couloni* (McQuillan and Ireson, 1987). The distribution of the insect hosts may be related to the biotic factors measured here.

Strain distribution and abundance

While the distribution and abundance of *M. anisopliae* var. *anisopliae* is little affected by soil environment, strains of the fungus can clearly be separated into environmental niches (Fig. 11.2). Of the four most abundant strains (3,4,14,15), strain 4 was more commonly isolated from loam soils and low rainfall zones. Rainfall was the dominant strain separation factor (Fig. 11.2) along axis 1 which accounted for 69% of the variation amongst strains. The four strains could not be separated on pH, conductivity or altitude. CVA also showed that the separation of these four strains on a pH/soil type axis (axis 2, Fig. 11.2) was minimal.

Isoesterase analyses of *Beauveria bassiana* (Poprawski *et al,* 1988) and *M. anisopliae* (Riba, Bouvier-Fourcade and Caudal, 1986) have been able to separate some strains of these fungi based on geographic distribution. In the case of *M. anisopliae*, Brazilian strains were differentiated from other strains collected from around the world. This study is more detailed than these other studies because, having previously distinguished (Yip *et al*, 1992) strain differences in the Tasmanian *M. anisopliae* flora, This study associated them within environmental niches within a fixed geographical area.

The differentiation of strains into environmental niches does not necessarily suggest that the strains are adapted to the specific environmental factors studied here. Like the earlier discussion of *M. anisopliae* abundance, the distributional differences may in fact be due to the preferred environment of the unknown hosts.

This study highlights the distributional changes occurring within strains of *M. anisopliae* var. *anisopliae* and in doing so adds some ecological weight to the appropriateness of the earlier taxonomic classification of these strains (Yip *et al,* 1992). While the results give an insight into the natural distribution and abundance of *M. anisopliae,* they raise concerns about the persistence of potential biocontrol strains. If a strain 4 isolate was a candidate fungus, what is its persistence in the unfavourable environments common to strains 14 and 15? Further studies are in progress to assess the effect of these soil environment factors on the persistence of the candidate *M. anisopliae* isolate (DAT F-001) for *A. couloni* control.

Impact on biocontrol using *M. anisopliae* DAT F-001

This study has shown that isolates of strains closely related to DAT F-001 (ie. strains 1, 2 and 3) are widely distributed in Tasmanian pastures but were found in only 16 of 419 soil samples. Of the 16 isolates only eight were pathogenic to *A. couloni* (Yip *et al.*, 1992). Consequently, fungi similar or identical to DAT F-001 can be regarded as scarce, suggesting that natural control of *A. couloni* by DAT F-001 would rarely occur. In this context, the use of *M. anisopliae* DAT F-001 can be seen as a 'new introduction'. The strategies for using entomogenous fungi are detailed in Chapters 7 and 9.

The occurence of strain 1, to which *M. anisopliae* DAT F-001 belongs, is common to all environmental niches (Fig. 11.2) and it is expected that survival of this strain would be little effected by incorporation into other environments. While the on-going studies mentioned above highlight this area, the data presented here give no evidence of detrimental environmental effects on the survival of DAT F-001 in any specific soil environment. This data will also be useful in the registration of DAT F-001 for *A. couloni* control.

CHAPTER 12

12.0 RECAPITULATION, CONCLUSIONS AND DIRECTIONS FOR THE FUTURE.

Metarhizium anisopliae DAT F-001 is currently being commercialized by Bio-Care Technology Pty Ltd and it is expected that the fungus will be available to farmers in 1993. The research in this thesis provided the basis for the commercial development. A recapitulation of the experimentation, together with conclusions and directions for the future, follows.

12.1 Laboratory experiments

Screening experiments

Several isolates of entomogenous fungi, including *M. anisopliae*, *M. flavoviride* and *Beauveria bassiana*, were screened for pathogenicity to L3 larvae and adult *Adoryphorus couloni*. *M. anisopliae* DAT F-001 was pathogenic to L3 larvae at spore concentrations of 10^4 to 10^6 spores/g sand-peat and to adults at 10^5 and 10^6 spores/g sand-peat. Other isolates showed varying pathogenicity to larvae and adults. Only *M. anisopliae* DAT F-141 was as pathogenic for larvae (at 10^6 spores/g) as DAT F-001, whereas *M. flavoviride* DAT F-133 was more virulent to adults than DAT F-001 at a spore concentration of 10^4 spores/g sand-peat. DAT F-001 was more virulent than DAT F-133 at 10^6 spores/g sand-peat.

These studies highlighted the host specific nature of *Metarhizium* and insect-pathogenic fungi in general. McCoy (1988) reviewed the host specificity of insect-pathogenic fungi and concluded that "fungal species and/or pathotypes that are morphologically very similar may possess different host ranges". They cited *Beauveria bassiana* and *M. anisopliae* as examples of this. However, it must be remembered that insect-pathogenic fungi are not unique in this context; pathogens of plants and animals, whether fungi, bacteria, viruses or others, exhibit differing degrees of host specificity (which may or may not be related to host resistance).

The early screening study (section 4.2) must be compared to the recent study of Yip, Rath and Koen (1992) who screened 184 isolates of *M. anisopliae* for pathogenicity to *A. couloni*. While they found that only 10% of the isolates they had collected from soil were pathogenic, all the pathogenic isolates occupied the same distribution range as the host, suggesting a close host-pathogen link.

Milner (1989) had also shown that only a "minority of isolates from soil are pathogenic to the larvae of the predominant insect host". For this reason many researchers tend to test *M. anisopliae* isolates originating from the host or closely related insects.

Wherever the isolates originate from, variation in host susceptibility/pathogen virulence dictates that as many *M. anisopliae* isolates as possible must be screened as early as possible in the program. Further, there is no hard-and-fast rule which will determine which isolates are likely to be most virulent.

Lack of pathogenic isolates is not reason why there is only one registered *M. anisopliae* bioinsecticide in the world, as there is a plethora of papers on *M. anisopliae* bioassays against insect pests. The reasons for this is more likely to be related to the host-pathogen interactions with the environment, and this will be discussed further in following sections.

Further research needs to be directed at determining the factors governing the pathogenicity of fungi to insects (Gillespie, 1988) as these studies may speed research and development of new bioinsecticides as well as expand the host ranges of existing "products". The research of Dr. Ray St. Leger *et al.* at Cornell University may help to elucidate the pathogenicity factors.

Spore concentration bioassays

Further investigations of the virulence of DAT F-001 for L3 larvae showed that spore concentrations as low as 10^1 spores/g sand-peat (at 20°C) could kill 100% of L3 larvae provided the incubation time was of sufficient duration. In these experiments, the LT_{50} ranged from 19 days (at 10^7 spores/g) to 83 days (at 10^1 spores/g). This suggested that, in the field, low spore concentrations would not give 'short-term' control but that the fungus may exert a controlling influence on the L3 population even at very low spores concentrations. This is important in relation to field efficacy as it is likely that field applied DAT F-001 will, over time, decrease in concentration. Additionally, as cost is one of the problems in the development of a *M. anisopliae* bioinsecticide (Bartlet and Jaronski, 1988), low concentrations would mean a cheaper product.

The life-cycle and ecology of the pest is critical for the successful use of low concentrations. Low concentrations require more time to be effective which

means that any target insect must have a sufficiently long life-cycle, matched to a relatively 'lax' crop damage criteria. An insect which causes crop damage over a short period combined with several generations a season may not be possible to control with fungi, even if very large concentrations of fungi were used. Control of *A. couloni* with *M. anisopliae* was aided by the two-year life cycle of the host combined with a high damage threshold and the extended time period over which the damage occurred. Consequently, given the appropriate fungal isolate, control of other scarabs and other pests in soil should be possible. This is now being shown to be true for *Melolontha melolontha* with *Beauveria brongniartii* by Keller (1989).

Concentration studies also raise the question of pathogenicity. How many spores are required to infect and kill a pest? Does the insect's immune system confer resistance to low 'challenges'? Many of these questions are being addressed by other researchers, however, it is likely that host-pathogen interactions change from one system to another, requiring continued research.

Temperature studies

The temperature at which L3 larvae were exposed to *M. anisopliae* DAT F-001 had a marked effect on virulence. Germination studies showed that the fungus would germinate at temperatures from 2° to 25°C though the germination rate was increasingly retarded as the temperature declined. Infectivity studies showed a correlation between these germination rates and the LT_{50} of L3 larvae. The fungus was pathogenic at all temperatures from 5° to 15°C (ambient field conditions) with the LT_{50} ranging from 189 days to 36 days (respectively, at a spore concentration of 4.1×10^6 spores/g sand-peat). A fluctuating day/night temperature regime had no effect other than that attributable to the mean daily temperature.

These studies showed that the temperatures at which the fungus grew and infected *A. couloni* was matched to that of the environment. Gillespie (1988) stressed that it was "important to select fungal isolates able to grow rapidly at the temperatures prevailing after spore application". From these experiments it could hardly be said the DAT F-001 grew rapidly at 5°C, however, the long life-cycle of the pest again aided the efficacy of the fungus.

It is debatable whether growth at 5°C was a necessary requirement for the success of DAT F-001. If there was no growth at low temperatures (below 10°C) modelling (Chapter 9) shows that control of the pest is only marginally delayed and that as a consequence it probably has no effect on overall efficacy. I now have an isolate of *M. anisopliae* (DAT F-401) which is equally pathogenic to DAT F-001 at 20°C, but which does not grow at 5°C. It would be interesting to test the model's prediction by comparing the field performance of these two isolates.

Temperature-matching with the environment does not guarantee efficacy. Other studies in this laboratory (Wright and Rath, unpublished data) have shown that it is growth rate of the fungus and not germination that is a critical determinant of efficacy. The inability to grow fast enough can make a fungus avirulent. Further studies have been directed to assessing the growth rates of candidate fungi for a new project on control of winter-active pests. For these pests the ability of the fungus to regulate the populations at 5°C will be crucial. In a further search for cold-adapted isolates with fast growth rates, I have been awarded an Australian National Antarctic Research Expedition grant to undertake an ecological survey of *Metarhizium* and *Beauveria* on subantarctic Macquarie Island during the summer of 1992/93. This island has a constant winter/summer temperature of 4-6°C and may provide isolates for world-wide use. Isolates such as these could bring *Metarhizium* control of pests out of the glasshouses and the tropics and into widespread temperate use.

Modelling

The temperature and concentration experiments indicated that, under field conditions, the prevailing temperatures would be more important in determining the time required to kill 50% of larvae than the spore concentration. A simple model was developed (using pilot laboratory data) to predict the timing a mortality in field experiments when an application rate of ~ 100 kg/ha (10^{14} spores/ha) was used. The model was useful because it enabled the examination of the effect of application timing on larval survival. It predicted that application in autumn would not save winter pasture productivity, but, all application times examined would result in the reduction of the L3 population before the end of the year. This simple model was accurate in predicting the long lag-phase between application and the LT_{25}, though the predictions of the times to the LT_{50}'s and LT_{75}'s were proximal and optimistic.

The incorporation of application rate into a second model enabled greater examination of the potential outcomes of *M. anisopliae* application. This latter model was more accurate than the earlier model in its predictions of the times required to kill larvae in field experiments established in 1989.

Both these models were not entirely accurate in predicting the outcomes of field experiments, however, their major advantage was that they enabled the mathematical exploration of the fungus-host interaction. This allowed field experiments to be established with more confidence and more efficiency because strategies that could not possibly work were eliminated. The models also helped to encapsulate the biocontrol system and in so doing, helped to conceptualize the factors which impose on the system.

Moisture

The effect of moisture on pathogenicity was not studies in this thesis, yet one of the hypotheses for the reduced field efficacy of the fungus in 1991 was the prevailing dry conditions. I hypothesized in this thesis that the major problem with low moisture was not lack of fungal efficacy but the delayed development of the larvae. The delayed development was possibly due to lack of movement (as the soil was so hard) which would in turn mean a reduced probability of the larvae contacting the fungal spores. Hence reduced efficacy.

Moisture is critical not only for the above reason but also for its' effect on survival of the fungus in the soil.

Further studies are underway which address both issues. To date the study (Pung and Rath, unpublished) has shown that fungus efficacy is optimal at the 20-25% level (20% moisture was used in these experiments).

12.2 Field experiments

In these experiments, the fungus was drilled into the soil at a depth of 20 mm and at 150 mm spacings. In 1989, application of the fungus into pastures infested with L3 larvae resulted in up to 84% reduction of the larval population in 1989, and up to 96% reduction of the adult population prior to mating flights in 1990. The level of mortality in the L3 larvae in the year of application depended

on application timing (the earlier the application the greater the percentage mortality). Applications of fungus in spring resulted in no mortality of L3 larvae by early summer but a 50% reduction in the adult population by the following autumn. None of these experiments resulted in visible pasture differences in the year of application.

M. anisopliae DAT F-001 reduced the numbers of L2/L3 larvae in March by 60 - 70% in both the second and third years after application. This resulted in visible productivity increases in the third year when the numbers of larvae in untreated plots averaged 900/m^2. This level of control was due to the maintenance of the fungus in the pasture soils. This meant that the adults, eggs and L1 larvae would become exposed to, and die from, the fungus over the spring/summer period. At field sites where *A. couloni* was present, the fungus was maintained at concentrations close to that applied, or higher, for 12 - 36 months. The CFU concentrations in the soil at these sites suggests that *A. couloni* control should continue for many more years.

Application of the fungus to pastures in spring (Nov. 1990), when ovipositing females and eggs were present, could not reduce the L2/L3 population by March 1991. Placement of the fungus at a depth of 20 mm, in spring, would have no effect on egg survival as there would be no contact between the fungus and the immobile eggs. It was expected that L1 larvae would encounter the fungus over summer, however, the dry topsoil (0 - 80 mm) over that period may have reduced the contact between larvae and fungus.

By comparison, the earlier experiments may have had a more even distribution of the fungus due to movement of fungus by L3 larvae (alive or mummified) the previous year. This even distribution and the presence of the fungus from initiation of oviposition was probably responsible for the greater efficacy of the fungus in years subsequent to the year of application.

Application of low rates (1, 10 and 25 kg/ha) of DAT F-001 in the autumn of 1991 did not reduce the larval population in contrast to that predicted by the modelling. During 1991, the larvae were stressed by soil moisture deficiencies and failed to develop from the L2 to the L3 stage when expected. The lack of moisture reduced movement of the larvae and consequently reduced larval contact with the fungus, and fungal efficiency. The retardation of larval development limited pasture damage (by the larvae not by the drought).

Consequently, it is predicted that the fungus will control the larvae in most years, however, in drought years, when pasture productivity is low, the larval damage will be limited by environmental factors. Fungal survival was not adversely affected by the dry conditions, and consequently fungal control will continue be maintained in more productive years.

Metarhizium survival

The long-term maintenance of the fungus in the soil is crucial to the success of this control measure. The fungus would be too expensive to apply repeatedly to large pastoral areas. Consequently, one application must last "several" years. The data presented in the thesis shows that survival is good for two to three years. However, it is probable that the fungus level will decline in the soil due to soil type or pH effects, leaching, microbial competition, agricultural chemicals or fertilisers, or invertebrate feeding. Studying these factors should help to determine which factors are detrimental and which aid fungal survival. I have commenced research which examines some of these effects.

Pasture productivity

Pasture productivity was not measured during these experiments, though there were marked differences in discernible damage at the Lester site during 1991. The ultimate goal of pest control is to increase the productivity of the agricultural crop (in this case pasture). Further research needs to be directed at the productivity improvements resulting from the use of *Metarhizium*. The reductions in L3 larvae numbers recorded in some of these experiments suggests that productivity improvements should be achievable. If there is no gain in the longer-term the product is obviously not sellable.

Non-target invertebrates

M. anisopliae DAT F-001 had no adverse effect on the numbers of non-target invertebrates in the soil after 12 months. The numbers of another pest, the blackheaded cockchafer, increased in experimental blocks in the third year following the reduction of *A. couloni* numbers earlier in the autumn. *A. couloni* competitively exclude blackheaded cockchafer from pastures when *A. couloni* larval densities are high, and it was expected that blackheaded cockchafer would reinfest pastures once *A. couloni* was controlled.

Non-target information is important to ascertain the 'environmental friendliness' of biological products. This information is essential to registration of an insecticide in Australia and in many other countries. The data could have been improved if the densities of non-target organisms had been higher, however, the nature of *A. couloni* infestations is that there are few other invertebrates present. While further research could be directed at generating more information on non-target susceptibility, I do not believe that it is warranted. If DAT F-001 had been a chemical insecticide it is probable that there would have been a large negative effect on the non-target population.

12.3 Environment

A final study examined the distribution and abundance of *M. anisopliae* strains in Tasmanian pastures. Part of this study showed that strains pathogenic to *A. couloni* were found both rarely and in low concentrations. This implied that natural *A. couloni* control is not possible without the further introduction of DAT F-001 into pastures.

The study also showed that Tasmanian strains of *M. anisopliae* are differently distributed in the soil environment (particularly with respect to rainfall, pH and soil-type). However, the strain to which DAT F-001 belongs (strain 1) was placed in a mid-range environmental niche which overlapped most other strains. This suggests that DAT F-001 should not be adversely affected by being placed in differing pasture soil environments.

Studies on the ecology of insect-pathogens are rare in the literature. Studies of this type are useful for registration and will probably increase in frequency simply for this reason. They are relevant to biocontrol studies and should become and integral part of any biocontrol program because they can help to explain the interactions between the host, the pathogen and the environment. Without such studies, insect-pathogens would be released into a 'black-box' and reasons for failure (or success) may not be quantifible.

12.4 Commercialisation

The data used in this thesis will form the basis of the submission for registration of *M. anisopliae* DAT F-001 as a biocontrol agent. Registration will enable the

commercial sale of *M. anisopliae* in Australia, however, the success of this research will be measured by the length of time *M. anisopliae* is commercially available.

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$$P = [e^{(a+bx)}]/[1 + e^{(a+bx)}],$$

where **P** is the proportion of (1) larvae surviving, or (2) spores germinated.
e is the base of the natural logarithms,
x is the independent variable (time of measurement in this case)
and **a** and **b** are coefficients to be estimated.

The particular transformation which linearises the logistic curve is the logit:

$$z = \text{logit}(p) = \text{Ln}[p/(1-p)] = a+bx$$

The appropriate error distribution for a proportion surviving or germinating is binomial rather than the Normal. This error distribution, together with the logit linking transformation, specifically form the basis of the GLM technique used to analyze these counted proportions (Healy, 1988). The GLIM statistical package was used.

The use of the logit link function is a compromise between the use of a composite link function (Thompson and Baker, 1981) and the analysis of the data at each specific time. This compromise is necessary because the experimental design is one of repeated measures. While the composite link may be a more appropriate model to fit to the data, software for it is not readily available and there are still some questions as to the appropriateness of some of the underlying assumptions of the model. Analysis of the data at each time, while overcoming the statistical requirements of the repeated measures design, is unable to address the structure of the data in relation to the decline of L3 survival with time and dose (for example). This is best illustrated by using a comparison of the LT_{50}'s of two doses. Standard analysis (such as probit analysis, Finney, 1971) may indicate that the two LT_{50}'s (the time required to produce a 'response', in this case death, in half the population) are not different,

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Appendix 1

Rath, A.C. (1989). Developments in the use of *Metarhizium anisopliae* for control of subterrean red-headed cockchafer Coleoptera: Scarabaeidae: *Adoryphorus couloni.* In *Proceedings 5th Australasian Grassland Invertebrate Ecology Conference* (ed. P.P. Stahle). Melbourne University. 15 - 19 August, 1988. pp. 88 - 95. D & D Printing: Victoria.

DEVELOPMENTS IN THE USE OF *METARHIZIUM ANISOPLIAE* FOR CONTROL OF SUBTERRANEAN RED-HEADED COCKCHAFER (COLEOPTERA: SCARABAEIDAE: *ADORYPHORUS COULONI*).

A.C. Rath

Entomology Section, Department of Agriculture, St. John's Avenue, New Town, Tasmania, 7008 Australia.

ABSTRACT

Metarhizium anisopliae isolate DAT F-001 has shown potential as a control agent for the red-headed cockchafer (*Adoryphorus couloni*) in laboratory and field experiments. The isolate was cold adapted (at 5°C, 80% of spores germinated within 17 days) and highly virulent for third instar red-headed cockchafer - doses from 10^4 to 10^6 spores/g sand-peat mix, caused between 80% and 100% mortality in the laboratory. Mortality in the field can exceed 60% if *M. anisopliae* is introduced below the soil surface level. The short-term and long-term survival and effectiveness of the fungus is being studied.

INTRODUCTION

Larvae of the red-headed cockchafer *Adoryphorus couloni* (Burmeister) are serious root-feeding pasture pests in south-eastern Australia. They are distributed over three million hectares of pastoral land in Victoria and over 200,000 hectares of improved pasture in Tasmania. The distribution is expanding in Tasmania, New South Wales and South Australia and the insect has become established in New Zealand (McQuillan and Ireson, 1987).

"Substantial" loss of pasture dry matter occurs when the number of larvae exceeds 300/m^2 (Allen, 1986), however, total denudation of pasture occurs in many areas of Tasmania as the numbers of larvae reach 700 - 1100/m^2 (Rath, unpubl. data). Larvae damage the pasture by severing the roots during feeding and through physical disruption of the roots during underground movement.

Rath (*J. Invert. Path.* 1989, in press) has shown in laboratory studies conducted at 20°C, that greater than 90% mortality of third instar red-headed cockchafers was possible when the larvae were exposed to doses of 1 x 10^4 spores/g sand-peat (1:1), or higher, of the entomopathogenic fungus, *Metarhizium anisopliae* (DAT F-001). At 10°C, there was 75% mortality of larvae after 70 days exposure to a *M. anisopliae* dose of 1.46 x 10^6 spores/g sand-peat.

This paper presents the results of two laboratory and five preliminary field studies. The laboratory experiments investigated (a) the interaction of fungal dose and exposure time on cockchafer survival and mummification and (b) the effect of temperature on the germination rate of the fungus. The field studies, which were designed to confirm laboratory experiments, examined the survival and mummification of cockchafers in soils which had been treated in different ways with *M. anisopliae* (DAT F-001).

MATERIALS AND METHODS

Laboratory Studies

A Effect of dose and exposure time on the pathogenicity of *M. anisopliae*.

Third instar larvae of *A. couloni* were collected from an infestation on the property 'Glen Morey' at Woodbury in the Midlands region of Tasmania. Larvae were taken by hand from the soil which had been greatly loosened by larval feeding activities, placed into galvanised-iron trays with lids (400x300x80 mm) filled with sand-peat (1:1) and held in the laboratory for 14 days at 10°C prior to experimentation.

The *M. anisopliae* (isolate DAT F-001) inoculant and the dosed sand-peat was prepared as described by Rath (op. cit.). 75 larvae (0.60 ± 0.05 g) were placed into each of two replicate galvanised-iron trays with lids, for each of the four doses and the uninoculated controls.

The effect on survival and mummification of increasing the time to which the larvae were exposed to the doses of *M. anisopliae* (10^2 - 5.0 x 10^5 spores/g sand-peat) was measured after 28 and 51 days incubation at 20° ± 2°C.

B. Effect of temperature on the germination rate of *M. anisopliae*.

The germination rates of spores of *M. anisopliae* were measured at 5°, 12°, 16°, 20° and 25° ± 1°C. Spores were harvested from SDA + YE plates which had been point inoculated with *M. anisopliae* spores and allowed to grow for 14 days at 25 ± 1°C. Harvested spores were suspended in 1/4 strength Ringer's solution and serially diluted until a suspension containing about 1 x 10^7 spores/ml was obtained. 25 petri dishes containing 15ml of Czapek-Dox medium were inoculated with 0.1ml of the fungal spore suspension (spread-plate technique), giving five replicate plates at each temperature. The numbers of germinated and ungerminated spores were counted for two fields of view on each plate under the microscope (400x) at times between 18 hours and 70 days. Regression analysis was used to determine the time required to attain 80% germintion at each temperature.

Preliminary Field Studies

Description of experimental site.

All field studies were undertaken at the 'Glen Morey' infestation. Cockchafers had been active from early February 1987 and by late July 1987 when the experiments commenced the field had a 'ploughed' appearance due to the grub searching activities of birds. The temperature at the site was measured with a Goerz(R) thermoscript buried 10cm below the soil surface.

Plot pretreatment.

Fibreglass insect mesh was used to surround the plots (2m x 2m) and to divide these plots into 2m x 1m subplots. The fibreglass was stretched from the surface to a depth of 15cm to prevent movement of larvae into and out of the plots.

Experiments 1 - 3: The soil from each $4m^2$ plot was removed to a depth of 15cm, the larvae were removed and counted prior to returning half the larvae to each subplot as described for each experiment. Separate treatment and control plots were used.

Experiments 4 - 5. The $4m^2$ plots were divided into two $2m^2$ split-plots (one control and one treatment). Neither the soil nor the larvae were removed from the plots and due to the small size of the plots there was no estimation of the initial number of larvae.

The numbers of treatment and control plots together with the initial number of cockchafers per plot are tabulated (Table 1). The M. anisopliae inoculum which was grown on millet grain as described by Rath (op. cit.), produced on average 1.43×10^8 spores/g grain. Experiments which used the fungus millet mix at a rate of $100g/m^2$ gave in the top 10cm of soil an effective dose approximately 1.43×10^5 spores/g soil.

TABLE 1. The Number of Treated and Control Subplots Together with the Number of Cockchafers/m^2 ($\bar{x} \pm$ S.E.) at establishment (23-30.7.87).

	Controls		Treatments	
Exp.#	#subplots	# larvae	#subplots	# larvae
1	2	570	6	648±59
2	2	531	2	610
3	2	649	6	516±57
4	4	*	4	*
5	2	*	2	*

* not estimated

Experiment 1: Individually dosed insects: Larvae were rolled in either live (treatment) or heat killed (control) M. anisopliae spores. The larvae were spread over the plots after the return of 10 cm of soil; the larvae were covered with the remaining soil.

Experiment 2: Dosed soil: After removal of the larvae, the plot soil was mixed with live or heat-killed M. anisopliae (100g spores + millet mix/m^2) in a cement mixer for ten minutes. The larvae were spread over the plots after the return of 10 cm of the mixed soil and then covered with the remaining 5 cm of mixed soil.

Experiment 3: Layered soil: After removal of the larvae, 10 cm of the plot soil was replaced. The larvae were spread over the surface prior to the addition of another 2.5 cm layer of plot soil. Live or heat-killed M. anisopliae (100g spores + millet mix/m^2) was then spread evenly over the surface of the plot before the addition of the last 2.5 cm layer of plot soil.

Experiment 4: Metarhizium deposits: Each $4m^2$ plot was divided into two $2m^2$ plots of which one was treated with live M. anisopliae spores and the

other with heat-killed spores. In each plot, 4g of M. anisopliae + millet mix was added to the soil at 10x10cm intervals. A knife was used to open the soil to a depth of 2.5cm to allow the depositing of the M. anisopliae. The holes were then closed with soil.

Experiment 5: Surface spray application: Each $4m^2$ plot was divided into two $2m^2$ plots of which the treatment plot was sprayed with an aqueous suspension of M. anisopliae spores and the control plot received 2 litres of water/m^2. A pressurised spraying unit was calibrated to deliver 100g of fungus-millet mix in 2 litres of water/m^2 to the treatment plots.

Analysis of the field experiments took place 110 days later (11.11.87) when the soil in all 32 $2m^2$ plots was removed to a depth of 15cm. All insects were removed and the number of alive and M. anisopliae dead insects were counted.

RESULTS

Laboratory Studies

A. Effect of dose and exposure time on the pathogenicity of M. anisopliae.

Larval survival decreased as the dose and the exposure time was increased (Figure 1A). Doses higher than 1×10^4 spores/g sand-peat reduced survival to less than 15% after 51 days exposure. Doses between 10^2 and 10^3 spores/g sand-peat had minimal effect on survival even after 51 days. Mummification of larvae increased with both dose and exposure time to a maximum of 63% (Figure 1B).

B. Effect of temperature on the germination rate of M. anisopliae.

At 5°C, 80% of the spores had germinated by 17 days while at 12°C, 16°, 20° and 25°C, 3.1, 2.8, 1.8, and 1.1 days were required (Figure 2). The time (D days) to 80% germination of spores at different temperatures (T°C) was expressed by the linear equation $D = 101.2(T^{-1}) - 3.65$ ($r^2 = 0.97$).

Preliminary Field Studies

Soil temperature.

The soil temperature at the commencement of the experiment ranged from 2° - 12°C, and at the conclusion, from 11° - 25°C (Figure 3).

Experiments 1-3.

The application method had no significant effect ($P>0.1$) on larval survival in the first three experiments, consequently the data were pooled. M. anisopliae (DAT F-001) reduced ($P<0.005$) the cockchafer survival to less than 40% of tnat shown in the control plots and 20.3% ± 2.2% of cockchafers were mummified (Figure 4).

Experiments 4 and 5.

M. anisopliae, deposited in the soil in small clumps or applied to the

surface with water, did not reduce the number of larvae when compared with the controls (P>0.05) (Table 2), however, mummification levels in the small clump plots, which reached 40 mummified cadavers/m^2, were significantly greater than in the corresponding control plots (P<0.01).

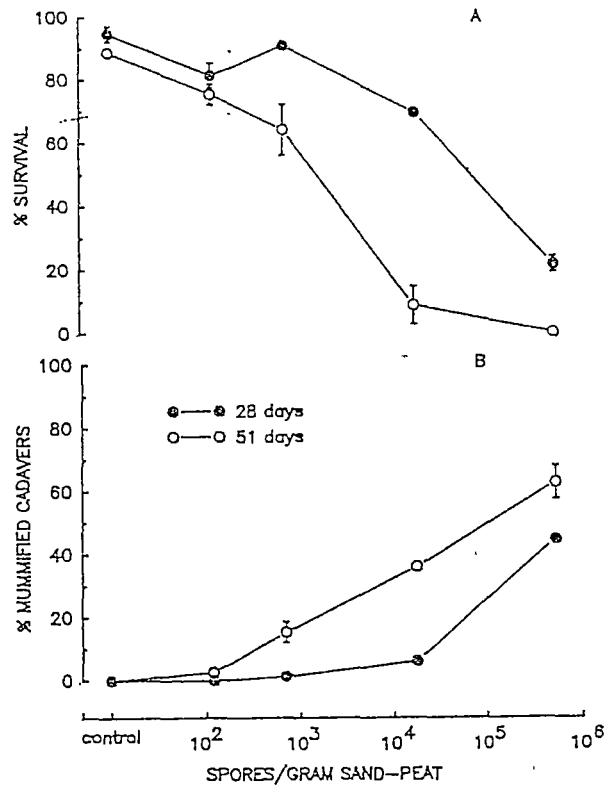

FIGURE 1. The Effect of Exposure Time and Dose Rate of M. anisopliae on (A) Survival and (B) Mummification of Larvae of A. couloni after 28 and 51 Days at 20°C ($\bar{x} \pm$ S.E.).

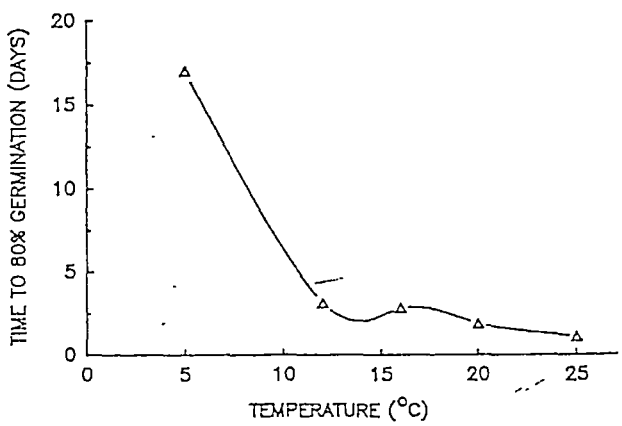

FIGURE 2. The Effect of Temperature on the Mean Time Taken for M. anisopliae to Reach 80% Germination when Grown on Czapek-Dox Agar.

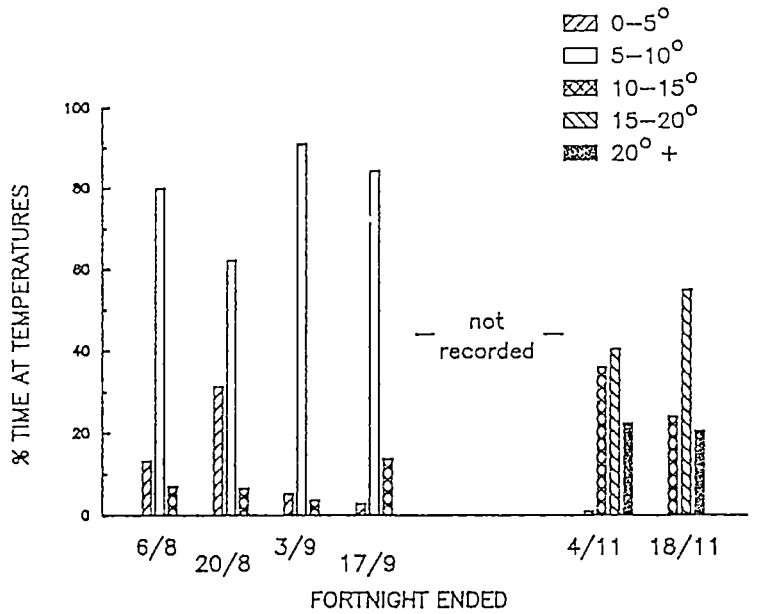

FIGURE 3. Frequency of Soil Temperatures 10cm Below the Surface in Fortnightly Intervals, at the 'Glen Morey' Field Site During the Experimental Period.

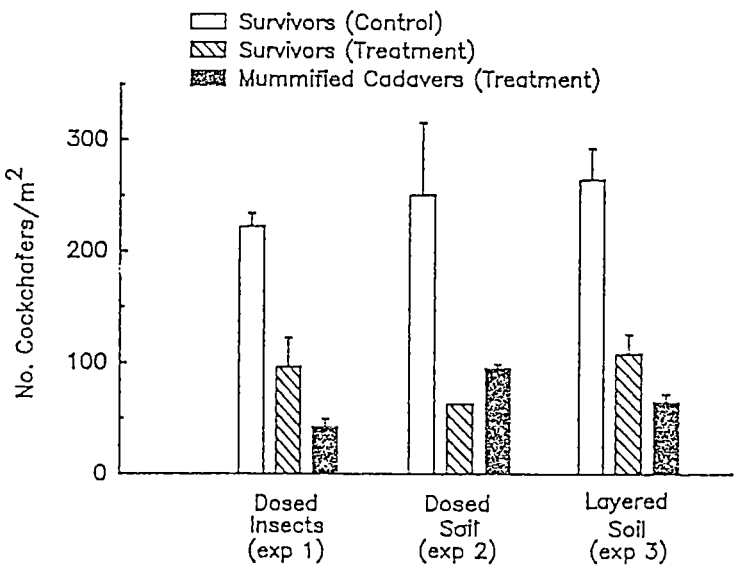

FIGURE 4. The Effect of the Method of Dosing the Soil with M. anisopliae on Survival and Mummification of larvae of A. couloni.

TABLE 2. The Effect of M. anisopliae applied (A) in 4g Deposits in the Soil (Exp. 4) or (B) applied in an aqueous Suspension to the Soil Surface (Exp. 5) on Survival and Mummification of A. couloni larvae[1].

		Control			Treatment		
			Mummified			Mummified	
Exp.#	rep.	Alive	cadavers	Total	Alive	cadavers	Total
4	1	146	3	149	112	40	152
	2	119	1	120	108	9	117
	3	120	2	122	78	9	87
	4	135	2	137	146	32	178
5	1	71	1	72	84	3	87
	2	85	1	86	105	5	110

[1] per m^2

DISCUSSION

M. anisopliae was highly virulent for third instar A. couloni larvae. Larval survival decreased as both dose and time increased (Figure 1), consequently low doses were as effective in killing larvae as high doses if given sufficient time. Though these experiments were conducted at 20°C, M. anisopliae should cause mortality at 5°C as the fungus can germinate at this temperature (Figure 2), however, larval mortality would not be initiated within 70 days (Rath, 1989 in press.).

Mummification of larvae increased when both time and dose were increased, although Rath (op. cit.) has shown that doses higher than 1×10^6 spores/g sand-peat give a reduction in the level of mummification when compared with doses less than 1×10^6.

At the most effective doses (higher than 10^4 spores/g sand-peat) the number of surviving larvae and the number of mummified cadavers did not total the number of remaining control larvae; 40% to 50% of larvae at these doses were 'missing' (Figure 1). The 'missing' cockchafer larvae appear to die of a bacterial septicaemia induced by penetration of the insect integument by the fungus (Rath, unpublished data). Coles and Pinnock (1982) have shown a similar bacterial septicaemia in black-headed cockchafer (Aphodius tasmaniae) after application of M. anisopliae.

The field experiments show that the survival of cockchafers can be reduced by the application of M. anisopliae below the soil surface (Figure 4), consequently, the fungus has the potential to control red-headed cockchafer. The number of survivors and the number of mummified cadavers totalled (as in the laboratory experiments) only 60% of the number in the controls. Large-scale field experiments (planned for 1989/91) will use survival of cockchafers as the criterium upon which the usefulness of M. anisopliae is based because:

A. M. anisopliae reduces the population of cockchafers (both in the

laboratory and in the field) to a greater extent (about 40% more) than indicated by the number of mummified cadavers, and

B. Graziers will judge the effectiveness of the fungus by the reduction in the numbers of living cockchafers (by whatever means) and not by the percentage of larvae killed.

Further research will be directed towards continuing the studies on the distribution and abundance of M. anisopliae biotypes in Tasmanian pastures, the short-term and long-term survival and effectiveness of the fungus as well as the development of a 'home-brew' kit for on-farm use.

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Appendix 2

Rath, A.C. and Yip, H.Y. (1989). Long-term control of the root-feeding cockchafer *Adoryphorus couloni* (Coleoptera: Scarabaeidae with the entomogenous fungus *Metarhizium anisopliae*. In *Proceedings of A Soil-Invertebrate Workshop*. (ed. L.N. Robertson and P.G. Allsopp). QDPI conferences and wordkshop series QC 89004. pp. 2 - 5.

LONG TERM CONTROL OF THE ROOT-FEEDING COCKCHAFER ADORYPHORUS COULONI (COLEOPTERA: SCARABAEIDAE) WITH THE ENTOMOGENOUS FUNGUS METARHIZIUM ANISOPLIAE

A.C. RATH and H-Y. YIP

Tasmanian Department of Agriculture, St. John's Avenue, New Town 7008.

Abstract
Metarhizium anisopliae isolate DAT F-001 is highly virulent to larvae and adults of the redheaded cockchafer, Adoryphorus couloni, over a wide range of temperatures. Production technology and application technology have developed to the stage where effective control of one generation of cockchafer will be achieved using 25 kg of powdered fungus direct-drilled into pasture. Satisfactory control of cockchafer will however, only be achieved if the population causes little damage to winter pastures for a period of up to 10 years. Current data show a rapid reduction in fungal levels in soil after 12 months, though only to near the natural maintenance levels associated with Tasmanian pastures.

Introduction

Redheaded cockchafers (Adoryphorus couloni) are serious subterranean pasture pests in south-eastern Australia. They infest over 3 million ha of pastoral land in Victoria and half of the 900,000 ha of improved pastures in Tasmania. The distribution of the insect is expanding in Tasmania, New South Wales and South Australia and it has become established in New Zealand (McQuillan and Ireson 1987).

Beetles are present in the soil for 10 months of the 2-year life-cycle while the root-feeding larvae are active in the soil for 12 months. "Substantial" loss of pasture dry matter occurs when the number of larvae exceeds 300 m^{-2} (Allen 1986), however, total denudation of pastures occurs in many areas of Tasmania as the number of larvae reach 700-1100 larvae m^{-2} (Rath unpubl. data). Larvae damage the pasture by severing the roots during feeding and through physical disruption of the roots during underground movement. Miller (1989) estimates that in Tasmania the direct short-term pasture loss caused by redheaded cockchafer totals over $1million annually.

M. anisopliae Control of Cockchafer

Several isolates of M. anisopliae have been found in Tasmania which are pathogenic to redheaded cockchafers. One isolate (DAT F-001) is pathogenic to larvae and adults at doses greater than 10^3 spores cm^{-3} soil, while two other isolates (DAT F-054 and DAT F-120) are only pathogenic to adults (but no more so than F-001). The most studied isolate, DAT F-001, germinates at temperatures as low as 2°C (Rath unpubl.) and sporulates at 5°C (Rath unpubl.; G. Riba 1989 pers. comm.). The growth of the fungus at low temperatures is essential for control of cockchafer in Tasmania as the major damage to pastures occurs in autumn and winter (the mean mid-winter temperature 50mm below the soil surface is 7°C). In laboratory studies, the effect of reducing the temperature (from 25°C to 10°C) at which cockchafer are exposed to the fungus, is to increase the time taken to kill larvae but not the number killed (Fig. 1). A field trial established in July 1987 has shown that a dose of 1×10^5 spores cm^{-3} soil can reduce the population by 60% in 110 days (Rath 1989).

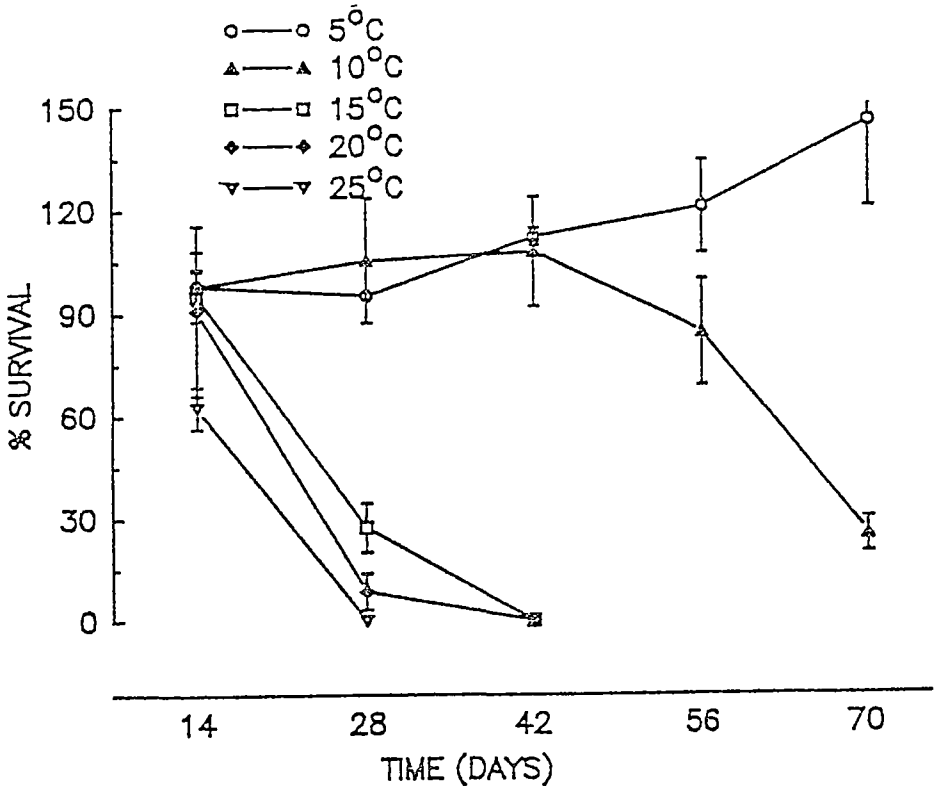

Figure 1. The effect of temperature on survival of third instar A. couloni when exposed to M. anisopliae DAT F-001 at a dose of 1.46 x 10^6 spores/g soil expressed as a percentage of adult survival (mean ± SE)

Production Technology

The Rural Credits Development Fund is supporting a project examining the production of small quantities of M. anisopliae "on-farm". The technology has progressed to the stage where to achieve a dose of 10^5 spores cm^{-3} soil (in the top 10 cm), only 25 kg of unprocessed fungal product/growth medium is required per hectare. To date over 800 kg of Metarhizium product has been produced using 20 litre drums (production units of 225 litres are planned).

Application

Spores of Metarhizium must be placed below the soil surface for effective control of the cockchafer (Rath 1989). Experiments are currently in

progress which are examining application through the seed and fertiliser bins of seed drills both during pasture renovation and by direct-drilling existing pastures.

Long-term Control

The success of this control project will rely on the number of years, after application, over which M. anisopliae can control the cockchafer populations to below the economic threshold (about 100 larvae m^{-2}) because:
(a) The application methods are equivalent to the total renovation of a pasture which would not normally be done for 7-14 years;
(b) M. anisopliae kills the population slowly and even application early in the year will not stop economic damage being caused by the larvae in the autumn;
(c) The longer the fungus can control the population, the greater the economic benefit.

Table 1. Decline in M. anisopliae DAT F-001 spore level following application to a cockchafer infestation in July 1987

Months after application	Spore level per g soil (mean ± SD)
0	1.43×10^5
4	$1.05 \pm 0.3 \times 10^4$
7	$3.12 \pm 1.9 \times 10^3$
10	$3.72 \pm 1.3 \times 10^3$

Prospects for Long-term Control

Survival of Metarhizium spores from the 1987 field trial show a rapid reduction (10-fold) of spores in the first 4 months followed by 6 months where a concentration of 3×10^3 spores cm^{-3} was maintained (Table 1). Rath (1989) has shown that doses of 10^3 to 10^4 spores cm^{-3} soil can effectively reduce the larval population. The Metarhizium (DAT F-001) levels present 10 months after application are very similar to the levels of all M. anisopliae isolates found in Tasmanian pastures (Table 2). Consequently we feel that following an application of Metarhizium (DAT F-001) to a pasture, even though the spore level will drop quite quickly, this new level will still be virulent for larvae and adult redheaded cockcahafer and that the dose should persist for 2-3 years. However, after this time the distribution of Metarhizium (DAT F-001) will become very patchy across a pasture.

Table 2. Colony counts of soil samples yielding M. anisopliae (all isolates) in Tasmanian pastures ($x10^3$)

Count	Frequency
<2	30
2-10	30
11-20	17
21-40	13
41-50	4
51-100	2
>1000	2

Acknowledgments
This research is partly funded by the Wool Research and Development Fund of the Australian Wool Corporation and the Rural Credits Development Fund of the Reserve Bank of Australia.

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Miller, C.S. (1989). Cost of pasture depredation by corbie and cockchafer larvae. Dept. Agric. Tasmania: Hobart.

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Appendix 3

Report of an insecticide field trial using two granulated organophosphates against L3 larvae of *Adoryphorus couloni*.

Redheaded Cockchafer field trial

Conducted for : Cyanamid Australia Pty Ltd.

Conducted by : Bill Fulkerson and Andrew Rath, Department of Primary Industry, Tasmania.

Establishment date : 7.6.90

Report date : 19.12.90

Chemicals tested : 1. COUNTER (R) 150G (0.6kg ai/ha)

: 2. THIMET (R) 150G (0.6kg ai/ha)

Summary:

The redheaded cockchafer is a subterranean beetle grub that causes extensive damage to pastures in Tasmania and Victoria. There are currently no registered insecticides for this pest. A trial was established in June 1990 to examine the efficacy of two granulated insecticides (Counter(R) and Thimet(R)) drilled into two dairy pastures supporting heavy cockchafer numbers (> 250/m²). Examination of the plots 12 weeks after chemical application showed that neither insecticide caused any reduction in cockchafer numbers.

Background:

The distribution, damage, life cycle and current recommendations for control are attached as Appendix 1.

The agreed experimental design is attached as Appendix 2.

Statistical analysis:

The data was analysed using the standard analysis of variance package 'MINITAB'. Analysis of the effect of the chemicals on the insects were performed using both the original counts as well as the difference in the counts from establishment to 12 weeks within each block.

Results:

After application of the two chemicals there was no difference between the control and the two treatments on either farm 1 - Andersons (P = 0.723) or farm 2 - Gracie (P = 0.977). The number of insects was higher on farm 1 (256/m²) than on farm 2 (172/m²) (Table 1).

After 12 weeks there was a reduction in cockchafer numbers in the control plots of both farms (Table 1). In all cases the reduction in the chemically treated plots was less than the control reduction, though sample time was not a significant factor (P = 0.103). This indicates the great variation in cockchafer densities

There was no significant difference (P > 0.552) in the numbers of cockchafers between treatment and control plots on either farm at 12 weeks. Neither of the two treatments reduced (P > 0.825) cockchafer survival over

the 12 week period (Table 1).

Conclusions

Thimet and Counter have had no effect on the survival of cockchafer over the twelve weeks from June to September of 1990. While it had been previously pointed out (Appendix 1) that the cockchafers enter a non-feeding period in mid winter and that they range from 25-150mm below the surface, and hence the chemicals may be less efficacious, we believe that this is not the reason for lack of mortality. The results shown here clearly show that these two products induced no mortality in redheaded cockchafer populations at the time tested. We would have expected at worst a 25% kill if these products were to provide effective control of cockchafers earlier in the season. Consequently, we see no reason for Cyanamid to continue investigation of these chemicals for redheaded cockchafer control.

Table 1: Effect of Thimet and Counter of larval populations of redheaded cockchafer.

	Farm 1 - Anderson's			Farm 2 - Gracie		
treatment	immed post application	12 weeks	% reduction	immed. post application	12 weeks	% reduction
nil	246±31	190±25	22.8	175±24	159±22	9.1
thimet	279±41	239±30	14.3	169±23	159±21	5.9
counter	244±32	205±36	16.0	173±26	179±24	-3.5

* larvae/m^2 ($x \pm s.e$; $n = 20$)

A sampling method to determine the field mortality of the subterranean pasture pest Adoryphorus couloni (Coleoptera: Scarabaeidae) exposed to Metarhizium anisopliae

T.B. Koen and A.C. Rath

Department of Primary Industry, Tasmania.
St. John's Avenue,
New Town.
Tasmania.
Australia. 7008.

The variation in the abundance of the subterranean pasture pest Adoryphorus couloni within a pasture necessitated the tailoring of an experimental design and sampling plan for determining the efficacy of the control agent Metarhizium anisopliae.

The design consisted of paired treatment and control plots (each 10 x 25m) which were randomly assigned within each of four replicate blocks (*see Figure 1*). Where treatments comprised different application times of M. anisopliae, each treatment had its own neighbouring control plot. Each plot was divided into five strata (10 x 5m), from which one randomly located soil sample was taken at any sampling occasion (20 soil samples/treatment/sampling time). Sampling coordinates within each strata were randomly chosen without replacement, and were identical for that specific treatment and control stratum. This strategy of sample pairing, both between strata and between plots within blocks, was adopted to minimise the influence of natural changes in insect abundance upon the results of the experiments.

Each soil sample measured 1.0x0.2x0.15(-0.3)m (width.length.depth). This large quantity of soil was chosen because preliminary studies had shown that the sampling variance declined as the sample unit size increased (*Figure 2*). Twenty such units were taken per treatment (*Figure 3*).

Analysis of preliminary data from this experimental design indicated that significant differences ($P<0.05$) in larval survival between control and M. anisopliae treated plots would not occur until there was a 30% difference in the population levels. In practice, the design was better than predicted (*Table 1*) as one experiment detected ($P=0.022$) a 20% reduction in the survival of insects from M. anisopliae treated plots, and in another experiment, a 30% difference ($P=0.003$). In general, there was no effect of M. anisopliae on an A. couloni population until after a sufficient lag time (based on ambient temperature) had passed. After this, the level of mortality in treated insects would change from 0% to 30-35% between sampling periods. Consequently, this experimental design was well suited to analyse the effect of M. anisopliae on A. couloni.

Further details attached

Development of a Cockchafer Sampling Strategy

In defining a sampling strategy to count cockchafer grubs, a question which arose quite early in the study was: *how large a soil sample should be taken?* A secondary question to the first was: *how many of these sampling units should be taken?*

The following field layout was used:

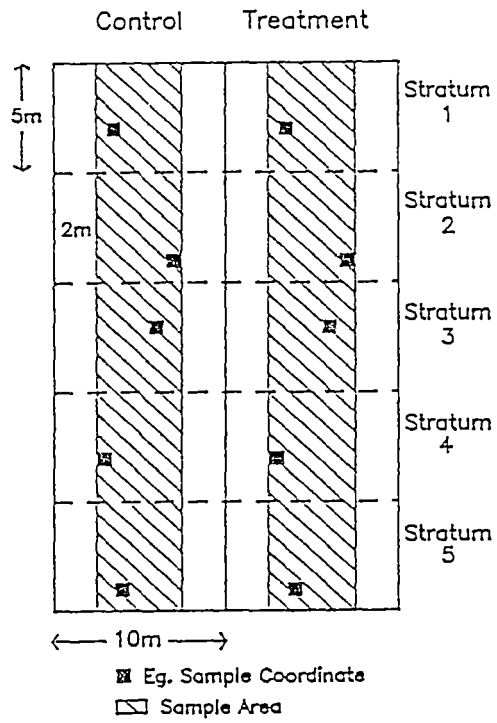

Figure 1

In preliminary samplings, three (3) immediately adjacent 0.2x0.2m quadrats were dug, and cockchafer density counted separately for each quadrat unit. This action permitted an investigation to be made of the changes in count variability as sample unit size increased, namely either 0.2x0.2 or 0.2x0.4 or 0.2x0.6m quadrats. The data for 10 such samplings of three quadrats follows:

Sample no.	Number of grubs per 0.2x0.2m unit of soil		
	Quadrat 1	2	3
1	14	4	1
2	6	0	1
3	5	4	4
4	0	4	1
5	4	9	7
6	6	24	5
7	10	11	9
8	4	9	16
9	6	3	6
10	1	6	4

The previous table gives 3^{10}(=59049) ways of "sampling" single 0.2x0.2m units. I have chosen only 3, namely those data for quadrats 1, 2 and 3. The table offers 2^{10} combinations of "sampling" 0.2x0.4m units; I have chosen to form the sum of only 3, namely 1&2, 1&3 and 2&3. The table only gives 1 "sample" of 3 adjacent plots, which I have used.

The following calculations have been performed after adjusting the grub counts to numbers/square metre.

Data set	mean	s.e.	s.e.(% μ)	CV	average CV
(Column 1 x 25)	140.0	32.1	(23%)	73	
(Column 2 x 25)	187.5	52.1	(28%)	88	83
(Column 3 x 25)	132.5	37.3	(28%)	89	
(Col 1 + 2)/2 x 25	163.7	31.3	(19%)	60	
(Col 1 + 3)/2 x 25	136.2	23.8	(17%)	55	63
(Col 2 + 3)/2 x 25	160.2	36.8	(23%)	73	
(Col 1+2+3)/3 x 25	153.3	26.8	(17%)	55	55

These average CV values were graphed against the size of the sample unit, producing the following display, *Figure 2.*

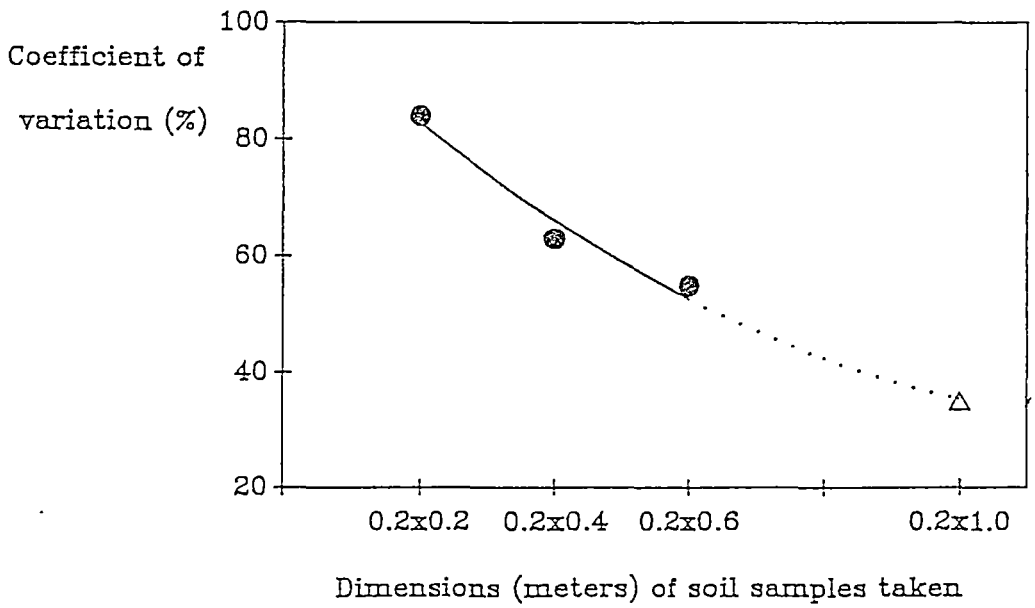

Figure 2. Increasing the dimensions of the soil sample unit decreased the C.V.%. A 0.2x1.0m quadrat was chosen as the size of the sampling unit throughout the trials.

The judgement was made that the CV% would continue to decline in the form of a hyperbola, and therefore a sampling unit of 0.2x1.0m was chosen. Any larger size unit than this was considered not worth the extra resources it demanded. *(Note: On the basis of what follows, a trial was established, and pre-treatment sampling of 20 samples of 0.2x1.0m plots gave an average CV% of 35%, a figure which fits suitably into the shape of Figure 2).*

Making use of the concepts of Variance Components, an Analysis of Variance table was calculated from the original data matrix, resulting in the following table:

Source	DF	SS	MS	
Between sampling sites	9	310.1	34.46	$= s_u^2 + 3s_b^2$
Between sample units within sites	20	457.3	22.87	$= s_u^2$
Total	29	767.5	26.46	

This implies that the between sampling unit variance $s_u^2 = 22.87$
and that the between sampling sites component of variance $s_b^2 = 3.8635$

These components of variation allow the creation of a graph to aid in the establishment of a sampling plan. Since

$$\text{variance}(\mu) = (s_u^2 + Us_b^2) / B*U$$

$$= s_u^2/B*U + s_b^2/B$$

and U (the size of the sample unit) has been chosen to be 5 units (i.e. 0.2x1.0m), it remains to solve this equation over a range of B values (the number of sample sites).

Interpretation of this graph is made simpler if the Y-axis is changed from variance(μ) to the standard error(μ) as a percentage of the mean. The necessary calculation is

$$\text{S.E.}(\mu)\,\% = 100/6.133 \; * \; \sqrt{\left\{ \frac{22.87}{5*B} + \frac{3.8635}{B} \right\}}$$

The accompanying ***Figure 3*** suggests that 20 samples of a 0.2x1.0m sampling unit will give estimates of cockchafer density with standard errors of approximately 10% of the mean.

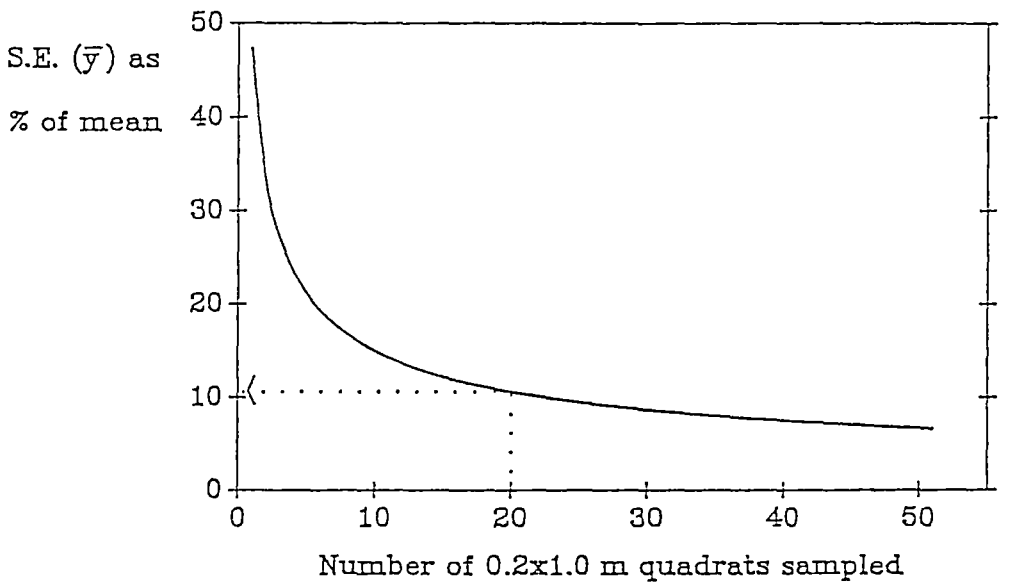

Figure 3. Sampling variance declined with increasing number of quadrats taken. 20 such samples were used per treatment per time of sampling.

Interest now lay in examining how sensitive this sampling strategy had been. The mechanics of how this is done is shown below for the "Les July" site using the statistical software MINITAB. The 20 plots for both the control and treated groups are expressed as percentages of the immediately pre-treatment grub counts. Since the plots were paired, a 'paired t-test' is used.

ROW	control	treated	con - trt
1	36.538	60.6690	-24.1310
2	69.231	43.9330	25.2980
3	48.077	37.6570	10.4200
4	36.538	25.1050	11.4330
5	46.154	31.3810	14.7730
6	73.298	20.0620	53.2360
7	69.372	44.7530	24.6190
8	103.403	67.9010	35.5020
9	54.974	78.7040	-23.7300
10	49.738	60.1850	-10.4470
11	61.983	63.6600	-1.6770
12	27.893	43.7670	-15.8740
13	41.322	43.7670	-2.4450
14	87.810	26.5250	61.2850
15	61.983	47.7450	14.2380
16	45.082	36.1990	8.8830
17	114.754	44.1180	70.6360
18	76.503	38.4620	38.0410
19	81.967	31.6740	50.2930
20	96.995	45.2490	51.7460

```
MTB > describe c1 c2 c3


                N      MEAN    SEMEAN
control        20     64.18      5.37
treated        20     44.58      3.40
con-trt        20     19.60      6.35
```

```
MTB > divide 19.60 by 6.35, store in k1

    ANSWER =        3.0866
```

.....this calculates the t statistic

```
MTB > divide 19.60 by 64.18 ,store in k2

    ANSWER =        0.3054
```

.....thus the 30.5% observed reduction

```
MTB   cdf 3.0866;
SUBC> t 19.

    3.0866     0.9970

MTB > subtract 0.9970 from 1, hold in k2

    ANSWER =        0.0030
```

.....the probability of this t statistic being due to chance is 0.003

```
MTB > invcdf 0.95;

SUBC> t 19.

    0.9500     1.7291
```

To be significant at P=0.05 on a one tail t-test, the ratio of the mean difference to its standard error should equal 1.7291. Thus, if one assumes that the standard error remains unaltered; then the minimum difference required to be deemed significant is the product of 1.7291 and the standard error.

```
MTB > multiply 1.7291 by 6.35, k1

    ANSWER =          10.9798.....the minimum difference in % points

MTB > divide 10.9798 by 64.18, k1

    ANSWER =           0.1711.....the minimum % reduction required over the
                                  controls to be judged significant is 17%
```

Data from other sites have been similarly analysed to produce the following table:

Site	Control	Treated	% Reduction	Significance Probability	Minimum % reduction at P=0.05
Les July	64.2	44.6	30.5%	0.0030	17.1
Les Sept	67.5	41.9	37.9%	0.0001	13.5
Bur ST3	58.4	46.2	20.9%	0.0220	16.6
Web Lo	54.4	25.8	52.6%	0.0003	21.9
Web Hi	54.4	24.7	54.5%	0.0002	22.3

Table 1. Based on the first statistically significant data from these experiments, M. anisopliae reduced A. couloni numbers an additional 20-55% over the natural mortality in the control plots. This sampling strategy is capable of detecting (P=0.05) reductions of 13-22% over the controls.

Appendix 5

AAVCC response to a preliminary submission for the clearance of *Metarhizium anisopliae* for control of *Adoryphorus couloni*.

STRALIAN AGRICULTURAL & VETERINARY CHEMICALS COUNCIL

G90/298
329-1-888

Telephone contact: (06) 2716373

3 December 1990

Mr A.C. Rath
Insect Pathologist
Department of Primary Industry
New Town Research Laboratories
Gpo Box 192B
HOBART TAS. 7001

Dear Mr Rath

DATA REQUIRED FOR CLEARANCE AND REGISTRATION OF
METARHIZIUM ANISOPALIE 5 OCTOBER 1990

Following on from our telephone conversation of 15 November 1990, discussing the regulatory requirements for Metarhizium anisopalie, your submission protocol has been assessed and we offer the following comments on your data :

- the composition of the material which is used for coating the pasture seeds should be identified

- the data in appendices 5, 6 and 8 relating to toxicological potential are in the form of a brief review of previous reports; copies of these previous reports are required

- a discussion is needed on the possibiliity of translocation of the fungus into the crop and the likelihood of residues in animals which ingest the organism.

Overall, the "protocol" you have provided seems fairly sound.

In the event that you decide to market your product, Federal clearance will be necessary and you will need to conform to the Agricultural and Veterinary Chemicals Act 1988 and Regulations 1989 and the " Requirements For Clearance of Agricultural and Veterinary Chemical Products 1989"; (if you do not already have these documents they are available from the Australian Government Publishing Service bookshop in Hobart).

Usually, I suggest contacting the State Registrar to obtain the name of a reputable consultant to assist with the "finer" points of a clearance application, however as you are part of the Tasmanian Department of Primary Industry, Mike Norman (Pesticide Registrar for Tasmania) may be able to assist you directly.

mmonwealth Department of Primary Industries & Energy
P.O. Box 858 Canberra A.C.T. Australia 2601
-o1 6 2723933 Fax. +o1 6 2724753

A fee is payable in respect of an application for clearance of a chemical product and the appropriate application fee can be determined from the Regulations (probably $20,000).

I have enclosed a Checklist for Agricultural and Veterinary Chemical Submissions which will assist you when you are preparing a submission for clearance.

Please pay careful attention to the development of clear, consise claims for use, proposed labelling and the application for clearance.

Yours sincerely

Jeanine Crowther
Senior Product Manager
for Chemicals Co-ordinator.

Appendix 6

Distribution of soils sampled for *Metarhizium anisopliae* in Tasmania (Fig. 1) and the distribution of soils which yielded *M. anisopliae* (Fig. 2)

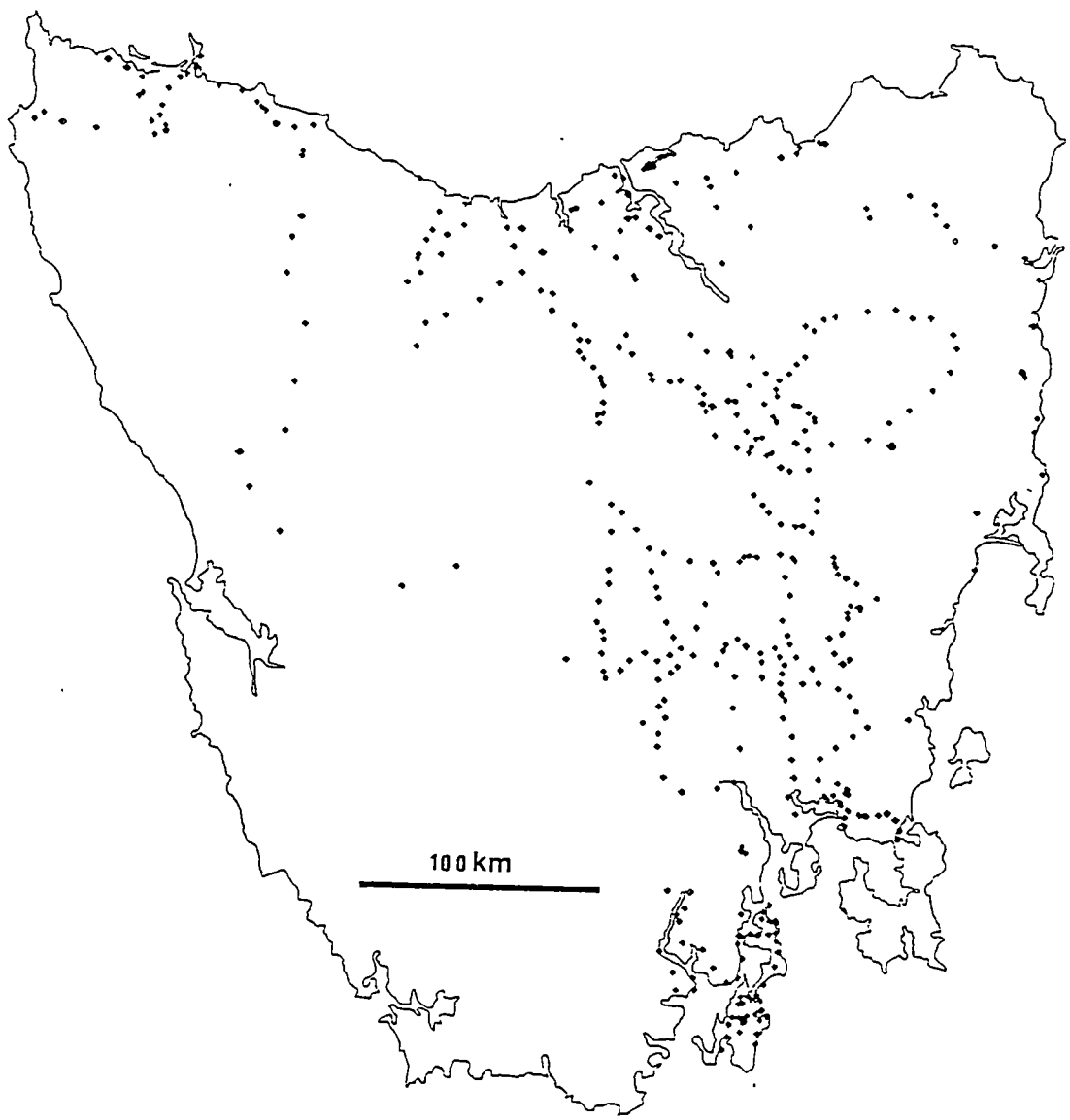

Figure 1 Distribution of soils sampled for *Metarhizium anisopliae* in Tasmania

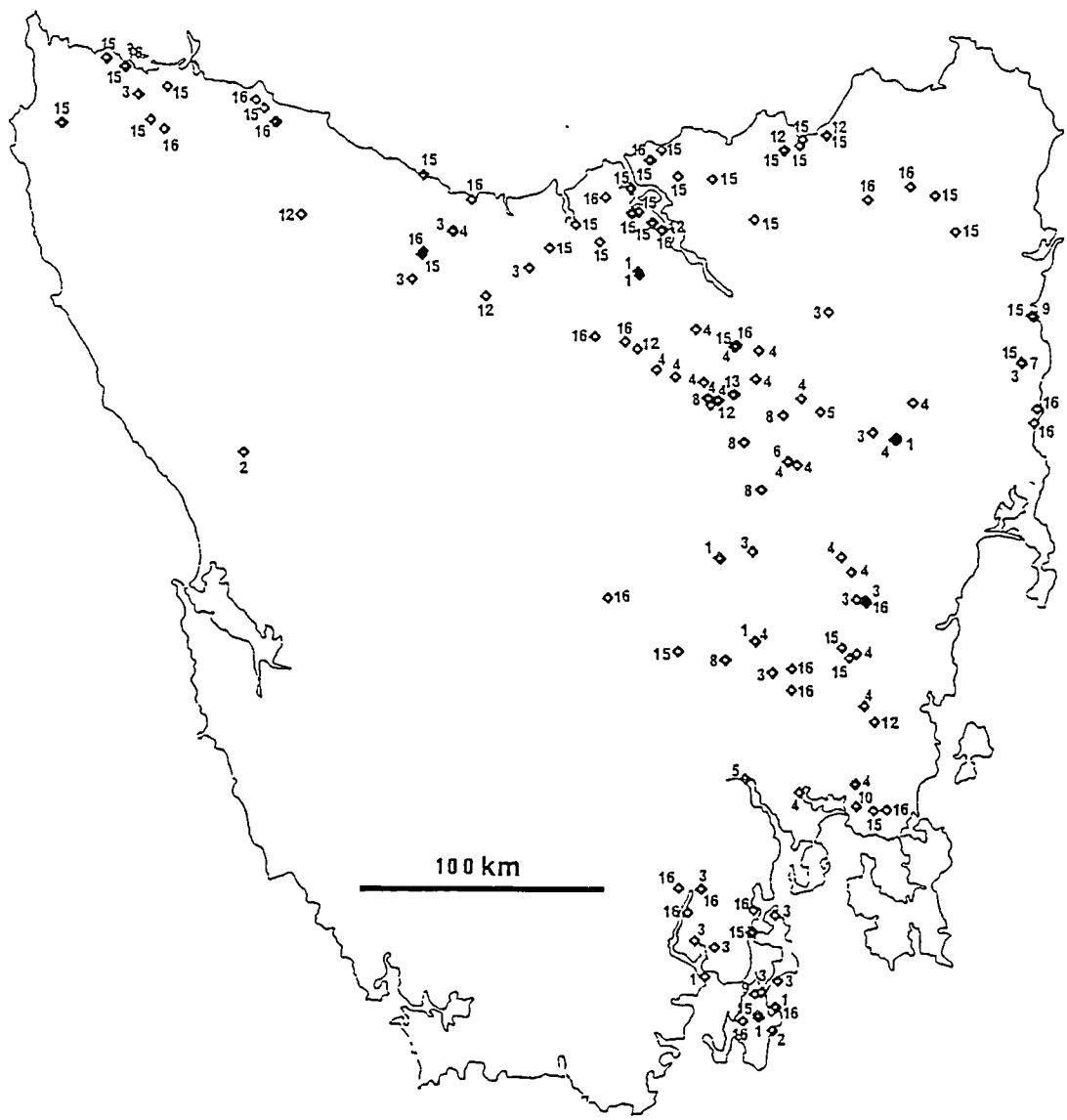

Figure 2 Distribution of soils which yielded *M. anisopliae*

Appendix 7

Rath, A.C., Pearn, S. and Worladge, D. (1990). An economic analysis of production of *Metarhizium anisopliae* for control of the subterrean pasture pest *Adoryphorus couloni.* In *Proceedings of Vth International Colloquium on Invertebrate Pathology and Microbial Control.* (ed. D.E. Pinnock). p.13. 20 - 24 August 1990, Adelaide, Australia

An Economic Analysis of Production of *Metarhizium anisopliae* for Control of the Subterranean Pasture Pest *Adoryphorus coulonı*.

A.C. Rath, S. Pearn and D. Worladge

Department of Primary Industry, Tasmania.
St. John's Avenue, New Town. Tasmania. Australia. 7008.

As a part of our study of *Metarhizium anisopliae* control of *Adoryphorus coulonı* we have been developing a fungal mass production system which requires very low technological input. The system utilizes local grains and differs significantly to that used in Brazil. Spore production is typically 1 x 10^9 spores/g grain. Application of 10-100 kg grain-fungus mix/ha of pasture is required to achieve a soil dosage level of between 1 x 10^4 and 1 x 10^5 spores/g soil. Field experiments using this formulation of *Metarhizium* at doses of 4.5 - 16.0 x 10^4 spores/g soil have given 60-80% control of *A. coulonı*.

A. coulonı are distributed over ca. 350,000 ha of Tasmania's 900,000 ha of improved pasture. It has been estimated that ca. 16,000 ha of the improved pasture is damaged (medium to severe) by the pest in an average year, costing graziers A$1.1 million in short-term productivity loss. Longer-term costs are probably 3-5x higher. Up to half of this damaged area may require pasture renovation within the following four years, leading to the renovation of 8,000 ha/yr as a direct consequence of *A. coulonı* infestation. We believe that the most appropriate application method is by direct-drilling with seed at the time of pasture renovation. The Australian Bureau of Statistics currently estimates that 40,000ha of improved pasture is resown in Tasmania every year. An initial 25% penetration of this market is possible. Either market analysis suggests that the fungus would be sown into ca. 10,000 ha/yr.

250 tonnes of our grain-fungus product would be required to treat the 10,000 ha/yr of pasture being renovated. We have estimated the cost of this production at A$46/ha, of which 33% is labour cost and only 10% is substrate cost. These costings do not include establishment costs such as product registration, factory incubation space, machinery or office requirements. The cost is double that of chemical insecticides used to control other pasture pests (*A. coulonı* has no registered insecticides) and would add ca. 30% to the costs of pasture renovation.

Control costs for the 16,000 ha damaged by *A. coulonı* amount to ca. A$750,000 but *M. anisopliae* has shown the potential to control the pest for 5-10 years. This lowers the control costs to between A$75,000/yr and A$145,000/yr or only 10% of the costs associated with short-term damage by the pest (A$1.1 million).

This assessment has shown that the costs of control of *A. coulonı* with *M. anisopliae* is significantly lower than even the short-term production loss due to pest damage. However, for commercial production of this select *M. anisopliae* strain to succeed, industry-government co-operation will be necessary to help establish production in Tasmania.

Appendix 8

Rath, A.C. and Headlam, N. Lack of enhancement of the efficacy of *Metarhizium anisopliae* against the soil scarab *Adoryphorus couloni* by the addition of the chemical insecticides Chlorpyrifos and Fenitrothion. (unpublished data).

SCIENTIFIC NOTE

Lack of enhancement of the efficacy of *Metarhizium anisopliae* against the soil scarab *Adoryphorus couloni* by the addition of the chemical insecticides Chlorpyrifos and Fenitrothion.

A.C. Rath and N. Headlam
Department of Primary Industry, Tasmania
St. John's Avenue
New Town. Tasmania. 7008
Australia

Metarhizium anisopliae DAT F-001 is a cold-tolerant isolate which is able to induce mortalities of greater than 70% in field populations of the redheaded pasture cockchafer, *Adoryphorus couloni* (A.C. Rath, T.B. Koen, D. Worladge, G.C. Anderson. *Proc. Vth Int. Coll. Inverebr. Pathol. Microbial Control.* (1990). However, DAT F-001 is slow-acting and application of the fungus during a cockchafer infestation would save little pasture in the year of application (A.C. Rath and S.G. Pearn, In "*Weed, Invertebrate and Disease Pests of Australian Sheep Pastures*" E. Delfose (Ed) 1990).

In seeking a more rapid cessation of pest damage, we tested the efficacy of DAT F-001 in combination with the chemical insecticides chlorpyrifos and fenitrothion. There are currently no chemical insecticides registered for this pest in Australia.

Preparation of each treatment (Table 1) and Generalised Linear Modelling statistical analysis followed the method of A.C. Rath, G.C. Anderson, D. Worladge and T.B. Koen (in review, 1990). The treatments were prepared by mixing the fungal spores and/or the liquid insecticide with 22.5 kg of sand-peat (1:1). The moisture content was adjusted to 20% (w/w). Each treatment and an untreated control were divided into three replicate galvanised iron trays (400x300x80 mm) with lids (7.5 kg/rep.) and 75 final instar (3rd) larvae of *A. couloni* were added to each tray (1350 larvae). The experiment was incubated at 20° ± 2°C. Larval survival was expressed as a percentage of the untreated control at each time.

Fenitrothion reduced the larval survival more quickly ($P<0.0001$) than DAT F-001 (Fig. 1A) but after 21 days the fenitrothion-induced mortality leveled off and by 28 days the survival of DAT F-001 treated larvae and fentrothion treated larvae was the same. There was no difference ($P=0.542$) between the survival of larvae treated with the combination of DAT F-001 and fenitrothion, and those

treated with only fenitrothion. However, the time x treatment interaction was significant (P=0.011) and this can be seen in the crossing over of the curves (Fig. 1A).

The decline in larval survival in the chlorpyrifos treatment (Fig. 1B) was less (P<0.0002) than either the DAT F-001 treatment or the fenitrothion treatment. There was no difference (P=0.359) in larval survival between the DAT F-001 treatement or the combined DAT F-001 + chlorpyrifos treatment.

Mummification was reduced in both insecticide + fungus treatments when compared to the fungus alone treatment. At 28 days, there were 57.3 ± 5.3% (x ± s.d.) mummified larvae in the DAT F-001 treatment, while only 27.2 ± 4.3% of larvae were mummified in the combined DAT F-001 + fenitrothion treatment, and 37.6 ± 13.8% in the DAT F-001/chlorpyrifos treatment.

A. Yevlakhova (*Proc. 1st Joint US/USSR Conf. on Production, Selection and Standardization of Entomopathogenic Fungi.* 1978) reports that the addition of sublethal doses of pesticides can increase the suceptibility of insects to pathogenic fungi. The lack of enhancement of mortality in our experiements, when either of the chemicals is combined with the fungus, is difficult to explain. C.M. Ignoffo, D.L. Hostettler, C. Garcia and R.E. Pinnell (*Environ. Entomol.* 4,765-768. 1975) have shown that chlorpyrifos marginally inhibits the growth of the fungus *Nomuraea rileyi*. A direct germination or mycelial growth inhibition by the insecticides would have effectively reduced the DAT F-001 dose in the combined treatments. Perhaps the reduced mummification levels found in the combined insecticide/DAT F-001 treatments is evidence of this.

Another possibility is due to the behavioural change that occured in larvae exposed to either insecticide. In these trays, after 4-7 days, larvae were exhibiting nerve spasms and unlike the control or DAT F-001 treated larvae, were

often found on the surface of the sand-peat. These larvae were not actively moving through the soil and would encounter a lesser dose of DAT F-001. The nerve spasms occured later and were less prevalent in the chlorpyrifos treatments than in the fenitrothion treatments. This argument is supported by the higher mummification in the DAT F-001 + chlorpyrifos treatments.

The agricultural implication of this study is that there is no advantage in adding these insecticides to the DAT F-001 formulation. In the field the mortality rate in a population treated with insecticides would be slower than indicated here as a granulated formulation would be required for drilling into the pasture soil. At the rates used in this experiment, the cost of the fungal product would be US$1,500/ha (A.C. Rath, S. Pearn, D. Worladge. *Proc. Vth Int. Coll. Invertebr. Pathol. Microbial Contr.* p. 13 1990), compared to the insecticides at US$30/ha. However, A.C. Rath, T.B. Koen, D. Worladge and G.C. Anderson (op. cit.) have achieved 74% control of larval populations in the field using a dose of only 4.0×10^4 spores/g soil (US$60/ha). The higher cost of the fungal product would be offset by the potential long-term control available.

KEYWORDS: *Metarhizium anisopliae, Adoryphorus couloni,* chemical insecticides, chlorpyrifos, fenitrothion, bioassay, synergism, antagonism.

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TABLE AND FIGURE LEGENDS

Table 1: Survival-time regression equations and LT_{50} values for third instar larvae of *A. couloni* exposed to sand-peat mixes treated as listed and incubated at 20°C.

Figure 1: Survival-time regression equations and mean values for third instar larvae of *A. couloni* exposed to sand-peat mixes treated with DAT F-001 and (a) fenitrothion and (b) chlorpyrifos.

Table 1

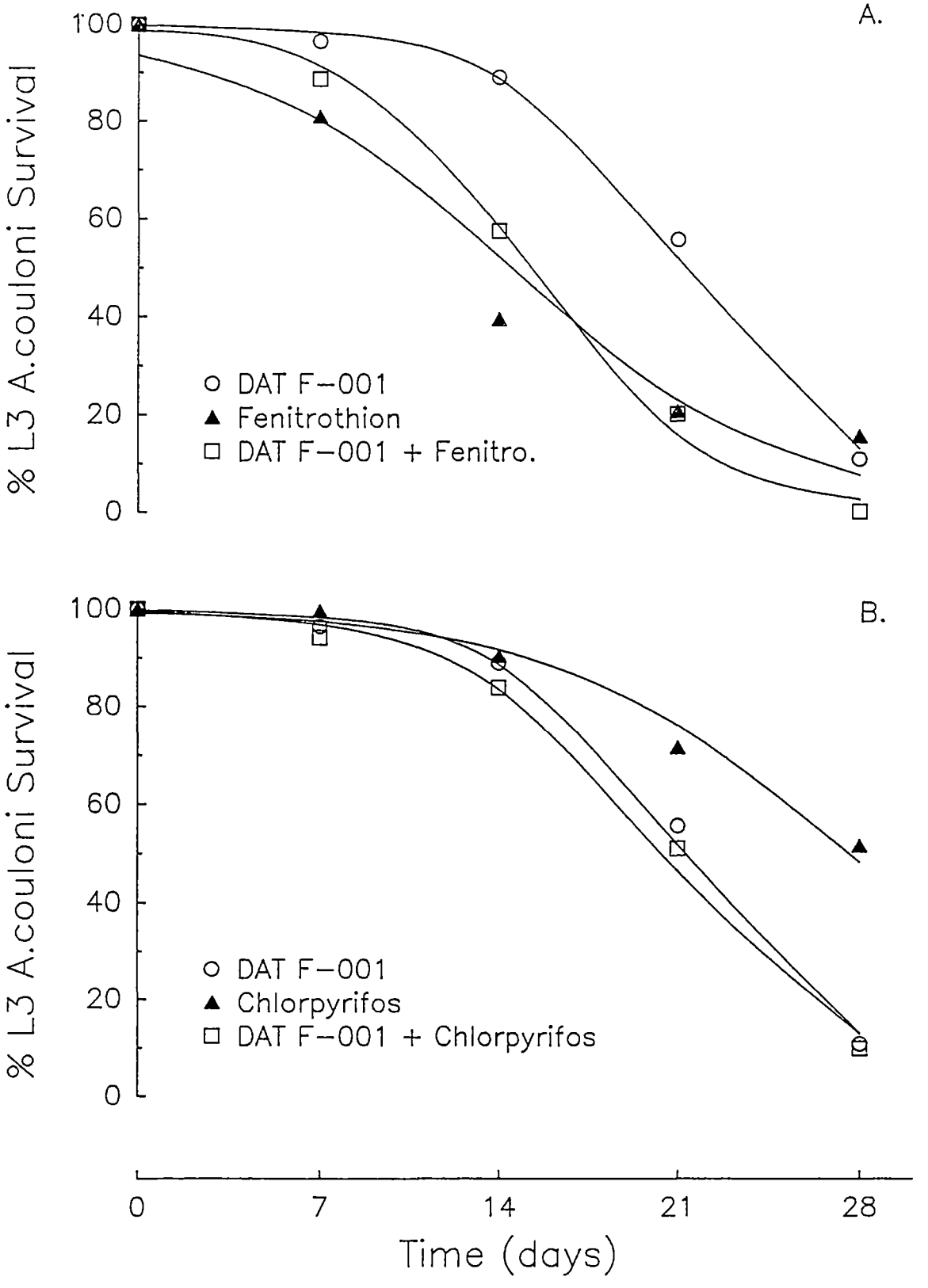
Treatment	Regression equations[1] a estimate	a S.E.	b estimate	b S.E.	sig.[2]	LT_{50} (days)
anisopliae (AT F-001) (10^6 sp/g)[3]	5.99	0.317	-0.282	0.015	a	21.3
nitrothion (0.014g/10kg)[3]	2.68	0.349	-0.185	0.017	b	14.5
lorpyrifos (0.009g/10kg)[3]	4,84	0.420	-0.175	0.019	c	27.7
T F-001 + nitrothion	4.35	0.390	-0.287	0.020	b	15.2
T F-001 + lorpyrifos	5.15	0.414	-0.252	0.020	a	20.4

$$\text{Proportion surviving} = \frac{e^x}{1 + e^x}$$

where $x = a + b \times \text{time(days)}$

Significance: Equations followed by the same letter do not differ significantly ($P<0.05$).

Quantities are per weight of a sand-peat (1:1) mix.


A.
100
80
60
40
20
0
% L3 A.couloni Survival
DAT F-001
Fenitrothion
DAT F-001 + Fenitro.
B.
100
80
60
40
20
0
% L3 A.couloni Survival
DAT F-001
Chlorpyrifos
DAT F-001 + Chlorpyrifos
0
7
14
21
28
Time (days)

Appendix 9

Papers arising from this research

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