CROP PROTECTION PROGRAMME

DEVELOPMENT OF MYCOPESTICIDES AND PHEROMONES FOR COCOA MIRIDS IN GHANA

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FINAL TECHNICAL REPORT

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EXECUTIVE SUMMARY

Cocoa is the main export crop of Ghana, and the livelihoods of over six million people depend on it. Mirids are the most important insect pests causing losses estimated at 100,000 tonnes or 25% of the crop. Currently conventional insecticides provide the only effective methods of control, but the recommended spray programmes are rarely used by farmers because of expense and difficulty of application, as well as being environmentally and ecologically damaging.

The Purpose of the project was to develop and promote improved methods for control of insect pests of cocoa. The project aimed to contribute to this Purpose by developing effective mycoinsecticides against the two main mirid pests of cocoa in Ghana, *Distantiella theobroma* and *Sahlbergella singularis*, and identifying the sex pheromones of these species. Mycopesticides and pheromones are non-toxic to animals and plants and specific for the target pest. Mycopesticides can be used as replacements for contact insecticides. Pheromones can be used in traps to monitor and control pest populations. There are also possibilities for using the two approaches together to lure insects to a source of mycopesticide.

Research activities included carrying out surveys for entomopathogenic fungi, characterising the pathogens found, mass-producing candidate pathogens and developing suitable formulations for evaluating these in the field. Collections of pheromone were made from live mirids and analysed to isolate candidate pheromone components. These were identified and synthesised, and slow-release formulations developed for evaluation of the synthetic materials in field trapping experiments with natural and synthetic pheromone. The UK institutes provided training to CRIG counterpart staff in UK and Ghana.

Research outputs included isolation of a strain of *B. bassiana* from one of the mirid species. This was shown to be highly pathogenic to *S. singularis* and had good rate of growth, sporulation and long-term viability characteristics. The pathogen was mass-produced on rice and various water- and oil-based formulations tested so that the material is now available for field evaluation. Volatiles were collected from males and females of both mirid species and analysed using gas chromatography coupled to electroantennographic recording. The same two active compounds were detected with both species and these were eventually identified and synthesised as novel compounds, despite the small amounts produced by the insects. Slow-release formulations were developed. In trapping tests, production of a pheromone by female *S. singularis* was demonstrated for the first time. Blends of the synthetic compounds were shown to be attractive to male *S. singularis* and probably also *D. theobroma*, although populations of the latter were very low. Two CRIG staff were trained in insect pathology and pheromone analysis in UK, and on-the-job training was given during 12 visits by UK staff to Ghana.

The project outputs provide new technologies that can now be evaluated for use in control of cocoa mirids in Ghana and neighbouring countries. These biorational approaches will help minimise use of conventional pesticides and should be more appropriate for use in developing countries and more sustainable. Cost-effective improvements in productivity of cocoa will benefit the livelihoods of over six million people in Ghana alone who depend directly or indirectly on cocoa.

BACKGROUND

Cocoa is the main export crop of Ghana, and the livelihoods of over six million people depend directly on it. Mirids (Heteroptera: Miridae) are the most important insect pests causing losses estimated at 100,000 tonnes or 25-30% of the crop. Currently conventional insecticides provide the only effective methods of control, but the recommended spray programmes are rarely used by farmers because of expense and difficulty of application, as well as being environmentally and ecologically damaging.

Cocoa mirids

There are two main mirid species in Ghana, *Distantielle theobroma* Dist. and *Sahlbergella singularis* Hagl., and two very minor ones, *Helopeltis lelandei* Carayon and *Bryocoropsis laticollis* Schum. They occur at low overall densities and often aggregate in "pockets" where conditions are particularly favourable, e.g. a break in the shade canopy. The nymphs and adult bugs feed on the stems, branches and pods, and stems and branches wilt above the point of attack. The feeding punctures can also serve as points of attack by plant pathogenic bacteria and fungi. Because both nymphs and adults probe the plant very frequently and each feeding puncture is injurious, serious damage can be caused at low population densities.

The Cocoa Research Institute, Ghana, (CRIG) has shown that effective control of mirids can be achieved with four sprays of insecticide such as Lindane or Propoxur during the main capsid season in August - December. The cost of chemical control was estimated to be between £24 and £30 per ha in 1991, but in surveys conducted by the CRIG Farming Systems Unit (FSU) only 0.7% of cocoa farmers had sprayed anything and only 0.4% had applied the recommended number of sprays, often not at the appropriate time. Cost of insecticide was a major factor, but capital outlay for a mistblower sprayer and the difficulties in carrying out the spraying also contributed towards the extremely low uptake of the recommendations.

Entomopathogenic fungi

Entomopathogenic fungi are particularly suitable as an alternative to chemical control for pests such as mirids which can be controlled effectively by spraying contact chemical insecticides. In addition to direct initial contact, insects can become infected by subsequent contamination from spores which remain viable for several days on plant surfaces and it is possible to have extended periods of control because of secondary cycling in which an infected insect dies and sporulates, releasing more conidia into the environment to infect other hosts (Thomas *et al*., 1995, Wood and Thomas 1996). Individual isolates of these fungi have restricted host ranges, making them more specific than chemical insecticides and they are generally undemanding in their growth requirements so that production is possible on locally-available materials in the country where they will be used.

Mirids are subject to infection by several entomopathogenic fungi, especially the widespread and well-known *Beauveria bassiana*. Lim *et al* (1989) showed that a Malaysian isolate from *H. theobromae* was ineffective to this insect by dusting or spraying. Two isolates from cocoa

mirids in Papua New Guinea are held at CABI and the USDA-ARS ARSEF Collection also holds isolates from Miridae. In Ghana, a bacterial pathogen of cocoa mirids was identified by Bolton (1973) and initial work on entomopathogenic fungi showed that *Entomophaga grilli*, *Fusarium* sp. and *Rhizopus* sp. infect both *D. theobroma* and *S. singularis* (Brew, 1992; Padi *et al.*, 1996(a); Padi and Ackonor, 1997).

Recent advances have demonstrated that entomopathogenic fungi can survive more adverse conditions than previously suspected: Formulation can survive at least two months at 50°C with minimal loss of viability (Morley-Davies *et al*., 1996). Ultra-violet irradiation is the most serious hazard (Moore *et al*., 1993, 1996a), but less of a problem in the shaded cocoa ecosystem. Under optimal conditions conidia remain viable for years and even under tropical temperatures can remain viable for many months (Moore *et al*., 1995, 1996b).

A limitation on the effectiveness of these pathogens as control agents has previously been the erratic success in infecting the target following application of conventional, water-based formulations. In recent years, successful field trials using ULV, oil-based formulations of *Metarhizium* and *Beauveria* have been carried out against locusts and grasshoppers in Africa. ULV application reduces the labour required to spray large areas of crop, and the oil-based formulations are more infective than comparable water-based formulations, particularly at low humidities. In the humid cocoa ecosystem this may not be so important and cheaper, water-based ULV formulations may be adequate.

B. bassiana and *Metarhizium anisopliae* have been subjected to mammalian safety testing (Laird *et al*., 1990). No hazards are associated with *M. anisopliae* and with *B. bassiana* only occasional allergic reactions were reported following inhalation of dry spores. Both genera fall into hazard Group 1 in UK (Categorisation of pathogens according to hazard and categories of containment: Advisory Committee on Dangerous Pathogens of the UK Health and Safety Executive, 2nd edition 1990). This group is the lowest level of hazard and contains all fungi "most unlikely to cause human disease".

B. bassiana has a wide host range, but individual isolates have more restricted host ranges. It is often possible to demonstrate heterologous infection under laboratory conditions where behaviour patterns which prevent infection in the field cannot occur, but field infection of non-target hosts is likely to be much rarer. For example. no field infection of non-target hosts was observed in a field study on the use of *B. brongniartii* against cockchafers in Switzerland (Baltnerweiler and Cerutti 1986).

Pheromones

One of the two main mirid species found in Ghana, *D. theobroma*, was shown to produce a female sex pheromone by Andrew King (1973). No chemical work was done on this at the time, but recently the chemical compositions of the female sex pheromones of several mirid species have been shown to consist of blends of relatively simple chemical compounds (e.g. Smith *et al*., 1991; Miller *et al.*,1997; Miller and Rice, 1998).

A project, "Integrated Pest Management in the Ghana Cocoa Industry" was set up in August 1995 between CRIG and Longwood College, Virginia, USA, funded by the American Cocoa Research Institute (ACRI). The aim of this was to identify the pheromones of all four mirid

pests of cocoa in Ghana and to develop their application in the field. Little progress was made on pheromone identification, but facilities for insect rearing were established and work has focussed on plant volatiles affecting mirid behaviour.

Pheromones too are non-toxic to animals and plants and highly specific for the target pest. As such they are compatible with all other methods of pest control and ideal components of integrated pest management systems.

Pest management options

The approaches being explored in this project can be used separately in pest management, although they are mutually compatible and possibilities for their combined use will be explored.

Thus mycoinsecticides provide a relatively specific, environmentally acceptable replacement for conventional insecticides which are more amenable to local production and distribution. Pheromone traps provide simple, highly specific tools for pest monitoring that can be used to assess the need for application of any control measures, an essential ingredient of integrated pest management philosophy. Use of pheromone traps for monitoring one mirid, *Campylomma verbasci,* has been already demonstrated by McBrien, *et al*. (1994). Pheromones might be used for control by mass trapping given that both male and female adults and nymphs cause damage, or for control by mating disruption and preliminary experiments on mating disruption of *C. verbasci* were reported by Judd (1998).

There are various possibilities for combination of the two approaches. Mirid pests on cocoa occur at relatively low densities and populations are aggregated. Pheromone traps might be used to monitor the mirids so as to optimise the timing and location of application of a mycoinsecticide. A combined approach to control might use mating disruption with pheromones for prophylactic control of "normal" pest populations supplemented by local applications of mycoinsecticide in outbreak areas.

Further possibilities exist for luring insects with the pheromone to places where they might be treated with the mycoinsecticide either by conventional means or in a specially designed "trap". At IACR-Rothamsted a novel system is being developed for control of diamondback moth (Pell *et al*., 1993a; Pell & Wilding, 1994; Furlong *et al*., 1995) which involves attracting the target insects with a pheromone to a device that inoculates them with a lethal dose of the entomopathogenic fungus, *Zoophthora radicans*. The insect is released carrying the spores to susceptible larvae on the crop thus initiating or enhancing an epizootic.

Demand

The project contributes to the Capsid (Mirid) Thrust which is a major research programme at CRIG. Although control methods based on conventional chemical insecticides have been developed, these have not been taken up by growers, and the CRIG Social Science and Statistics Unit has recommended that research should be directed towards finding cheaper, simpler and more environmentally-acceptable methods.

A World Bank review (Report on the External Review of the Cocoa Research Institute of Ghana, CRIG, Project 1854 GH, March 1994) recommended that special attention should be given to the integrated management of mirids, and highlighted the need for development of effective monitoring systems, such as those based on pheromone traps.

A project funded by the American Cocoa Research Institute (ACRI) from October 1995-September 1998 was set up in collaboration with CRIG to identify the mirid pheromones, but little progress has been made on this and effort has focussed on investigation of plant volatiles affecting mirid behaviour. In the light of this, the Executive Director of CRIG requested specialist inputs from NRI and CABI to assist in development of pheromones and mycoinsecticides for the mirids.

At the STCP "West African Regional Cocoa IPM" Workshop" held in Cotonou, Benin, in November 2001, participants from the six main cocoa-growing countries in the Region unanimously placed mirids as one of the top two pests and diseases affecting cocoa for which there are no satisfactory control measures currently available.

Ghana used to be the major world producer of cocoa and has always produced high quality beans. Producers of cocoa-derived products in developed countries are known to be concerned to increase production levels in Ghana in sustainable, environmentally-acceptable ways, and this project was supported by Mars, Nestlé and the American Cocoa Research Institute (ACRI). The latter provided $10,000 per year to provide items of equipment, particularly those associated with rearing mirids at CRIG.

The focus country for this project was Ghana, but the two main target species of mirid are also pests of cocoa in Nigeria, Côte d'Ivoire, Cameroon, Guinea and Benin. Furthermore, other mirid species are pests of cocoa wherever it is grown in Africa, Asia and S America, so that the approaches developed in this project will have applicability in these countries.

CRIG will provide the main uptake pathway for project outputs. The immediate beneficiaries will be CRIG scientists who will have new technologies with which to develop more cost-effective methods for controlling mirid pests. The techniques themselves avoid the use of toxic chemicals and hence will not interfere with naturally-occurring predators and parasites that can help to control these and other pests.

If cost-effective methods for reducing losses due to mirid pests can be developed, ultimate beneficiaries will be cocoa growers. Cocoa is a rural small-holder crop with the average size of farms at 2.8 ha. CRIG report that there are over 350,000 cocoa farmers and their families in Ghana, and cocoa is the major export commodity and foreign exchange earner, directly or indirectly supporting the livelihoods of an estimated six million people. Current production is 400,000 tonnes with an export value of £400m, but the Ghana Cocoa Board aims to increase production levels to 500,000 tonnes by raising yields rather than destroying virgin forests in the west of the country.

PROJECT PURPOSE

The Purpose of the project is to develop and promote improved methods for control of insect pests of cocoa. The project will contribute to this Purpose by developing effective

mycoinsecticides against the two main mirid pests of cocoa in Ghana and identifying the sex pheromones of these species. These project outputs will make possible evaluation of new, environmentally acceptable approaches to monitoring and control of cocoa mirids that are appropriate for use in Ghana and elsewhere.

RESEARCH ACTIVITIES

Surveys to obtain fungal isolates from mirids in Ghana

Together with scientists from the Cocoa Research Institute of Ghana (CRIG), Drs J. Ackonor and I. Opoku, three surveys for pathogens of cocoa mirids were conducted in the cocoa growing regions of Ghana i.e. Volta, Eastern, Ashanti, Brong Ahafo, Western and Central Regions. The mirid season in Ghana occurs between August and December. Cocoa farms were selected and surveyed with the assistance of the local Cocoa Service Division (CSD) staff. At each farm, specimens of living and dead cocoa mirids (*S. singularis*, *D. theobroma*, *Helopeltis* spp. and *B. laticollis*) were collected. A total of about 3000 kilometres was covered in the first survey in September 1999, during which 146 farms in 22 different districts were visited. Sampling was done by examining cocoa plants with mirid symptoms and gently collecting the mirids thereon using a fine brush. However, mirids were observed and collected from only 72 farms (49.3%). The second survey, in July 2001, was conducted in the Eastern Region only and a total of 40 farms were visited. The third survey was carried out in November 2001 on 25 farms in and around Tafo-Akim in Eastern region. Mirids collected from each farm were maintained in labelled, separate plastic containers for subsequent laboratory evaluation.

During the surveys, dead insects were also collected from trees in the same family as cocoa (Sterculiaceae) as well as from other trees in forest ecosystems in Apesokobe, Volta region and in Afosu, Eastern region. Besides cocoa mirids, other dead arthropods collected from cocoa and other trees and shrubs were grasshoppers, spiders, ants, mealybugs, scale insects and flies.

Field collected specimens were maintained in the laboratory at CRIG in Tafo-Akim in Eastern Region. Live insects were maintained in the same plastic containers in which they were collected from the field until they died. To ascertain whether the insects died from mycosis, they were surface sterilised by dipping in 5% sodium hypochlorite for 2 minutes, rinsed in sterile distilled water and placed on moist filter paper in 9cm diameter plastic Petri dishes. Dead insects were maintained on a bench at room temperature and observed every other day (for a period of 10 days) for the presence of fungal growth. All pathogens which emerged were isolated and cultured initially onto a selective artificial media (SDA) containing antibiotics (streptomycin and penicillin G) to eliminate saprophytic fungi. All activities were done as planned.

Screening of mycopesticide isolates for activity

The potential of the *B. bassiana* isolated from Ghana (code numbered 382984) as a biopesticide was evaluated alongside those of 4 other isolates from cocoa mirids collected from Papua New Guinea (382762, 382811, 382812 and 335249). The isolates were screened on the basis of 4 characteristics.

Bioassays

Using novel techniques (see below) these fungal isolates were bulked on boiled rice, the conidia safely extracted, packaged at CABI UK and then shipped to CRIG, where the tests were done. This was done in accordance with Government phytosanitary regulations of both the UK and Ghana.

Using techniques learned during the training course at CABI-ARC (see below), CRIG scientists successfully sub-cultured the five isolates of *B. bassiana* on Potato Dextrose Agar (PDA) and V8 artificial media at CRIG. Sufficient quantitities of spores were obtained for use in other studies including bioassays to test the pathogenicities of the different isolates. These isolates are stored on Sabouraud Dextrose Agar (SDA) slants in the refrigerator at CRIG.

To test whether the imported and locally collected isolates of *B. bassiana* were pathogenic to cocoa mirids, it was important to ensure that following the application of the pathogens, the test insects did not die of starvation. Mirids are known to be difficult to rear in captivity and techniques for maintaining them alive in the laboratory had to be devised. A series of small laboratory experiments were conducted in order to improve the longevity of the mirid populations. Attempts to maintain mirids in the laboratory on detached cocoa pods and chupons (shoots) met with little success, as these plant materials wilted or rotted before the completion of experiments. Fruit of *Desplatsia dewevrei* (Tiliales: Tiliaceae), a tropical forest tree which occurs in West, Central and East Africa were tested and found to sustain *S singularis* in the laboratory better than cocoa pods or chupons. Adult mirids (*S. singularis*) were observed to survive on fruit of *D. dewevrei* for a period of up to 32 days, as opposed to 5 days on cocoa pods and chupons.

Mirids (both nymphs and adults of *S. singularis*) were collected from cocoa farms around CRIG, Tafo-Akim in the Eastern region of Ghana. The collected mirids were kept in plastic bowls (diameter 13.5 cm; depth 14 cm) with lids into which 4 x 6cm holes were cut and sealed with fine mesh. Chupons of cocoa trees and fruits of *D. dewevrei* were introduced into the plastic bowls as food for the insects. The plastic bowls containing the mirids were maintained in a screen house at temperatures ranging from 25º to 28ºC, for a maximum of two days before use in bioassays.

The five isolates of *B. bassiana* were cultured on Potato Dextrose Agar (PDA) in 9cm diameter Petri dishes. The spores of the isolates were extracted from four week-old cultures using sterile distilled water containing 0.05% Tween 80. Through serial dilutions, the spore concentration for each isolate was adjusted to 1 X 10^7 /ml using a haemocytometer. Chupons collected from pesticide-free cocoa trees were used in the bioassay due to the shortage of *D. dewevrei* fruits. Three batches of four chupons each were sprayed with one of the five isolates of *B. bassiana* and placed in three separate plastic containers (replicates) lined with filter paper. A control treatment (chupons treated with sterile distilled water plus 0.05% Tween 80) was also included. Twenty unsexed mirids (mixture of adults and nymphs of *S. singularis*) were introduced into each plastic container.

Mortality of the mirids was monitored daily for a period of 10 days. Dead mirids were counted, removed and surface sterilized in 70% alcohol followed by sterile distilled water. In order to stimulate external growth of *B. bassiana*, the insect cadavers were then incubated on moist filter paper in 9cm diameter Petri dishes with tight fitting lids. Only insects from which *B. bassiana* sporulated (i.e. those whose mortality was due to infection by *B. bassiana* isolate) were included in the statistical analysis.

After 10 days, cumulative percentage mortalities after 10 days were calculated for each isolate and subjected to the Generalised Linear Model procedure in SAS with isolates as the main treatment effect.

Rate of growth

The rates at which the 5 isolates of *B. bassiana* grow were compared by measuring growth on SDA culture medium with antibiotics (penicillin G and streptomycin). These studies were carried out in incubators (12L:12D photoperiod) at three different temperatures (23º, 28º and 32ºC). The five isolates of *B. bassiana* were grown on SDA slopes in universal tubes for a period of 26 days. To extract the conidia from the mycelia, 9ml of distilled water with Tween 80 (1 drop Tween in 10ml of water) was added into each tube. Serial dilution with distilled water and Tween 80 of the conidial solution (1ml in 9ml) was done and spore concentration was quantified by the use of an improved Neubauer haemocytometer. To obtain the spore concentration, the mean of four counts of conidia was taken and substituted in the formula [M x $5x10^4$ x 10^n] where M is the mean haemocytometer count, $5x10^4$ is a constant and n is the number of dilutions. The spore concentration of each isolate was standardized at 4.0 $x10^7$/ml.

For each temperature, 200 µl of the solution of each isolate was pipetted using a microapplicator onto clean SDA medium in 9cm diameter Petri dishes and spread out separately using a sterile glass rod for each isolate. The Petri dishes were then incubated at 28ºC for 48 hours, by which time uniform mycelia had grown on the surface of the media. Using a 6mm diameter cork borer, a hole was formed in the centre of clean SDA media in other 9cm diameter Petri dishes. These were also incubated at 28ºC for 48 hours and any medium with microbial contaminants was discarded. In a laminar flow cabinet, the tip of a cork borer was dipped in 95% alcohol, flamed, cooled and then used to transfer a 6 mm diameter agar plug with mycelial growth of each isolate separately into the holes in each of 7 Petri dishes with clean SDA media. These Petri dishes were incubated in an upright position for 1 day at the respective test temperatures. The Petri dishes in each incubator were arranged in a randomised complete block design with 7 replicates for each isolate. The Petri dishes were incubated at each temperature in an inverted position and the radial growth of each colony measured daily. Two lines were drawn at right angles on the bottom of each Petri dish such that the lines intersected at the centre of each colony. The average diameter measured on the two lines was taken as the size of the colony. The diameter of the initial hole (6mm) was subtracted from the measured colony diameter at each reading. The measurements were taken every 2 days for a period of 40 days.

The average daily diameter of each isolate was calculated at each temperature. The rate of growth (the period between days 2 and 26 were chosen to exclude days when growth rate slowed at 23º and 28ºC) and colony diameter after 40 days were separately subjected to Analysis of Variance (ANOVA) with isolate as the main treatment effect (Genstat 4.1). Data

were analysed for each temperature separately since isolates were randomised within each incubator but only one incubator was used for each temperature. The null hypothesis of "no difference between isolate means" was tested, applying ANOVA assumptions.

Sporulation experiments

A spore solutions of each isolate was prepared as described above, and the concentration standardised at 4.65 X 10^6 /ml.

The experiment was conducted at three different temperatures (23º, 28º and 33ºC), and each temperature was maintained in an incubator with a 12L:12D photoperiod. At a given temperature, a 100 µl solution of each isolate was dropped at the centre of each of five 5.5 cm Petri dishes containing SDA. These Petri dishes were placed randomly in each of the three incubators.

After 15 days of growth, the Petri dishes (replicates) with the sporulating cultures of each isolate were removed from the incubator and the quantity of spore produced was then assessed. To extract the spore, 9 ml of distilled water/Tween 80 was poured into each Petri dish and a triangular glass rod used to gently dislodge the conidia. The conidial suspension in each Petri dish was then sonicated for two minutes and passed through a sieve (106 µm) to remove any mycelia or other debris. The suspension was diluted and the spore concentration estimated as described above.

The mean number of spores produced by each isolate at each temperature was calculated. The mean number of spores produced after 15 days were normalized using the natural log transformation and analysed using an ANOVA with isolate as the main treatment effect (Genstat 4.1). Data were analysed for each temperature separately since isolates were randomised within each incubator but only one incubator was used for each temperature. The null hypothesis of "no difference between isolate means" was tested, applying ANOVA assumptions.

Viability

In this experiment the ability of the spores of the four isolates of *B. bassiana* to remain alive after storage at different temperatures was assessed. Spores from each isolate were separately scratched from 26-day-old cultures growing on SDA, onto 8 glass microscope slides. From one microscope slide, 0.01 g of the dry spore powder was suspended in 9 ml of distilled water/Tween 80 solution (1 drop Tween 80 in 100 ml distilled water). The solution was then sonicated for one minute and 200 µl spread on SDA in 9 cm Petri dishes and incubated at 28ºC for 24 hrs. The viability was assessed as the proportion of germinated spores assessed under the microscope at x200 magnification. This was taken as the initial viability (viability at week 0).

At weekly intervals, a sample of spores (0.01 g) was taken randomly from each slide and suspended in 9 ml of 0.05% Tween 80. The spores were incubated on SDA at 28ºC for 24 hours and the viability assessed as described above. The viability of spores was assessed weekly for 11 weeks

The average rate of loss in viability, between weeks 1 and 11, together with viability at week 11, of the spores of the isolates of *B. bassiana* were calculated at each temperature and compared using a similar analysis to that used in the 'rate of growth' experiment.

Development of mass production techniques for mycopesticides

A two-phase production system was used in which fungal blastospores and hyphae produced in a liquid medium were used to inoculate rice grains to promote spore production.

For the liquid phase, 75 ml suspension of yeast powder (20 g/l) plus sucrose (20 g/l) in tap water was autoclaved (121ºC, 120 kPa, 30 min) and inoculated with a 1 ml spore suspension of *B. bassiana* (10^7 spore/ml). The inoculated suspension was placed on a rotary shaker at 150 rpm at 24º-27ºC for 3 days. The suspension of hyphae and blastospores was then diluted by 50% with sterilized tap water and used to inoculate boiled and autoclaved rice grains to which water was added (300 ml tap water/kg of rice). The inoculated rice grains were incubated for 4 days in closed plastic bowls (24º-27ºC) that were thereafter opened to allow air-drying of the spore for 14 days. Extraction of the spores from the rice was previously achieved by shaking the medium through a sieves of increasingly finer sieves - a method that not only laborious and led to spore wastage, but was also a potential health risk because of the possibility of spore inhalation by technicians. To minimise loss and facilitate extraction of spores, a novel cyclone spore extractor (developed at CABI-UK) was used.

Development of a suitable mycopesticide formulation for use in laboratory and field

Studies to evaluate the effect of various locally available diluents on the viability of the spores of the five isolates of *B. bassiana* were conducted in the laboratory at CABI-ARC. The oils tested were Ondina (a lubricant), Sunflower, Vegetable, Corn, Kerosene and distilled water/Tween 80 as a control. To reduce the viscosity of Ondina, Sunflower, Corn and Vegetable oils, they were each mixed (50:50) with kerosene. An aliquot (9 ml) of each oil and water/Tween was placed into 25 universal bottles, which were then autoclaved at 121ºC at 15kPa for 20 minutes. For each diluent, 0.01 g of spores of each isolate was placed into five universal bottles and sonicated for 1 minute. Spore counts were then done using a haemocytometer as described above and the spore concentration standardized at 2 x10^5/ml. The initial viability of the spores was assessed as described above.

Spore viability of each isolate was assessed immediately after spores were mixed with the oil and thereafter every 3 days for 30 days. In the water/Tween 80 formulation, only spores which had not germinated when the solution was spread on SDA were considered in the assessment of germination.

The mean viability of spores (initial viability) in each diluent prior to exposure to different temperatures was calculated. The average rate of loss in viability, between day 3 and 30,

together with viability at day 30, of the spores of the isolates of *B. bassiana* was calculated at each temperature and compared using an ANOVA as described in previous sections. Evaluations of the different formulations in the field were not done due to the delays in receiving Government approval to import the exotic isolates into Kenya.

Pheromone collection

Insects

Cocoa capsids, *D. theobroma* and *S. singularis*, were collected from cocoa farms in and around CRIG. As far as possible, mirids were collected as late nymphs and allowed to develop through to give unmated adults of known age. Mirids were maintained on cocoa shoots and chupons, or on fruits of *Desplatsia dewevrei* (Tiliales: Tiliaceae), under ambient environmental conditions or in controlled humidity cabinets at 88-90% RH and 23ºC.

The fruits of *D. dewevrei* lasted longer than cocoa shoots. *D. theobroma* adults survived for over one month on the fruits, but laid no eggs. However, *S. singularis* laid eggs on the fruits and these hatched and developed through to adults, which then survived for up to 34 days (Padi *et al.*, 1996(b)).

Pheromone collection

One or two insects were maintained on cocoa chupons in a glass vessel (10 cm x 3 cm diameter). A diaphragm pump (Capex Mk II, Charles Austen, UK) was used to draw air (1-2 l/min) into the chamber through an activated charcoal filter (20cm x 2cm, 6-18 mesh) and out through a collection filter containing Porapak Q (200mg, 50-80 mesh, Waters Corp., MA 01757, USA), held between plugs of silanised glass wool in a Pasteur pipette. The Porapak was purified by soxhlet extraction with chloroform for 8 hr, and filters were washed well with dichloromethane immediately before use. Adsorbed volatiles were removed from the filters with dichloromethane (Pesticide grade; 1 x 0.5 ml, 1 x 1 ml).

The majority of pheromone collections were carried out by CRIG staff at CRIG in an air-conditioned room at approx 25ºC with ambient lighting. A few collections also made at NRI in a controlled temperature and humidity room at 50-60% RH and 23ºC on a 12L:12D cycle. Collections were run for 24 hr or 48 hr.

Isolation, identification and synthesis of pheromones

Gas chromatography (*GC*)

GC Analyses were carried out using a Carlo Erba Mega 5300 instrument equipped with fused silica capillary columns (25 m x 0.32 mm i.d.) coated with polar CPWax52CB (Carbowax 20M equivalent; Chrompack, London, UK) or non-polar CPSil5CB (methyl silicone; Chrompack, London, UK). The carrier gas was helium at 50 kPa, and the oven temperature was held at 60°C for 2 min then programmed at 6°C/min to 240°C. Injector and detector

temperatures were 200°C and 240°C, respectively. Injection was splitless and detection was by flame ionisation detection (FID). Data was captured and processed using EzChrom 6.1 software (Aston Scientific, Stoke-Manderville, Bucks, UK). Retention Indices (RI) were calculated relative to the retention times of straight-chain hydrocarbons.

For analysis of enantiomeric composition, a fused silica capillary column (25 m x 0.32 mm i.d.) coated with Chirasil-Dex CB (β-cyclodextrin Chrompack, London, UK) was used, operated at 140°C isothermal.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were carried out with a Carlo Erba Mega 5300 GC linked directly to a Finnigan ITD 700 Ion Trap Detector (Thermoquest, Hemel Hempstead, Herts, UK) operated in electron impact or chemical ionization (*iso*-butane) mode. GC columns and conditions were as above except that column i.d. was 0.25 mm. Spectra were compared with those in the NBS/NIH/EPA library, published spectra and a library generated from samples analysed previously at NRI.

High resolution GC-MS was carried out using a HP 5890 GC linked directly to a VG Autospec mass spectrometer with a fused silica column (30 m x 0.25 mm i.d.) coated with HP1 (methylsilicone, Hewlett Packard). Column temperatures were as above.

Electroantennography (EAG)

After anaesthetisation with carbon dioxide, the whole insect was placed on a plasticine block on its ventral surface, covered with a strip of filter paper and its antennae restrained with copper wire hooks. Glass capillaries (50 mm x 0.2 mm diameter) containing 0.5 M KCl and 1% polyvinylpyrrolidine were placed over silver wire electrodes attached to a DC amplifier (UN06, Syntech, Hilversum, The Netherlands). The electrodes were held in place by micromanipulators (Leitz, Wetzlab, Germany). The amplifier was connected to a PE Nelson 5300 interface, and the output captured and processed using Turbochrom 4 software (Perkin Elmer-Nelson, Beaconsfield, Bucks, UK). The indifferent electrode was placed in the connective tissue between the scape and pedicel of one antenna and the recording electrode in the connecting tissue of the two flagellal segments of the other antenna.

For linked GC-EAG analyses GC columns and conditions were as above, and effluent from the GC outlet was split equally between the FID and the insect preparation. The GC column effluent was blown at 15-sec intervals over the EAG preparation with a three second pulse of nitrogen (Cork et al., 1990). GC data was also recorded and processed using Turbochrom 4 software.

For EAG dose-response studies, the preparation was exposed to charcoal-filtered air (1 l/min.) through a glass Y-piece (i.d. 5 mm) positioned 1 cm away from the antenna. A glass Pasteur pipette containing the chemical stimulus was positioned in the remaining Y-piece arm, its tip 3 cm from the Y-piece exit. The test solution was placed on a piece of filter paper (Whatman No. 1, 1 cm^2) in the glass Pasteur pipette. The pipette was attached to tygan tubing leading to a nitrogen source pre-programmed to deliver a three second pulse of nitrogen (1 l/min). Immediately before attaching the pipette, the tubing was purged with a pulse of nitrogen.

Chemicals

All chemicals were synthesised at NRI by relatively standard methods and purified by flash chromatography and kugelrohr distillation. Compounds were characterised by their mass spectra, infrared spectra and NMR spectra (JEOL EX270: $CDCl_3$; ^{1}H 270 MHz, ^{13}C 67.5 MHz)

Development of pheromone slow release formulations

Dispensers tested were white rubber septa (20 mm x 10 mm o.d., Sigma-Aldrich, Gillingham, Dorset, UK, cat no. Z10,072-2) and polyethylene vials (26 mm x 8 mm x 1.5 mm thick, Just Plastics Ltd., London).

For measurement of release rates, dispensers were maintained in a laboratory wind tunnel (27°C, 8 kph wind speed). Duplicate samples were removed at intervals and stored in a freezer (-20ºC). These were then cut into small pieces and extracted overnight at room temperature in hexane (5 ml) containing pentadecyl acetate or decyl acetate (1 mg) as internal standard. The resulting solution was analysed by GC using the polar column as above. Volatiles emitted by lures were also collected using procedures similar to those used for collection of pheromone from insects. Collection filters were eluted with dichloromethane containing decyl acetate (5 µg) and analysed by GC on the polar column as above.

Field trials of pheromones

Trapping trials were carried out on and around the CRIG station. Traps evaluated were white sticky delta traps (Agrisense, 28 cm long x 20 cm wide x 14 cm side with stick base) and green "New Rectangular Traps". The latter were constructed from Correx sheet (38 cm long with cross section 10 cm wide x 14 cm high) with a Correx liner (28 cm long) coated with polybutene sticker (Tanglefoot) on the base and side surfaces. Unless otherwise stated, traps were suspended from cocoa trees approximately 2 m above the ground. Virgin female mirids used as bait were housed with a chupon in tubes (10 cm x 3 cm) closed at the ends with tygan mesh. These tubes and synthetic lures were suspended from the roof of the trap centrally with respect to length and cross section. Unless otherwise noted, traps were kept in the same position throughout the experiment.

Training of counterparts

Formal training in the UK

Dr Joseph Ackonor, an Entomologist at CRIG, attended a 4-week training course in insect pathology at CABI, UK Centre in 1999.

Mr Sammy Lowor was trained in pheromone collection, analysis and identification for three weeks at NRI in March 1999.

On-the-job training in Ghana

On-the-job training of CRIG staff was given during 12 visits to CRIG by staff from CABI and CRIG. In the mycopesticide work, techniques taught included the collection and maintenance of field-collected mirids for the pathogen survey, isolation and identification of entomopathogens and preparation of culture media. In the pheromone work, equipment was provided for pheromone collection and staff instructed in its use.

RESEARCH OUTPUTS

Surveys to obtain fungal isolates from mirids in Ghana

Details of mirids collected during the three surveys made in Ghana are shown in Table 1.

TABLE 1. Number of mirids collected during the survey for pathogens of cocoa mirids in Ghana.

Date	Farms visited	Farms infested	No. mirids collected			
			S. singularis	*D. theobroma*	*Helopeltis*	*B. laticollis*
Sept 1999	146	72	510	315	10	52
July 2001	40	8	15	0	25	1
Nov 2001	25	14	49	0	98	3

The entomopathogenic fungus *Beauveria bassiana* was isolated from a single field-collected *S. singularis,* in Tafo-Akim, Eastern region. Mirids in other species were not found to be infected. Other pathogens isolated included *Aschersonia aleyrodis* (Deuteromycotina: Hyphomycetes) from a scale insect collected from Afosu forest, *Entomophaga grylli* (Zygomycetes: Entomophthorales*)* from several grasshoppers and a *Cordyceps* sp. (Deuteromycotina: Hyphomycetes) from a fly. Besides pathogens, the parasitoid, *Euphorus sahlbergellae,* was also isolated, from 1.2% of the collected *S. singularis* specimens.

Based on previous work, *B. bassiana* was considered to have the most potential for further development into a biopesticide. The others are either obligate parasites or have specialised nutritional requirements, making it difficult to produce the pathogens *en masse* on artificial media. This Ghanaian isolate of *B. bassiana* is currently maintained in the laboratory at both CRIG and CABI and is available for further evaluation amongst other fungal isolates.

Screening of mycopesticide isolates for activity

Bioassay

All *B. bassiana* isolates tested were pathogenic to *S. singularis.* Dead mirids in the fungal treatments, but not the control, showed hyphal growth of *B. bassiana* emerging from the mirid cadaver. Mortality of the mirids (which started on day 4) in all treatments increases steadily up to day 6 (Fig. 1). At day 10, analysis showed that although the mortality of mirids treated with the fungal isolates was significantly higher than the control ($p<0.05$), there were no significant differences between the isolates themselves. However, the Ghanaian isolate, 382984, infected more mirids than the other, exotic isolates.

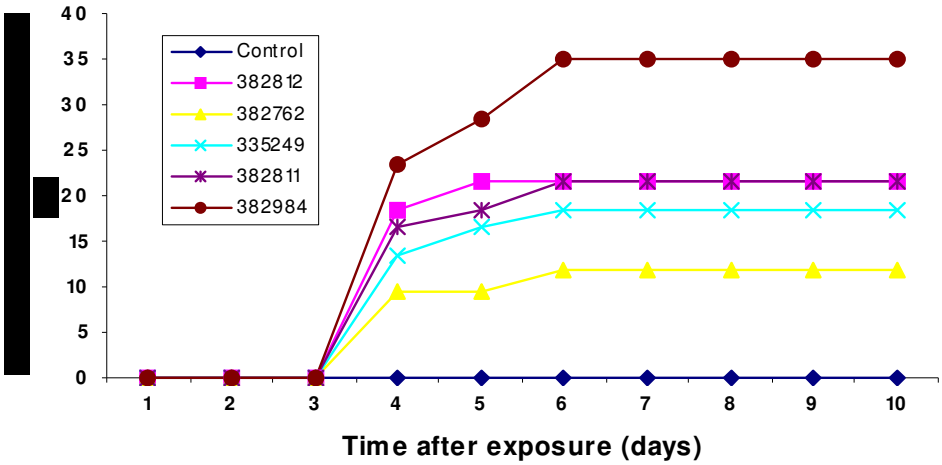

FIG. 1. Proportion of mirids dying of fungal infection after being exposed to cocoa chupons treated with different isolates of *B. bassiana*.

Rate of growth

The overall mean growth rate was 1.93mm/day. The mean growth rates at 23ºC (2.3 mm/day) and 28ºC (2.4 mm/day) were higher than at 33ºC (1.0 mm/day). The overall mean colony diameter after 40 days was 64.1 mm. The mean colony diameters after 28 days were 61.0, 67.0 and 29.0 mm at 23º, 28º and 33ºC, respectively.

There were significant ($p<0.001$) differences between the growth rates of the *B. bassiana* isolates at all temperatures (Fig. 2). Isolates 382811 and 382812 showed consistently slower growth rates compared to other isolates at all temperatures. The growth rate of isolate 335249, compared to those of isolates 382762 and Ghanaian isolate 382984 (which had similar growth rates), was significantly lower at 23^{O}C, equal at 28^{O}C and higher at 33^{O}C. The differences between the isolates with respect to the colony diameter on day 40, followed a similar trend to growth rate above.

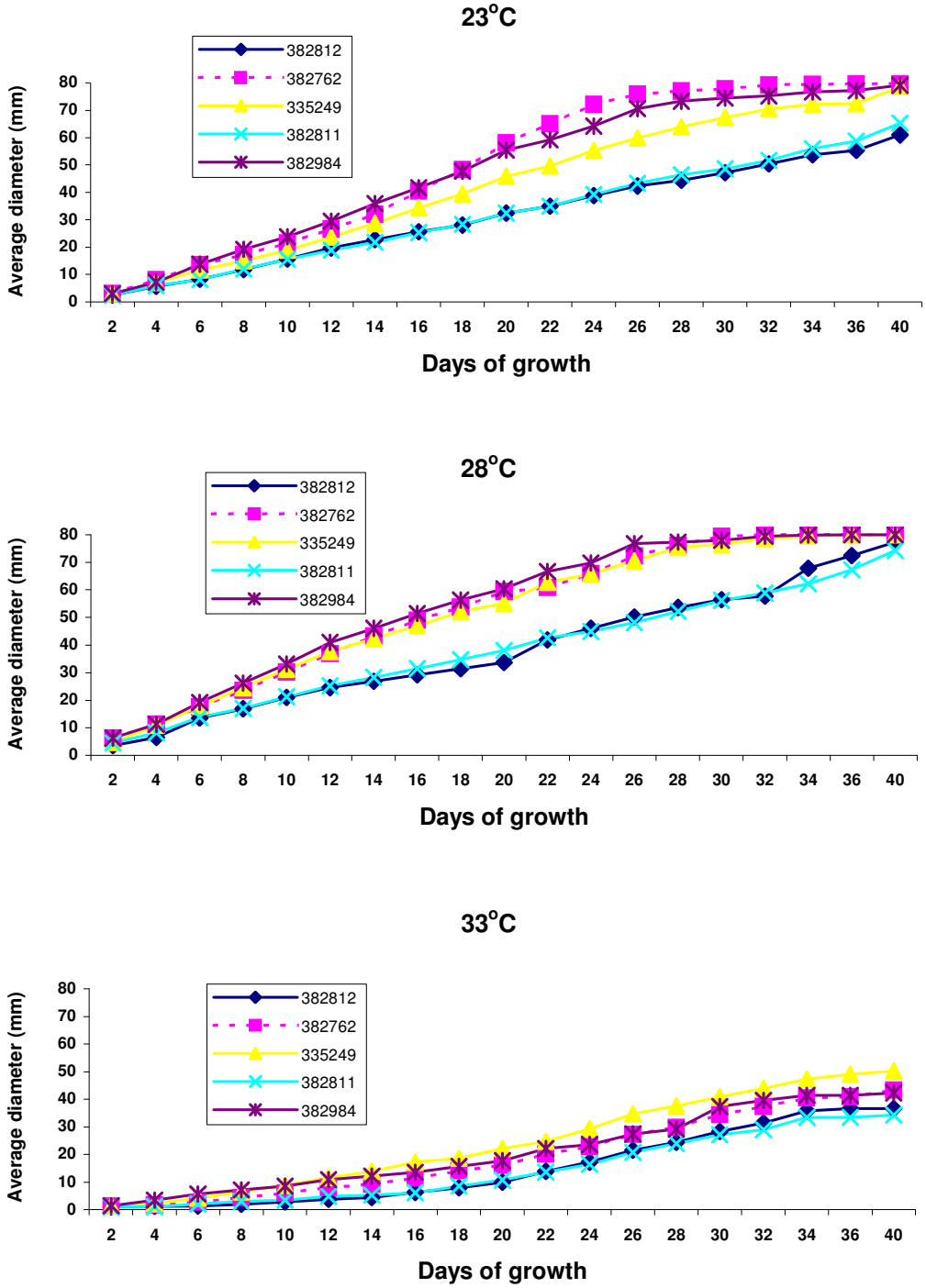

FIG. 2. Growth of different isolates of *B. bassiana* on artificial medium (SDA) at different temperatures.

Sporulation experiments

An overall mean of 8.05 X 10^7 spores were produced in each Petri dish. The isolates produced significantly ($p<0.001$) different numbers of spores at all temperatures. Isolate 335249 and the Ghanaian isolate 382984 produced substantially ($p<0.001$) more spores per dish (17.3 X 10^7 and 13.3 X 10^7, respectively) overall, compared with other isolates. Isolate 382811 produced the lowest number of spores at 23°C and 28°C and one of the lowest numbers at 33°C, compared with other isolates (Fig. 3).

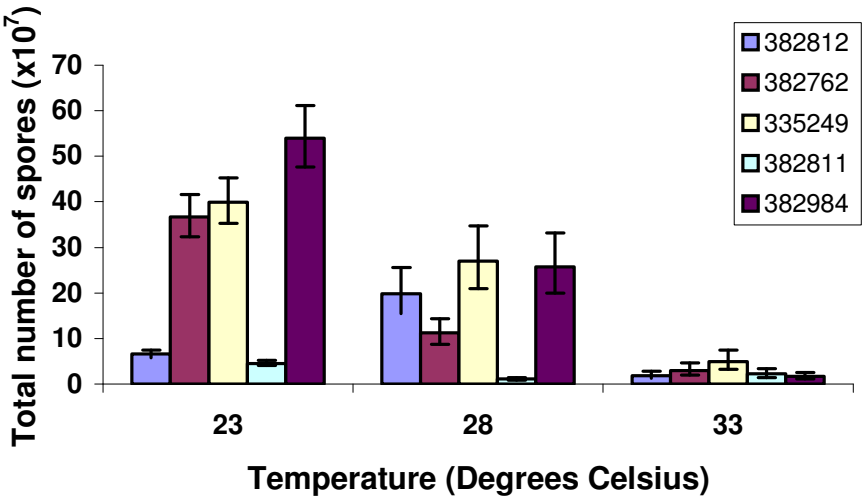

FIG. 3. Number of spores produced by different isolates of *B. bassiana* at different temperatures.

Viability

The overall loss of viability was 7.1% per week. Although isolates 335249 and 382811 lost their viabilities at significantly ($p<0.001$) faster rates than the other isolates at 23° and 28°C, this trend was not observed at 33°C (Fig. 4). The loss of viability between isolates 382762, 382812 and Ghanaian isolate 382984, was similar at 28°C. However, the latter isolate lost its viability at a significantly slower rate, and 382762 at a significantly higher rate, at 33°C. The overall losses of viabilities, across isolates, were 4.2, 3.9 and 4.6% per week at 23°, 28° and 33°C, respectively.

The relative viabilities of the isolates after 11 weeks of exposure to the different temperatures were significantly lower at 33°C (0.2%) than at 28°C (28.2%) or 23°C (21.3%). The overall mean spore viability after 11 weeks was 16.6%. The mean viabilities of isolates 335249 and 382811 (8.0 and 0.1%, respectively) were significantly ($p<0.001$) lower than the other isolates (23.8 – 26.4%). The isolates with the significantly ($p<0.05$) highest viabilities at the different temperatures were 382984 (at 23°C), 382984 and 382762 (at 28°C) and 382984 (at 33°C). However, across temperatures, Ghanaian isolate 382984 was significantly superior to the others.

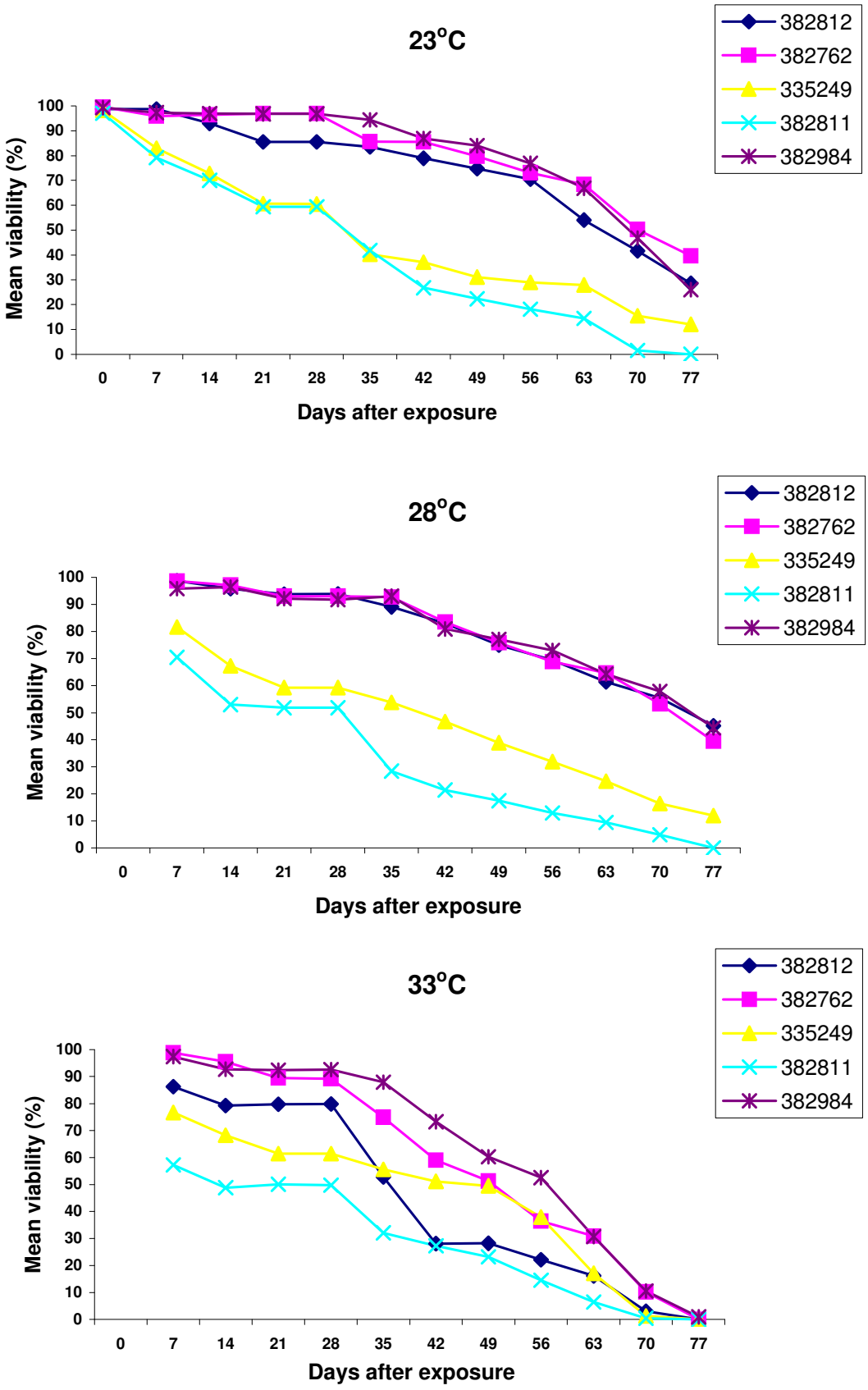

FIG. 4. The viabilities of different isolates of *B. bassiana* at different temperatures

Development of mass production techniques

Growing the entomopathogens on rice and extracting the spores by the use of the novel cyclone spore extractor, this efficient technique enabled the project to produce as much as 22 g of pure spores from 1 kg of rice. The pure spore were then collected and stored in airtight containers containing silica gel. Sufficient quantities of spores both for field trials and laboratory experiments were therefore made available.

Development of a suitable mycopesticide formulation for use in laboratory and field

The initial spore viability of the isolates of *B. bassiana* (in water/Tween) ranged from 97.2 to 99.6% (mean=98.5%). The viabilities of the spores of the isolates varied significantly ($p<0.001$) during the brief exposure (about 15 minutes) to the tested oils. The viabilities of spores, across oils, of isolates 382812 (97.9%) and 382762 (86.7%) were significantly higher than the others (79.4 – 82.3%) after this period. Vegetable and Corn oils led to significantly ($p<0.001$) lower spore viability (70.6 and 75.5%, respectively) compared to Kerosene (88.3%), sunflower (87.4%) and Ondina (91.7%) oils.

Generally, the viability of the spores in the different diluents and temperatures declined at a rate of 1.8% per day. The water/Tween solution always contained spores which had germinated to form mycelia even before they were plated onto SDA. Sunflower oil caused a significantly faster ($p<0.05$) rate of loss of viability (1.5% per day) compared with the other oils (1.5 – 2.0% per day) (Fig. 5).

Corn oil led to the slowest rate of loss of spore viability during the exposure period (1.5% per day), followed by kerosene (1.7% per day). The effect of oil however varied depending on the isolate ($p<0.001$).

Kerosene (38.0%) and Ondina (33.2%) supported the highest spore viability after 30 days exposure, although the effect of different oils on spore viability varied between isolates.

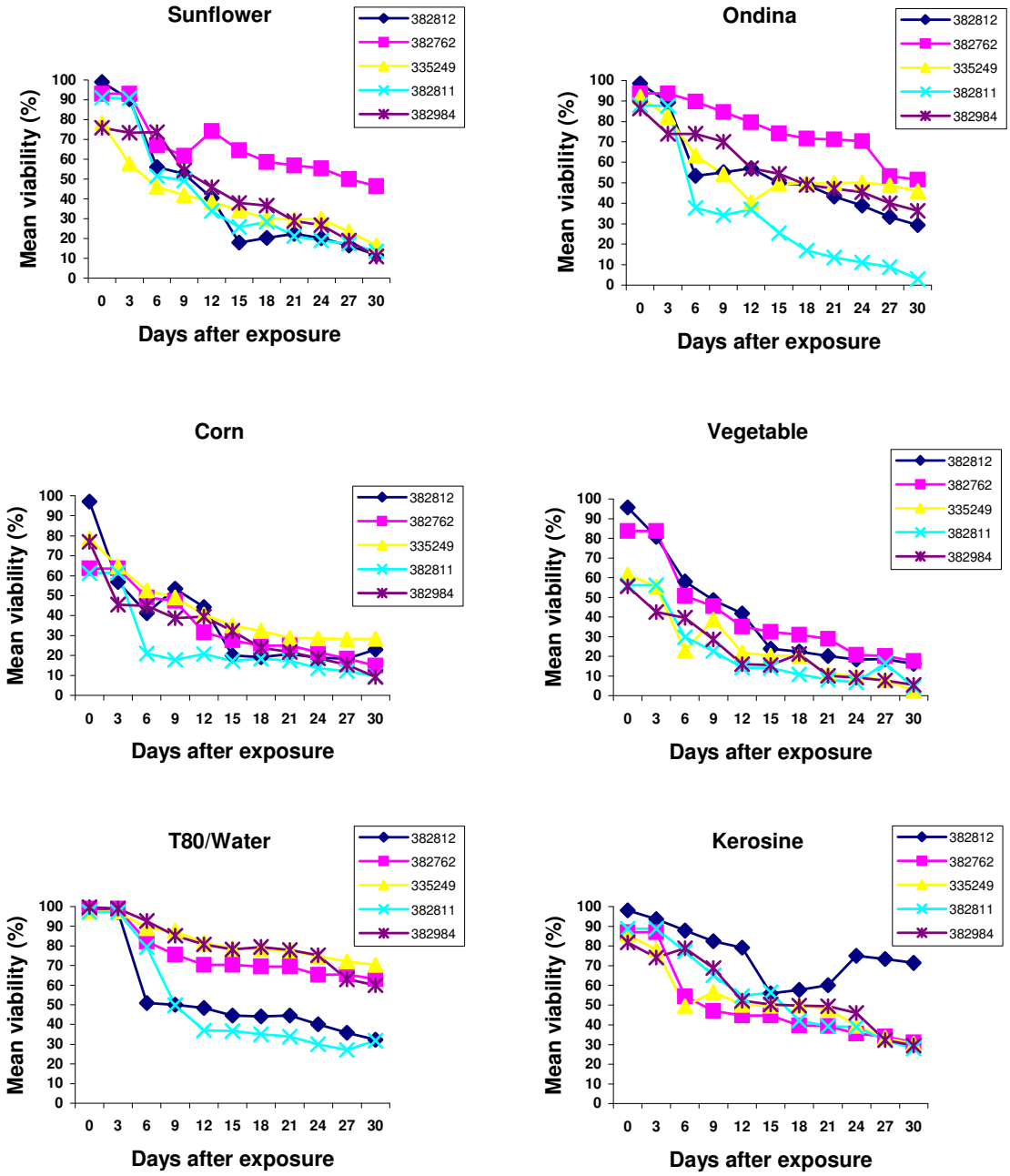

FIG. 5. The effect of different oils on the viability of different isolates of *Beauveria bassiana*

Pheromone identification and synthesis

Pheromone isolation

In initial pheromone collections at CRIG during 1998-1999, collections were made from female *S. singularis* (12 collections), female *S. singularis* (8 collections), female *D. theobroma* (10 collections), male *D. theobroma* (2 collections), cocoa chupons (2 collections) and cocoa pods (4 collections). Due to the difficulty of maintaining mirids for long periods at that time, insects from 0-4 days old were used. All collections were analysed by GC-MS and components identified as far as possible from library spectra or interpretation of mass spectra and GC retention times. Over 50 components were detected, but no obvious, consistent differences between sexes or species could be detected. Collections were bulked by species and sex and reanalysed, and results are shown in (Appendix 1). The major components in the collections were (*E*)-β-ocimene, linalool and dendrolasin from the cocoa chupons.

The bulked collections were analysed by GC-EAG using an insect of the same species and opposite sex for the EAG preparation. No significant responses were observed.

As it was reported that both mirid species only become sexually mature after 6-7 days (King, 1973), subsequent collections were done with older insects. During 1999-2000 and 2000-2001, 140 collections were made at CRIG using insects from 5 - 32 days old. The collections were analysed by GC-MS at NRI, but again there were no obvious, consistent differences between collections from males and females of either species.

Because of the poor survival of insects sent from CRIG to NRI, it was only possible to carry out a few GC-EAG analyses. In a few of these analyses with *D. theobroma*, a significant EAG response (> 10% above baseline response) was observed at KI 2220 on the polar GC column and KI 1678 on the non-polar column (Fig. 6).

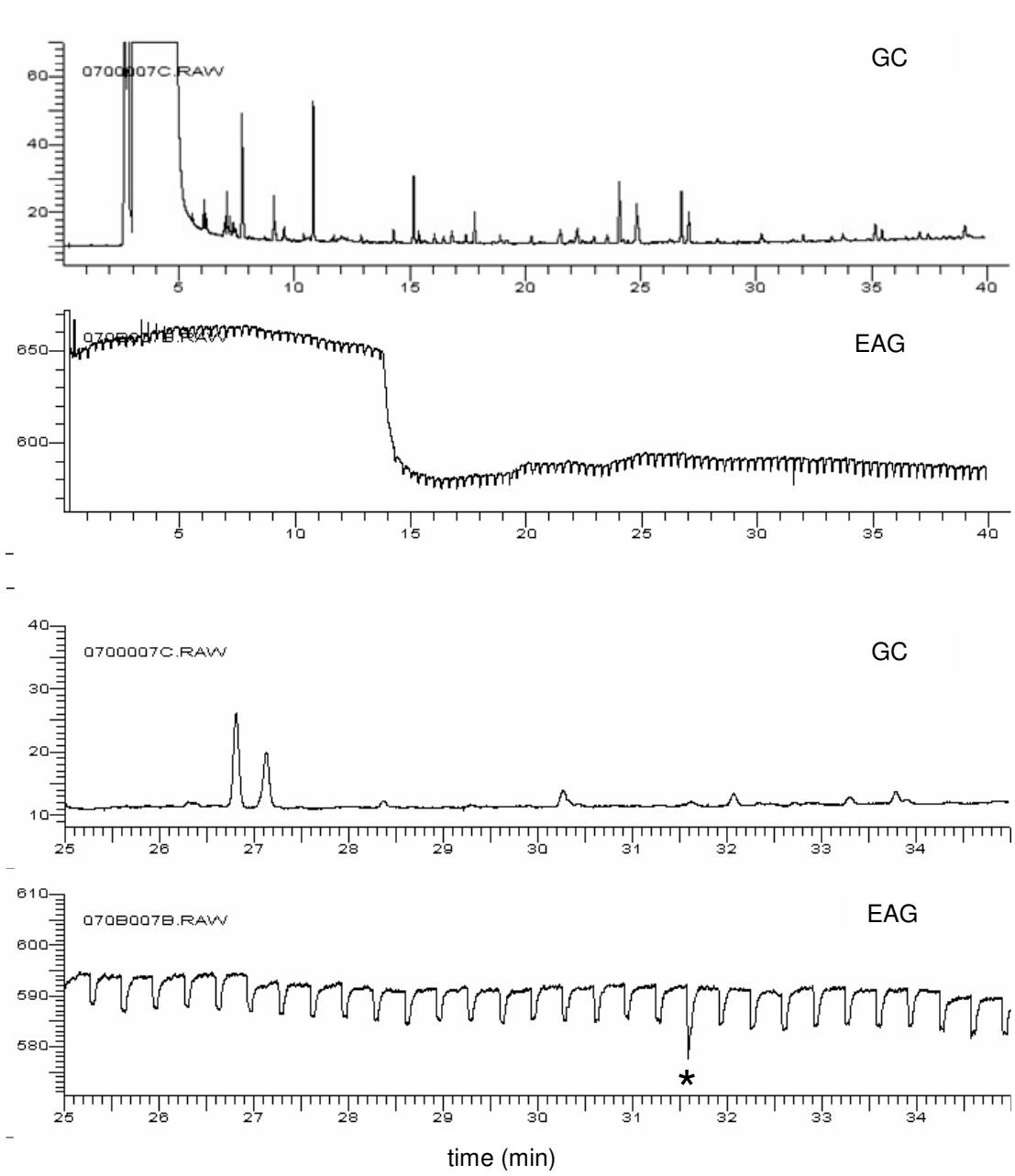

FIG. 6. Complete (upper) and expanded (lower) traces of GC-EAG analysis of volatile collection from *D. theobroma* female with *D. theobroma* male EAG preparation (* EAG response)

Subsequently in GC-EAG analyses of collections from *S. singularis* females with a male *S. singularis* EAG preparation, two responses were observed to amounts of material essentially undetectable above background by the GC FID (Fig. 7). The second of these had KI 2220 on the polar GC column similar to that of the EAG-active component from *D. theobroma.* The first had KI 1886.

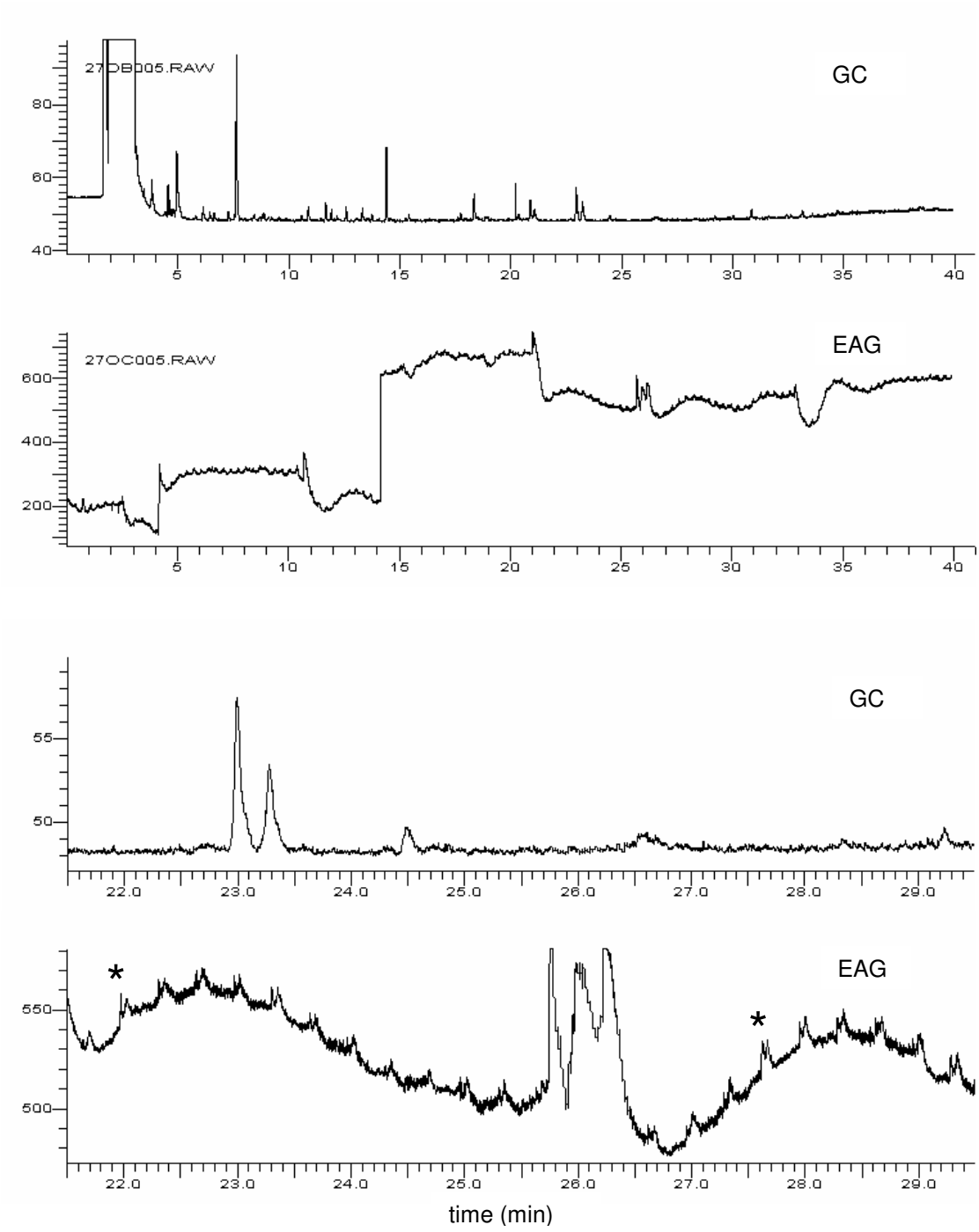

FIG. 7. GC-EAG analysis of volatiles from two *S. singularis* females, 7 days old with *S. singularis* male EAG preparation (* EAG response)

Pheromone identification

Subsequent examination of the active collections from *D. theobroma* by GC-MS showed that a component with the same mass spectrum could be detected at the retention times of the EAG responses on the two GC phases, greatly increasing confidence that a single active component had been detected. The amount present was extremely low, but EI and CI mass spectra were obtained (Fig. 8).

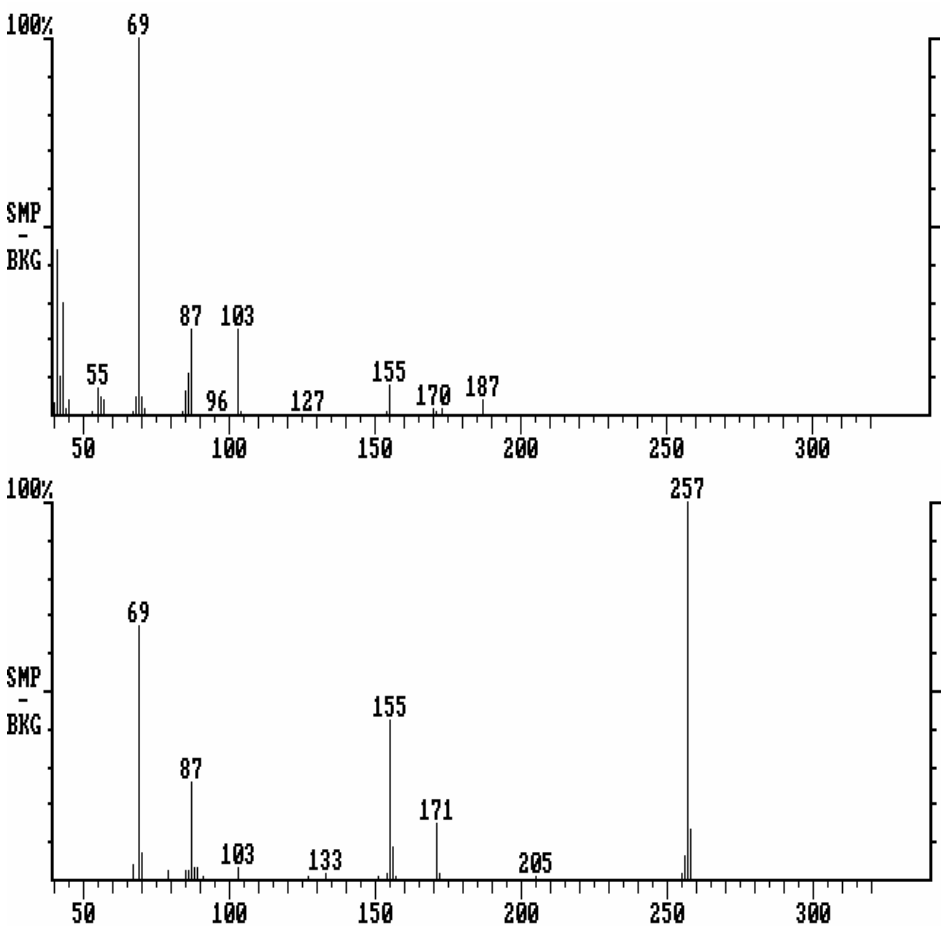

FIG. 8. EI (upper) and CI (lower) mass spectra of EAG-active component in volatiles from *D. theobroma*.

The mass spectra indicated a molecular weight of 256. Ions at m/z 69 (CH_3-CH=CH-C=O^+) and m/z 87 (CH_3-CH=CH-CO-OH_2^+) suggested the presence of a crotyl (2-butenyl) ester moiety. Ions at m/z 187 (M-69) and 171 (M-85) were consistent with this. The ions at m/z 103 (C_4H_9-$COOH_2^+$) and m/z 155 (M-101) suggested a pentanoate ester moiety.

A series of mixed crotonate and pentanoate esters of hexane diol isomers was synthesised (Appendix 2). Retention times of these were similar to those of the natural component, and the differences in retention times on the two phases (Δ) were also similar. The latter is a sensitive indication of the type of chemical structure.

However, none of the synthetic compounds had identical properties to those of the natural EAG-active compound, and it was difficult to see what further structural manipulation could be done to make retention data more similar. Furthermore, the mass spectra of these compounds all had a significant ion at m/z 85 (C_4H_9-CO^+) from the pentanoate moiety (e.g. Fig 9), and this ion was not prominent in the spectrum of the natural EAG-active component.

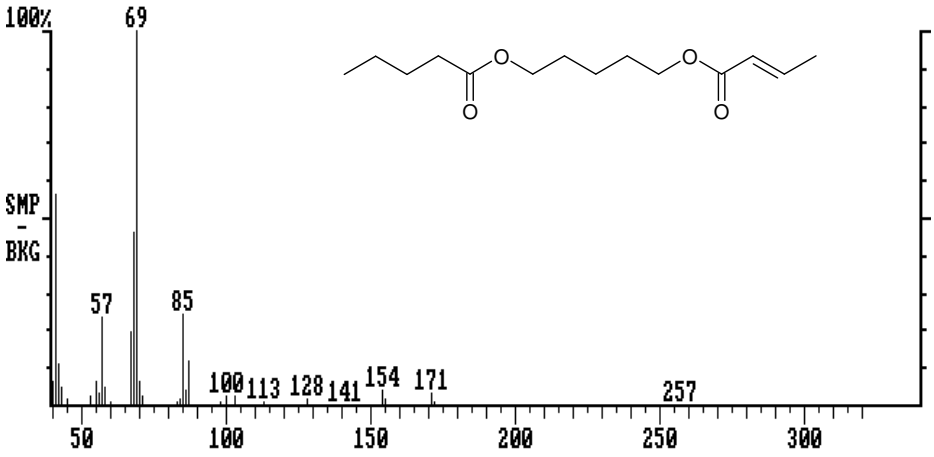

FIG. 9. EI mass spectrum of 1-((*E*)-2-butenoyl)-6-pentanoylhexane.

It was thought that replacement of the pentanoate ester by an oxygenated butyrate moiety might direct alternative fragmentations and reduce the intensity of the m/z 85 ion in the mass spectrum. A series of such derivatives was synthesised (Appendix 3). In the mass spectra of some of these, the ion at m/z 85 was much reduced, as predicted (e.g. Fig. 10), but, again, none had retention times and/or mass spectra matching those of the natural EAG-active component.

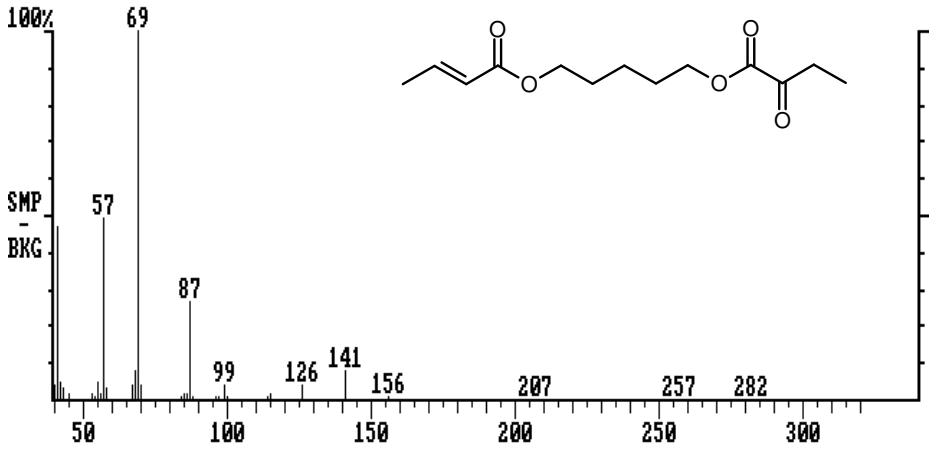

FIG. 10. EI mass spectrum of 1-((*E*)-2-butenoyl-6-(2-oxobutanoyl)hexane.

In order to make further progress, sufficient material was collected to obtain a high resolution mass spectrum. Results of this were not as accurate as hoped due to the small amount of material available. However, taking the best-guess interpretations (Table 2) the following interpretation could be made.

TABLE 2. High resolution mass spectral data on EAG-active component from *D. theobroma* (Observed(1) and Observed(2) are results from two measurements; Δ(1), Δ(2) are respective Observed-Calculated differences)

m/z	C	H	O	Calculated	Observed (1)	Observed (2)	Δ(1)	Δ(2)
187	13	15	1	187.1123	187.1210		0.0087	
	10	19	3	187.1334	187.1210		-0.0124	
171	10	19	2	171.1385	171.1391		0.0006	
155	8	11	3	155.0708	155.0659		-0.0049	
103	8	7		103.0548	103.0514	103.0375	-0.0034	-0.017
	4	7	3	103.0395	103.0514		0.0119	-0.002
87	4	7	2	87.0446	87.0459	87.0484	0.0013	0.0038
69	4	5	1	69.03404	69.0151		-0.0189	

The observed values for m/z 69 and 87 are consistent with those expected for the crotonyl moiety. The observed value for m/z 171 gives an excellent fit for $C_{10}H_{19}O_2$, and addition of the crotonyl moiety, $C_4H_5O_2$ (171+85=256) gives a molecular formula of $C_{14}H_{24}O_4$. If m/z 155 is $C_8H_{11}O_3$ as suggested in Table 2, then the 101 loss is represented by $C_6H_{13}O$, which is then not related to the m/z 103 ion as was thought previously. This suggested the alternative diester structure shown in Fig. 11 which is a hexyl ester of a hydroxybutyric acid then esterified on the hydroxy group with 2-butenoic acid.

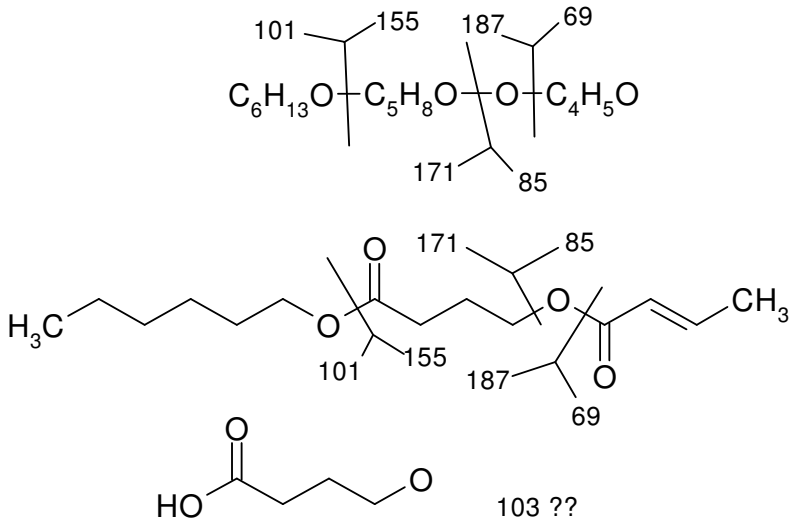

FIG. 11. Possible interpretation of high resolution mass spectral data on EAG-active component from *D. theobroma*

A further series of compounds based on this structure was synthesised (Appendix 4) and compound (I) (Fig. 12) had GC retention times and mass spectra identical with those of the EAG-active component produced by *D. theobroma*.

Re-examination of collections of volatiles from female *S. singularis* confirmed that compound (I) was also present in these and probably responsible for the second of the EAG responses in GC-EAG analyses. It was realised that the first of the EAG responses occurred at a retention time similar to that of the corresponding monoester (II) (Fig. 12) (Appendix 4), and the presence of this was confirmed by GC-MS analysis (Fig. 13).

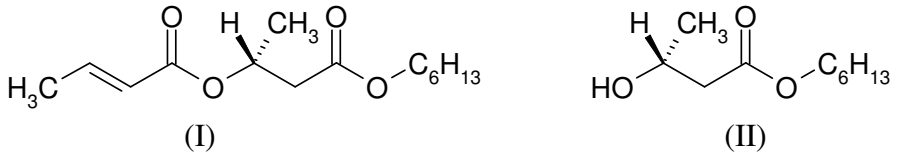

Fig. 12. Proposed structures of pheromone components of *D. theobroma* and *S. singularis*

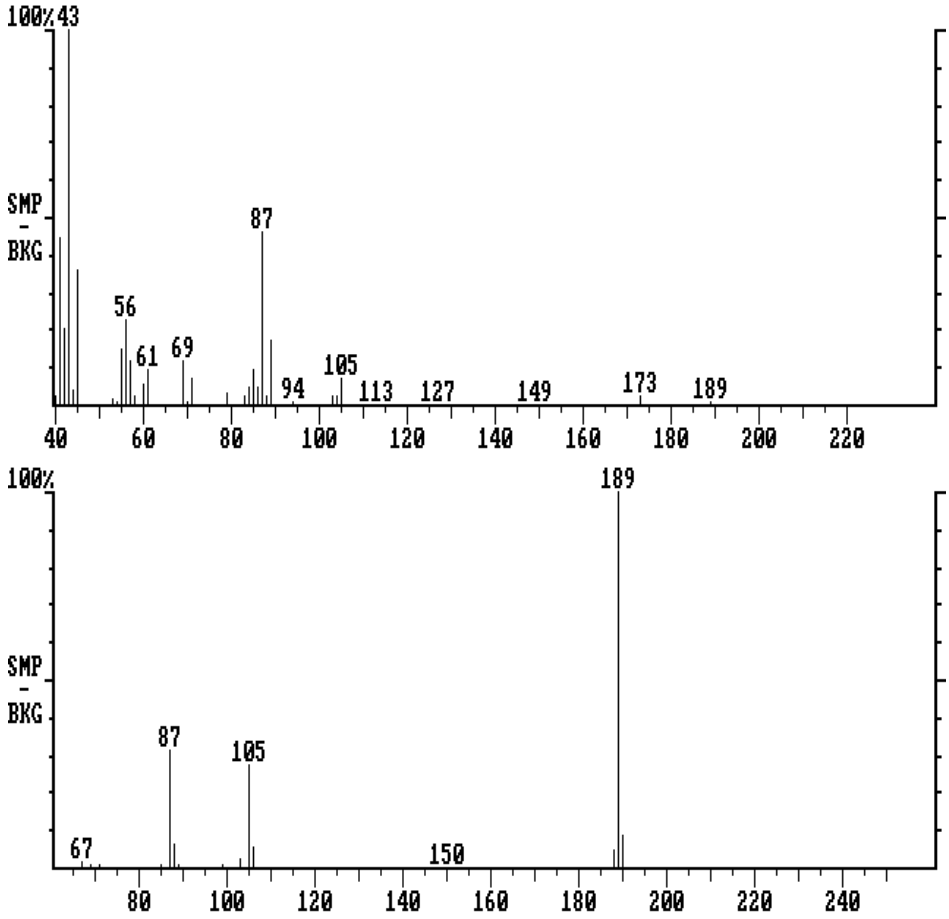

FIG. 13. EI (upper) and CI (lower) mass spectra of second EAG-active component.

The *R* and *S* enantiomers of diester (I) could be separated on a cyclodextrin GC column (Fig. 14). Examination of a collection of volatiles from *D. theobroma* showed that the naturally-produced compound co-chromatographed with the second, *R* enantiomer of (I) (Fig. 15) using

both FID or MS detection. Enantiomers of monoester (II) could not be separated on the cyclodextrin column.

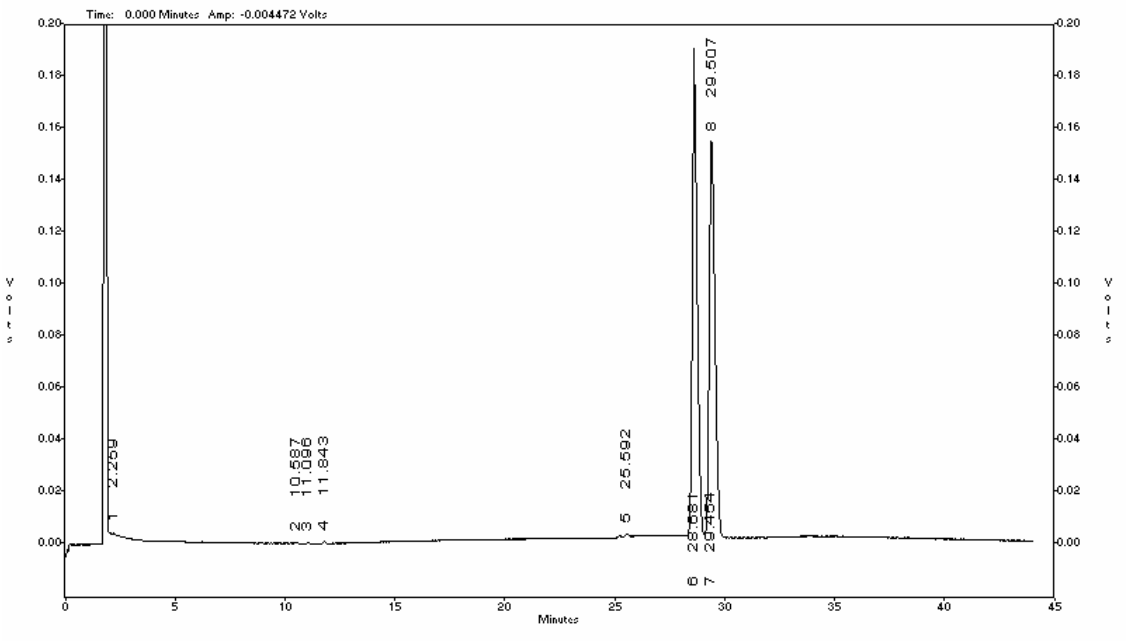

FIG. 14. GC analysis of racemic diester (I) on cyclodextrin column (140°C isothermal; *S* enantiomer eluted first)

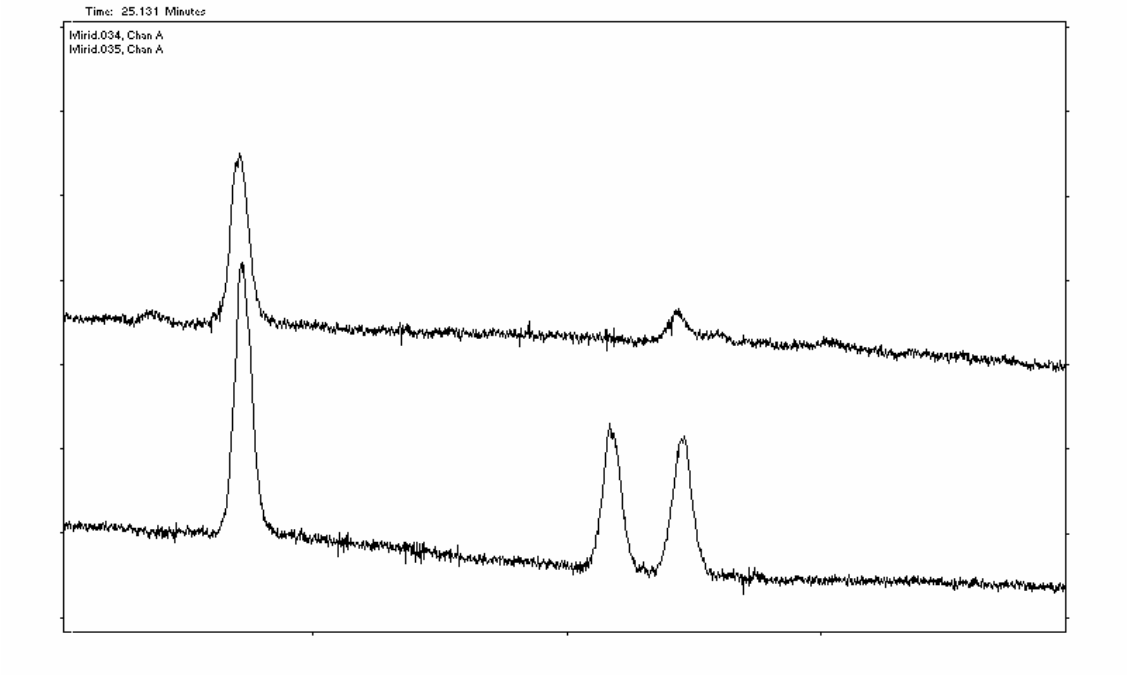

FIG. 15. GC analysis of natural (upper) and racemic synthetic (lower) pheromone component (I) on cyclodextrin column (first peak is internal standard, Z9-13:OH)

The synthetic compounds elicited EAG responses from a male *S. singularis* (Fig. 16). The response to the *R* diester (I) was significantly greater than that to the *S* diester or either enantiomer of the monoester (II). The response to *R*-(II) was significantly above response to solvent whereas the response to *S*-(II) was not significantly above that to solvent alone.

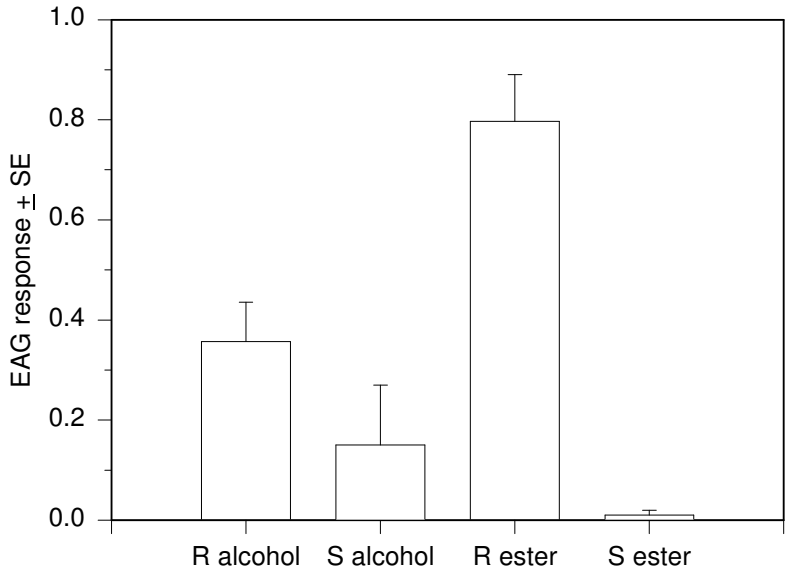

FIG. 16. EAG responses of male *S. singularis* to synthetic compounds (absolute EAG responses corrected for response to hexane; means ± SE for 3 replicates on different insects).

The amounts of pheromone components present in collections were generally very low. In the collections used for EAG work with both *D. theobroma* and *S. singularis*, the components were near the limit of detection for the GC-MS (< 5 ng/insect/24 hr). More was present in a few other samples with up to 100 ng/insect/24 hr of component (I) in collections from *D. theobroma* females between ages 6-12 d and *S. singularis* of 7-12 d old. None was detectable in collections from 1-4 d old or 20-30 day old insects.

Re-examination of collections from *D. theobroma* females showed that the second EAG-active component (II) was also present in these. Analysis of six of the best collections from each of the two species showed that the average percentage of (I) in the blend of (I) + (II) was 69% (range 57-80%) for *D. theobroma* and 72% (range 56-91%) for *S. singularis*.

Pheromone synthesis

Compounds (I) and (II) were initially prepared by esterification of hexanol with 3-hydroxybutyric acid in the presence of N,N'-dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) to give alcohol (B) (Neises and Steglich, 1978). This was then esterified with commercially-available crotonyl ((*E*)-2-butenoyl) chloride in the presence of pyridine (Fig. 17).

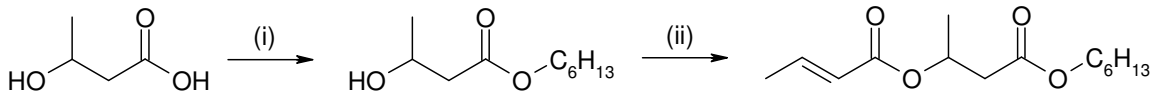

FIG. 17. Synthesis of pheromone components ((i) DCC/DMAP/dichloromethane/ hexanol 55%; (ii) (*E*)-2-butenoyl chloride/pyridine/dichloromethane/0ºC 25%).

3-Hydroxybutyric acid is prone to polymerisation and a route was developed by reaction of the more stable sodium salt with hexyl bromide in the presence of 18-crown-6 ether (Fig. 18). Both *R* and *S* enantiomers were prepared by this route.

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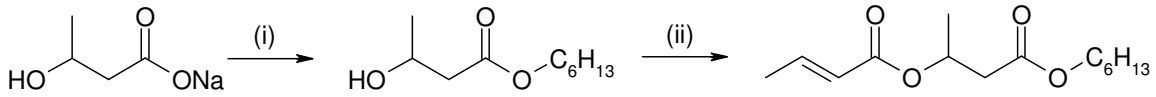

FIG. 18. Synthesis of pheromone components ((i) hexyl bromide/acetonitrile/18-crown-6/100ºC/3 days 90%; (ii) (*E*)-2-butenoyl chloride/pyridine/dichloromethane/ 0ºC 25%).

The most satisfactory route developed to date starts from relatively cheap biopolymer, poly-3-hydroxybutyric acid. Heating this with hexanol and catalytic sulphuric acid gives the monoester (II) cleanly and in good yield (Fig. 19). Despite much experimentation, the esterification with crotonyl chloride never gave good yields, and the product always contained > 5% of the *Z* isomer. Better yield was obtained using crotonic acid in the presence of DCC and DMAP. Furthermore, two recrystallisations of the crotonic acid gave material containing < 1% of the *Z* isomer. Analysis of the product on the cyclodextrin GC column showed it to be the *R* enantiomer with <0.1% of the *S* enantiomer.

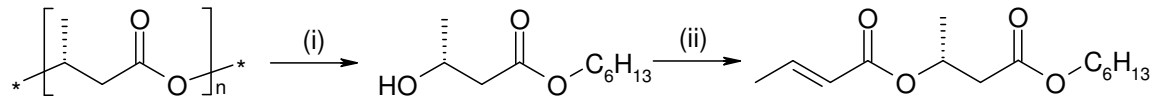

FIG. 19. Synthesis of pheromone components ((i) hexanol/catalytic sulphuric acid/120ºC/4 days 90%; (ii) (*E*)-2-crotonic acid/DCC/DMAP/dichloromethane/3 d 67%).

The racemic compounds were prepared from readily-available β-butyrolactone by acid-catalysed ring-opening with hexanol followed by acylation with (*E*)-butenoyl chloride

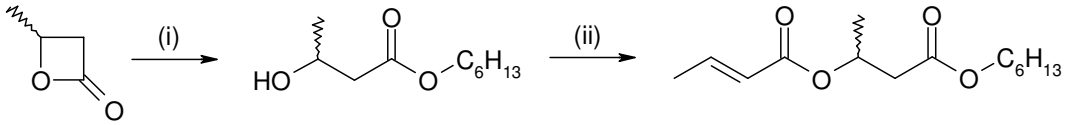

FIG. 20. Synthesis of pheromone components ((i) hexanol/catalytic sulphuric acid/100ºC/3 days 90%; (ii) (*E*)-2-butenoyl chloride/pyridine/dichloromethane/ 0ºC 25%).

Development of pheromone slow release formulations

Release rates of proposed pheromone component (I) from polyethylene vial and rubber septa dispensers were measures in the laboratory windtunnel at 27ºC and 8 kph windspeed. Release from the septa was faster ($t_{½}$ 20.3 days) than from the vials ($t_{½}$ 29.5 days) (Fig. 21).

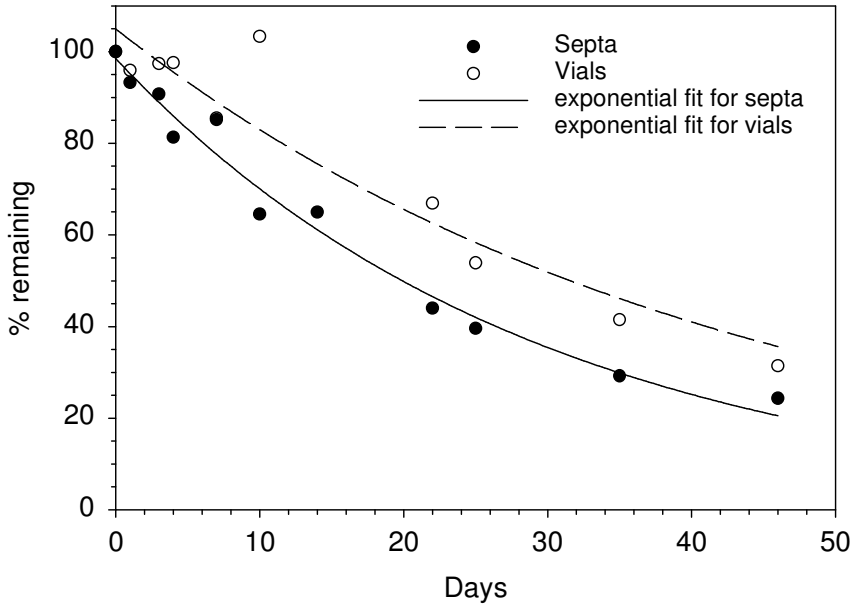

Fig. 21. Release of diester (I) from polyethylene vials and rubber septa in laboratory windtunnel at 27ºC and 8 kph windspeed.

Release of the monoester component (II) from the vials was much faster then that of the diester (I). Data on analysis of volatiles from a vial impregnated with 1 mg (I) and 0.5 mg (II) is shown in Table 3. In the initial week, release of monoester (II) was at least eight times faster than that of diester (I), although the relative amount of (II) in the volatiles decreased as the relative amount left in the vial decreased.

Table 3. Composition of volatile blend released by polyethylene vial impregnated with 1 mg (I) + 0.5 mg (II) at 27ºC.

Day	Release rate (µg/day)		Ratio (I)/(II)
	Monoester(II)	Diester (I)	
2	0.76	0.51	0.67
4	2.75	0.36	0.13
9	5.02	1.42	0.28
15	5.17	2.70	0.52
65	0.73	1.20	1.65

Release of the diester (I) from the vials with 1 mg loading was at least 20 times greater than the highest estimate of the amount released by the female insects (100 ng/insect/day)

In an initial field experiment carried out at CRIG (below), the tops were removed from the vials. The effect of this was investigated in the laboratory windtunnel, and the results (Fig. 22) showed release rate was increased with the diester (I) having half life 14.2 days compared with 29.5 days above. This experiment confirmed faster release of the monoester (II) with half life of only 3.9 days.

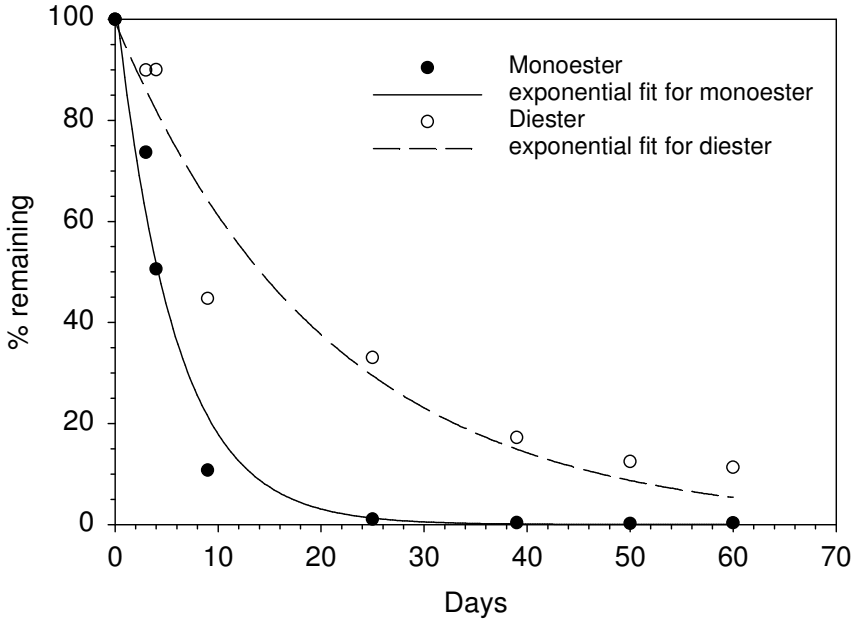

Fig. 22. Release of diester (I) and monoester (II) from polyethylene vials with tops off in laboratory windtunnel at 27ºC and 8 kph windspeed.

Field evaluation of natural and synthetic pheromone

Field evaluation of natural pheromone

Attraction of male *D. theobroma* to females was demonstrated previously by King (1973), with attraction occurring late in the afternoon. The same author could not catch male *S. singularis* in traps baited with conspecific females.

In experiments at CRIG carried out in 1999, evidence for a sex pheromone in *S. singularis* was obtained for the first time (Padi *et al*., 2000) (Table 4).

TABLE 4. Catches of male cocoa mirids in sticky delta traps and New Rectangular Traps (NRT) baited with virgin females, CRIG, 20 October – 1 November 1999

	D. theobroma		*S. singularis*	
	delta	NRT	delta	NRT
trapping nights	24	19	19	35
males caught	8	9	3	13
male/night	0.33	0.47	0.16	0.37

Trap catches were species-specific for the two mirid species, and more insects were caught in the New Rectangular Traps than in delta traps, although no statistical analysis was possible on these results because of the low catches.

In 2001, an experiment was run in three farmers' fields near to CRIG to examine the effect of trap height on catches of *S. singularis* in NRT traps baited with virgin females. Catches at 2 ft and 6 ft above ground were similar and higher than those at 9 ft (Fig. 23), although the differences were not significant at the 5% level after transformation to log(x+1), ANOVA and LSD test.

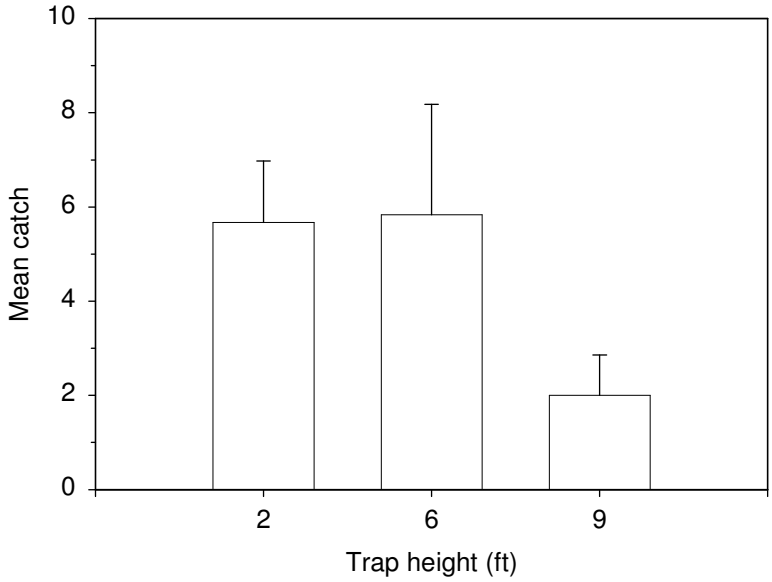

FIG. 23. Mean catch (± SE) per replicate of *S. singularis* males in NRT traps baited with virgin females at various heights (7 August – 30 October 2001; 6 reps).

In the course of this experiment, females between 8-54 days old were used. Because of differing numbers of each age group used and the low catches, it was not possible to make quantitative deductions on the relative attractiveness of the different ages, but males were

caught in traps baited with females between ages 9-42 days (Fig. 24). No males were caught in unbaited traps.

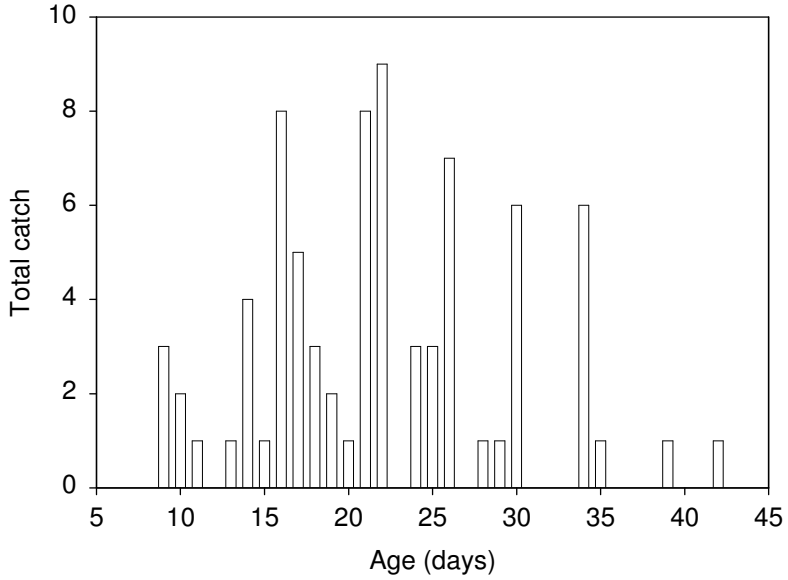

FIG. 24. Catches of male *S. singularis* in NRT traps baited with virgin females of different ages (7 August – 30 October 2001).

Field evaluation of synthetic pheromone components

The first synthetic lures were tested by CRIG staff during the above experiments. Polyethylene vials impregnated with blends of the *R* enantiomers of diester (I) and monoester (II) in amounts (µg) of 1000:500, 1000:5 and 1000:0 were evaluated in NRT traps with six replicates between 27 August – 30 October 2001, lures being renewed once after 10 days. Despite catches being observed in female-baited traps, only one male *S. singularis* was caught in the synthetic-baited traps right at the end of the period. The tops were removed from the dispensers during this trial, but, even so, the pheromone should have persisted throughout (Fig. 22), and the reason for the failure to catch is unknown.

The experiment was repeated with the same blends at 1000, 100 and 10 µg loadings of the diester (I) because laboratory release rate measurements suggested that the lower loadings might more closely simulate the release rate from female mirids (Table 3). Results in Table 5 showed that the synthetic lures were attractive to male *S. singularis*, with the 2:1 blends of diester (I) and monoester (II) probably less attractive than the other two blends. The 1000 µg loading was at least as attractive as the lower loadings. No insects were caught in unbaited traps and only very few in traps baited with a virgin female *S. singularis*. No female *S. singularis* were caught throughout. No *D. theobroma* were caught, and surveys had suggested that these were not present in the area.

In this experiment, catches were dominated by those in one particular trap baited with the 1000:5 blend in replicate (b) (Table 5). For the first two weeks of the experiment, traps were not moved, but during the second period the traps were moved on one position each day. The above trap continued to catch well (11 during the first two weeks, 17 during the second)

indicating that a favourable trap position was not the cause of the high catches. Analysis of residual pheromone in the lure indicated that there was nothing unusual about the lure, and, indeed, this trap continued to catch well in the next experiment with new lures.

TABLE 5. Catches of male *S. singularis* in NRT traps (CRIG, 19 November – 18 December 2001; all synthetic blends in polyethylene vials)

Component (µg)		Total catch per replicate					
Diester (I)	Monoester(II)	a	b	c	d	Total	Mean/trap
1000	500	4	0	1	0	5	1.3
1000	50	2	28	1	0	31	7.8
1000		1	8	1	1	11	2.8
100	50	0	0	0	0	0	0.0
100	5	1	0	0	2	3	0.8
100		9	1	0	2	12	3.0
10	5	0	0	0	0	0	0.0
10	0.5	1	0	0	2	3	0.8
10		2	0	0	0	2	0.5
virgin female		1	0	1	1	3	0.8
blank		0	0	0	0	0	0.0

At the same time as the above experiment, traps baited with five vials containing the 1000:500, 1000:5 or 1000:0 blends of (I) and (II) were evaluated nearby. Total catches in two replicates during 21 November – 18 December 2001 were 0, 2 and 2 *S. singularis* males respectively, indicating no obvious improvement in catches at the higher release rate.

In a third experiment, the same three blends at 1000 µg loading were compared with the same blends of the *S* enantiomers of diester (I) and monoester (II), and with rubber septa dispensers containing the blends of the *R* enantiomers at ten times the loading, which would be expected to give a very high release rate of pheromone.

The experiment was run from 18 December 2001 to 27 February 2002. As it is known from laboratory results (Table 3) that differential rates of release of the two pheromone components will cause the composition of the blend released to change over time, results are reported for the first 31 days as in the previous experiment (Table 6) and for the next 40 days (Table 7).

Results in Tables 6 and 7 show that the blends of the unnatural *S* enantiomers are completely unattractive and confirm the previous indications that increasing the release rate does not obviously increase catches. Catches were low in the first period and no statistical analysis was possible, but, as previously, the 2:1 blends of diester (I) and monoester (II) were less attractive to *S. singularis* males than the other two blends at the 1 mg level (Table 6). Catches were higher during the second period, and the 1000:50 (I):(II) blend was more attractive ($P<0.05$) than (I) alone. The 1000:500 (I):(II) blend was numerically, although not statistically, more attractive than (I) alone, although by this time the proportion of alcohol (II) in the blend released would have decreased markedly.

TABLE 6. Catches of male *S. singularis* in NRT traps (CRIG; 18 December 2001 – 18 January 2002; 10,000 µg blends in rubber septa, others in polyethylene vials)

Component (µg)		Total catch per replicate					
Diester (I)	Monoester(II)	a	b	c	d	Total	Mean/trap[1]
1000	500	1	0	0	0	1	0.3
1000	50	0	22	0	2	24	6.0
1000		0	3	0	1	4	1.0
1000 (*S*)	500 (*S*)	0	0	0	0	0	0.0
1000 (*S*)	50 (*S*)	0	0	0	0	0	0.0
1000 (*S*)		0	0	0	0	0	0.0
10000	5000	0	0	0	4	4	1.0
10000	500	0	0	0	1	1	0.3
10000		3	0	0	0	3	0.8
blank		0	0	0	0	0	0.0

TABLE 7. Catches of male *S. singularis* in NRT traps (CRIG; 18 January – 27 February 2002; 10,000 µg blends in rubber septa, others in polyethylene vials)

Component (µg)		Total catch per replicate					
Diester (I)	Monoester(II)	a	b	c	d	Total	Mean/trap[1]
1000	500	4	4	4	18	30	7.5 ab
1000	50	35	8	4	13	60	15.0 a
1000		6	3	1	6	16	4.0 b
1000 (*S*)	500 (*S*)	0	0	0	0	0	0.0
1000 (*S*)	50 (*S*)	0	0	0	0	0	0.0
1000 (*S*)		0	0	0	0	0	0.0
10000	5000	1	0	2	16	19	4.8
10000	500	9	0	0	2	11	2.8
10000		21	2	1	1	25	6.3
blank		0	0	0	0	0	0.0

[1] means followed by same letter are not significantly different after ANOVA and LSD test ($P<0.05$)

A few *D. theobroma* males were also caught towards the end of this experiment between 30 January – 18 February 2002 (Table 7). Catches were too low to make any conclusions at this stage.

TABLE 7. Catches of male *D. theobroma* in NRT traps (CRIG; 18 December 2001 – 27 February 2002; 10,000 µg blends in rubber septa, others in polyethylene vials)

Component (µg)		Total catch per replicate					
Diester (I)	Monoester(II)	a	b	c	d	Total	Mean/trap[1]
1000	500	0	0	1	0	1	0.25
1000	50	0	0	0	0	0	0.0
1000		8	0	0	0	8	2.0
1000 (*S*)	500 (*S*)	0	0	0	0	0	0.0
1000 (*S*)	50 (*S*)	0	0	0	0	0	0.0
1000 (*S*)		0	0	0	0	0	0.0
10000	5000	1	0	0	0	1	0.25
10000	500	0	0	0	0	0	0.0
10000		0	0	0	0	0	0.0
blank		0	0	0	0	0	0.0

A small trial was run during the same period in an adjacent area to investigate whether catches could be improved by placing the lures on the sticky base of the trap rather than suspended in the centre of the trap. During the whole period 18 December 2001 - 27 February 2002, mean total catches per trap of *S. singularis* males with the 1000:500, 1000:5 and 1000:0 (I):(II) blends were respectively 15.0, 6.5 and 9.0 with lures on the base compared with 7.8, 21.0 and 5.0 with lures suspended. These results would suggest the different lure positions do not affect the number of mirids captured.

Training of counterpart staff

Formal training in UK

After the training course in the UK, Dr Ackonor was able to:

- isolate fungi and nematodes from diseased insects;
- identify the most important entomopathogenic fungi and insects;
- design and interpret bioassay experiments;
- know how to produce large quantities of entomopathogens for field release;
- understand how to apply biopesticides and their use with other forms of control;
- access information resources on insect pathology and biopesticides for further study and research.

Mr Lowor learnt:

- methods for pheromone collection by trapping on solid adsorbents and solid phase micro-extraction (SPME;
- operation and maintenance of gas chromatography equipment;
- qualitative and quantitative GC analysis;
- methods for evaluation of pheromone formulations;
- basics of electroantennography.

On-the-job training in Ghana

On-the-job training empowered CRIG staff to identify and also manipulate entomopathogens in the laboratory.

Equipment was provided for pheromone collection and instruction given on how to use it and prepare samples for analysis. Guidance and advice were given on setting up and data collection in trapping experiments, and construction of different trap designs.

CONTRIBUTION OF OUTPUTS

Development of mycopesticides for cocoa mirids

In this project, extensive surveys of cocoa farms in Ghana yielded a single isolate of *B. bassiana* from the cocoa mirid, *S. singularis*. The cool shaded under-canopy of cocoa plantations offers an environment which is highly conducive for the collection and eventual use of fungal entomopathogens, and it was anticipated that more pathogens would be found. Given that cocoa is grown under a wide range of farming systems, extending the surveys to other countries such as Côte d'Ivoire, Nigeria and Cameroon could yield more isolates of *B. bassiana* or other entomopathogens.

In order to evaluate the potential value of the Ghanaian isolate as a control agent for mirids, it was characterised in comparison with four other strains of *B. bassiana* isolated from cocoa mirids in Papua New Guinea. All strains were highly pathogenic to *S. singularis*, and no significant differences in pathogenicity could be measured, possibly because the spore concentration of 1 X 10^7/ml used in the bioassay was too high and therefore not discriminatory. The Ghanaian isolate was one of the three whose colonies grew fastest and attained the largest colony sizes and one of the two producing the highest number of spores. After an exposure period of five weeks, the Ghanaian isolate showed significantly higher survival than other isolates over the temperature range 23º-33ºC that might be experienced in cocoa farms. Reduced survival at higher temperatures reported previously for *B. bassiana* (Walstad *et al*. 1970; Hong *et al*. 1997) was not observed here over the range tested.

These characteristics indicated that the Ghanaian strain of *B. bassiana* was highly suited to take forward for development as a biopesticide. It was successfully mass-produced on boiled rice, which is readily available. Further evaluation could be conducted to identify even cheaper media, e.g. maize or agricultural waste products like cocoa pulp, on which the fungi can be produced in order to facilitate adoption by farmers of the biopesticide.

Appropriate formulation and application of entomopathogens is critical to the success of a mycoinsecticide. The fungal spore is the active ingredient and needs to remain viable until it gets into contact with its host (pest). In formulation studies on the Ghanaian isolate of *B. bassiana,* the water/Tween solution supported the highest initial viability as well as viability after 28 days but did not maintain the fungus at the most resistant spore stage. Ondina oil also supported high initial and longer-term viability although its cost may preclude it as a candidate diluent. Of the locally available, cheap diluents, sunflower oil supported the highest initial viability as well as longer-term viability, although the latter was

significantly lower than that of Ondina. By mixing the spores with sunflower oil just before application the need to maintain the viability of the spores for periods longer than a day could be avoided.

Following training in the UK, the CRIG scientist was then able to design, carry out and analyse data from both laboratory and field experiments. They were also able to identify and mass-produce and manipulate the entomopathogens in the laboratory, hence ensuring sustainability of the project activities.

Development of pheromones for cocoa mirids

In this project, the production of a sex pheromone by female *S. singularis* was demonstrated for the first time during trapping experiments by CRIG (Padi *et al.*, 2000), complementing earlier reports of pheromone production by female *D. theobroma* (King, 1973). During this work an effective, locally-made trap was developed and traps at 2 ft or 6 ft above ground caught at least as many as traps at 9 ft.

Collection of volatiles from the mirids was effectively carried out at CRIG, and over 170 collections were made during the course of the project. Isolation and identification of the pheromone components at NRI was hampered by the difficulty of getting significant numbers of mirids to NRI for electrophysiological work, by the small amounts of pheromone produced and the novel chemical structures of the components. Nevertheless, two components were detected in volatiles from virgin female insects which elicited electrophysiological responses from the antennae of males. By consideration of mass spectral and chromatographic data and synthesis of more than 40 standards, these components were eventually identified as the diester (I) and corresponding monoester (II) (Fig. 12). The diester (I) was shown to have the *R* configuration by chromatography on a chiral GC column, and the monoester (I) was assumed to have the same configuration. The *R* enantiomers of the synthetic compounds elicited EAG responses from a male *S. singularis*, and the *S* enantiomers were much less active. This is the first time these types of structures have been reported as insect pheromone components, although a related compound was very recently reported to be produced in large quantities by certain wasp species (Clark *et al.,* 2001). Several synthetic routes to the pheromone components were investigated, the best starting from a readily-available biopolymer, giving the monoester and diester in successive steps with good yield and very high geometric and enantiomeric purities.

The rate of pheromone production by the insects seemed to be very variable, typically only 5 ng/insect/day, although in some cases up to 100 ng/insect/day. Nevertheless this is much less than has been reported for other mirid species, for example 10 µg/insect/day by *Lygus rugulipennis* (Innocenzi *et al.*, 2002).

The two mirid species seemed to produce the two pheromone components in essentially the same ratio, approximately 2:1 (I):(II). This was unexpected, as females of the two species attract only conspecific males. However, it is possible that they use similar pheromone blends and ensure species-specificity by different temporal patters of activity - *D. theobroma* is active and trapped in the late afternoon while *S. singularis* is active during the night. A similar situation was observed with two species of sweet potato weevils *Cylas puncticollis* and *C. brunneus* (Downham *et al.*, 1999). Although the two mirid species belong to different

genera, they are classified in the same tribe, the Odoniellini, and hence are relatively closely related (Entwhistle, 1972).
Dispensers for the synthetic pheromone components were developed. Closed polyethylene vials continued releasing the diester (I) for at least two months under laboratory simulation of field conditions. Rubber septa gave similar, slightly faster release, but these are much more expensive than the vials and were not investigated further. The monoester (II) is released faster from the vials, and this can lead to a change in the blend released from dispensers containing mixtures of the two components over prolonged periods.

Field trapping trials supported these findings. Both *S. singularis* and *D. theobroma* males were caught in traps baited with blends of the two synthetic pheromone components and catches were higher than in traps baited with virgin female mirids. Polethylene vials loaded with blends based on 1 mg of the pheromone component (I) were as attractive as dispensers with higher or lower loadings and hence release rates. Populations and trap catches of *D. theobroma* in Ghana are currently too low to make any deductions on the most attractive blend. For *S. singularis* indications are that, at least at first, blends of 1000:50 (I):(II) or (I) alone in polyethylene vials seem to be more attractive than a 1000:500 (I):(II) blend. This is consistent with release rate data as the latter blend in the polyethylene vial would initially release a blend of volatiles containing 1:4 (I):(II). Experiments to optimise the blend further are in progress at CRIG.

Trap catches were very variable. While this may be due in part to the known patchy distribution of mirids, there is evidence that catches are affected by details of trap design in that certain traps were found regularly to catch more than others. The cause of this could not be determined during this project, and the phenomenon needs to be further investigated.

Contribution of outputs

The planned outputs of the project have been achieved. A local isolate of an entomopathogenic fungus active against cocoa mirids has been isolated and characterised, produced on pilot scale and formulated so that it is now available for field evaluation. Pheromone components for both species of mirids have been isolated, identified and synthesised. Blends have been shown to attract one of the mirid species in the field and effective traps and dispensers are available for further evaluation. Staff at the main target institution, CRIG, have been trained in insect pathology and pheromone technology and will be able to carry on the work.

Progress has been slower than expected. Entomopathogens were much more difficult to find than anticipated from work on related species in other countries. The Ghana mirid species still cannot be reared in large numbers and they are very difficult to maintain and transfer between laboratories without heavy mortality. Prior to the project, *D. theobroma* was thought to be the dominant species, but now it is difficult to find compared with *S. singularis.* The pheromone work was hampered by the very small amounts of pheromone produced and the novel structures involved as well as by the factors above. The whole project suffered to some extent from the failure to agree contractual terms with CRIG, and this will be even more important in any future collaborative work.

Nevertheless, the project outputs provide new technologies that can now be evaluated for use in control of cocoa mirids in Ghana and neighbouring countries. Mirids were unanimously rated as one of the two main pest and disease constraints on cocoa production throughout West Africa at

the recent workshop "West African Regional Cocoa IPM". These biorational approaches will help minimise use of conventional pesticides and should be more appropriate for use in developing countries and more sustainable. Cost-effective improvements in productivity of cocoa will benefit the livelihoods of over six million people in Ghana alone who depend directly or indirectly on cocoa.

Future work

In discussion with the Manager of the CPP and Programme Advisor, it has been proposed to hold a workshop in Ghana to disseminate the outputs of this project and a parallel CPP project on control of *Phytophthora megakarya* (R7326). Participants will be invited from all cocoa-producing countries and organisations in West Africa, and this will also provide the opportunity to consider how best to take the two projects forward in a single cocoa IPM project aimed at the two main pest and disease constraints to cocoa production in West Africa.

Future work on the mycopesticide component would focus on:

- field evaluation of formulations of the Ghanaian *B. bassiana* isolate as a control agent;
- further improvement of production methods and investigation of use of cheaper media such as maize or agricultural waste products like cocoa pulp;
- further work to optimise the formulation and application techniques;
- if this approach is to be investigated in other countries in the Region, it may be desirable to survey for local fungal isolates to ensure maximal effectiveness and avoid quarantine restrictions.

Future work on the pheromone component would require:

- further refinement of the pheromone blend for *S. singularis*;
- further refinement of the pheromone blend for *D. theobroma*;
- further investigation of features of the trap/lure which determine effectiveness;
- better understanding of the dynamics of trapping in relation to mirid populations and distribution;
- development of trapping for monitoring and control of mirids.

In all future work there will be a need to resolve IPR issues concerning rights to the fungal isolate and identified pheromones. It will also be necessary to develop equitable methods for production of both mycopesticides and pheromones that are acceptable to both the inventors and commercial enterprises and which ensure the technologies are available to poor farmers.

Dissemination

Publications

PADI, B., HALL, D., FARMAN, D., DOWNHAM, M., LOWOR, S., & SARFO, J.E. (2000). Evidence of sex attractants in the cocoa mirids *Distantiella theobroma* (Dist.) and *Sahlbergella singularis* Hagl. (Heteroptera: Miridae) in field-trapping experiments. Proceedings of 13th International Cocoa Research Conference, Kota Kinabalu, Sabah, Malaysia, 9-14 October 2000, 6 pp. (presentation given and proceedings in press)

PADI, B., ACKONOR, J.B., OPUKU, I.Y., HALL, D., FARMAN, D., DOWNHAM, M., ODUOR, G., LOWOR, S., OWUSU-MANU, E., ADU-AMPOMAH, Y., SARFO, J.E., ADU-ACHEAMPONG, R., REICH, I. & ADUSEI, E.O. (2000). Recent advances in the development of an integrated pest management strategy for the control of cocoa mirids and mealybug vectors of the cocoa swollen shoot virus disease (CSSVD) in Ghana. INCOPED 3rd International Seminar on Cocoa Pests and Diseases, Kota Kinabalu, Sabah, Malaysia, 16-17 October 2000: 114-122.

Planned publications

- Paper on characterisation of Ghanaian isolate of *B. bassiana.*
- Paper on identification of pheromone components of *D. theobroma* and *S. singularis*.

Internal Reports

HALL, D.R. & FARMAN, D.I. (1998). VISIT TO Ghana to initiate project on biorationals for control of cocoa mirids, 3-17 November 1998.

HALL, D.R. (1999). Visit to Niger and Ghana, 8-18 June 1999.

ODUOR, G.I. (1999) Report on a survey of cocoa growing regions in Ghana for pathogens of cocoa mirids, Ghana, September 1999. Report No. 1. CAB International – Africa Regional Centre, Kenya. 2 pp. (BTOR)

DOWNHAM, M.C.A. & FARMAN, D.I. (1999). Visit to Ghana to undertake collaborative work on pheromones of cocoa mirids 13/10/1999 to 23/10/1999

ODUOR, G.I. (2000) Report on a visit to Ghana to conduct surveys for pathogens of cocoa mirids and discuss future activities, Ghana, July 2000. Report No. 2. CAB International – Africa Regional Centre, Kenya. 2 pp.

HALL, D.R. (2000). Report on visit to Ghana to develop pheromone trapping for cocoa mirids, 9-17 July 2000.

HALL, D.R. (2000). Visit to Cocoa Research Institute of Ghana, 29/10/2000 to 06/11/2000

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ODUOR, G.I. & GODONOU, I. (2001) Report on a visit to Ghana to conduct surveys for pathogens of cocoa mirids and initiate bioassay experiments, Ghana, July 2001. Report No. 3. CAB International – Africa Regional Centre, Kenya. 2 pp.

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HALL, D.R. (2001). Visit to Benin and Ghana 11/11/2001 to 24/11/2001.

HALL, D.R. (2001). West Africa Regional Cocoa IPM Workshop, November 13 – 15, 2001, Cotonou, Benin.

Other Dissemination of Results

Presentation given by D R Hall on behalf of NRIL to "West African Regional Cocoa IPM" Workshop, November 13 – 15, 2001, Cotonou, Benin: "Cocoa Research funded by DFID CPP".

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APPENDIX 1. GC-MS analyses of collections of volatiles from unmated *S. singularis* and *D. theobroma* adults, 0-4 days old, 1998 (individual samples bulked according to sex and species; SS *S. singularis*, DT *D. theobroma*, M male, F female, CHUP chupons, POD cocoa pod)

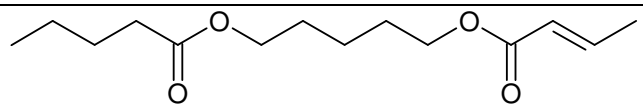
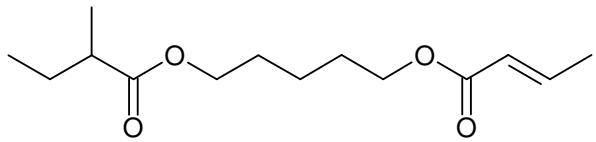
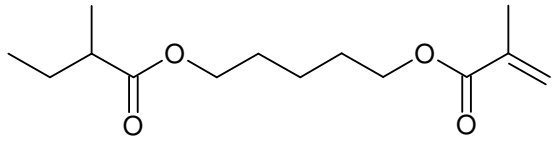
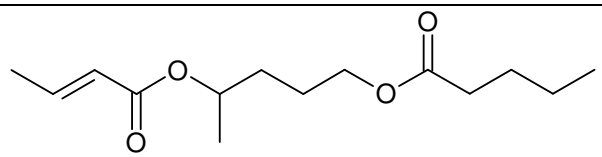
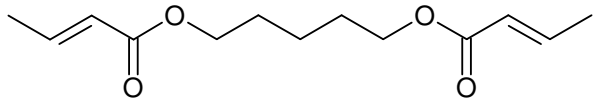
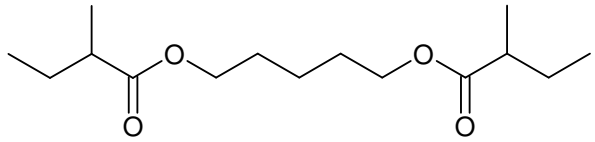
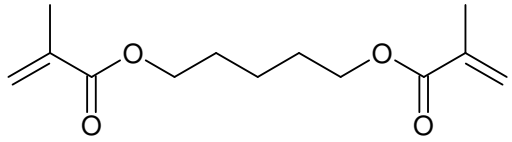
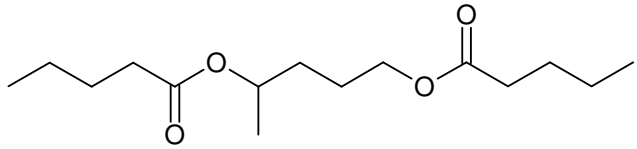
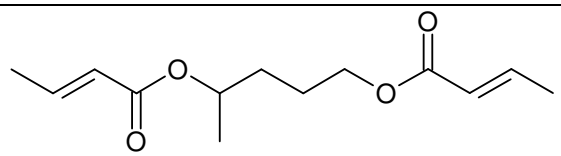
				Source (number of samples)							
Scan	Compound	Fit[1]	KI	SS M[2] (4)	SS F[2] (6)	SS M (4)	SS F (6)	DT M (2)	DT F (10)	CHUP (2)	POD (4)
307	2-octanol	658	1117	20.7%	17.8%	0.7%	6.1%	10.7%	5.2%		30.0%
360	myrcene	897	1162				0.3%	0.9%	0.5%		
381			1180	3.2%	3.4%		1.6%		0.8%		
398			1195						0.5%		
452	Ocimene/monoterpen	954	1235		1.4%	1.7%	1.1%		2.2%		
474	E-β-ocimene	964	1250		25.1%	33.0%	15.8%	25.6%	43.7%	6.2%	
520	a cyclohexanone?	889	1283						0.3%		
552	2Me,6methylene-1,7-octadiene-3-one	909	1306	1.8%	2.4%	4.2%	1.5%		1.1%		
611	alkene		1349						0.2%		
646			1374						0.3%		
654	3-hexenol		1380			1.6%	1.3%	1.5%	0.8%		13.7%
673	nonanal	934	1394	19.6%	1.8%		1.1%	2.2%	0.6%		13.3%
705	hexyl butyrate		1417						0.3%		
733	cis linalool oxide	942	1437		4.1%	2.4%	1.7%	1.5%	1.1%		
745	1-octen-3-ol									1.9%	
750			1449			1.3%	0.6%	1.5%	0.7%		
759	Z3-hexenyl butyrate		1456							3.3%	
764			1460	3.3%			0.7%		0.7%	1.4%	
771			1465	1.7%			0.3%		0.5%		
773	trans linalool oxide	940	1466		5.5%	4.0%	3.0%	4.8%	1.0%		
804	octanol	844	1489	1.7%	3.0%	1.4%		2.2%	1.3%		
818	decanal	944	1499	2.2%	2.1%	2.1%	0.6%	2.9%	0.4%		16.2%
878	linalool	921	1543		24.5%	8.5%	45.6%	10.8%	2.9%		
929			1580					1.9%			

935	bergamotene	925	1584				0.3%		4.4%		
938	B elemene	943	1586				0.5%				
945	caryophyllene	962	1591			0.7%	2.9%	0.7%	0.4%		
953	heptanone ((br)	965	1597			4.3%		4.0%			
1039	ethyl benzoate	946	1665			1.3%	0.6%				
1139	a-farnesene	950	1745					1.6%	2.5%		
1154	B elemene	941	1757				1.5%				
1175	methyl salicylate	945	1774			10.4%	3.3%				
1211	dendrolasin	899	1803	45.7%	3.3%	8.3%	6.2%	12.3%	18.7%	87.2%	
1463	tridecanal	922	2025						0.4%		
1474	E nerolidol	959	2035			4.3%	1.3%		0.6%		
1508	methyl 2MeObenzoate	971	2068						0.3%		
1525			2084						0.1%		
1616	hydrocarbon		2171		1.8%		0.7%	2.3%	0.5%		
1722									0.3%		
1732			2287						0.5%		
1761									0.3%		
1780			2335				0.7%	2.6%	0.7%		
1884	indole	964	2440		2.6%		0.7%		0.7%		
1909			2466		1.4%	5.0%		2.0%	0.3%		
1969			2526						0.3%		
1988			2545			4.7%		4.1%	1.7%		13.0%
2019			2577					3.7%	1.2%		13.9%
2048									0.3%		
2064									0.6%		
2092									0.2%		

[1] Fit of observed mass spectrum with library spectrum; 1000 is perfect fit
[2] These adults were possibly mated

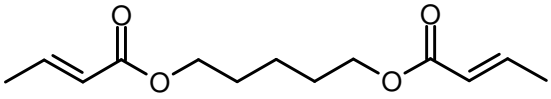
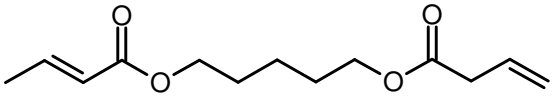
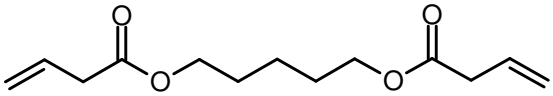
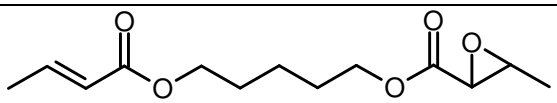
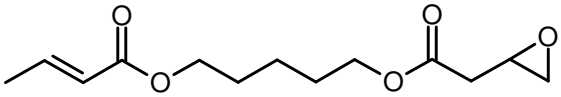
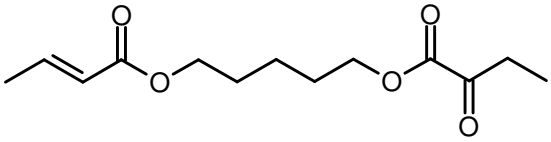
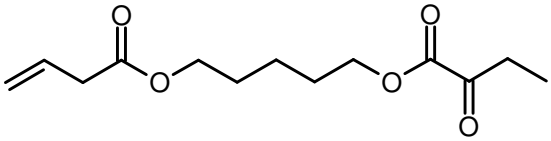
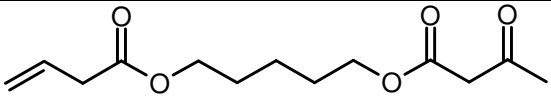
APPENDIX 2

GC retention data for first series of diesters on polar (Wax) and non-polar (Sil) GC phases (Δ is difference between RI on Wax and RI on Sil).

	RI		
	Wax	Sil	Δ
Distantiella theobroma EAG-active component	2220	1678	542
	2410	1780	630
	2323	1733	590
	2184	1675	509
	2272	1693	579
	2473	1733	740
	2178	1736	432
	2190	1609	581
	2208	1745	463
	2340	1642	698

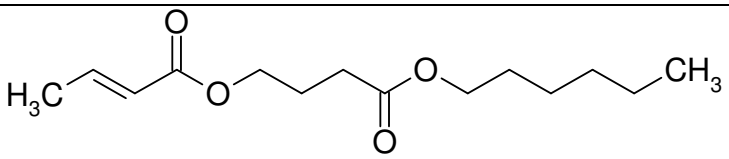
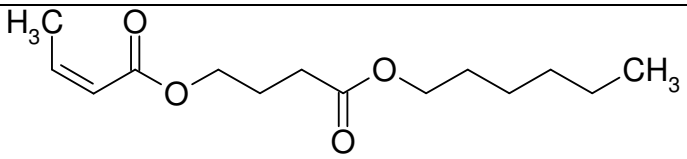
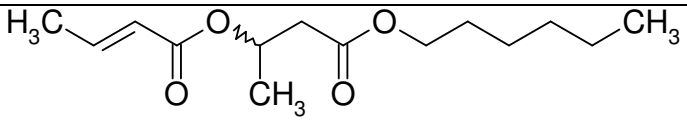
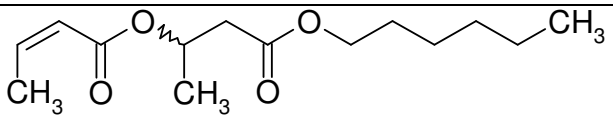
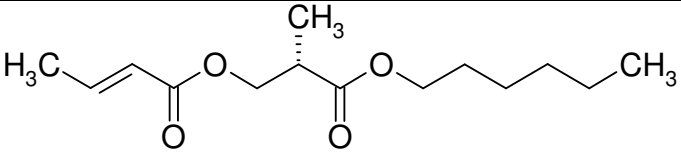
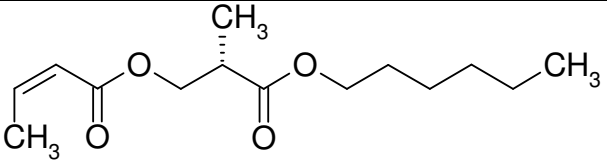
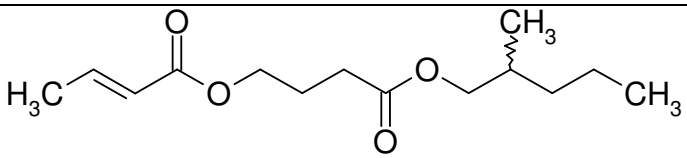
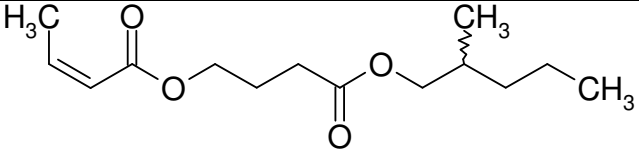
APPENDIX 3

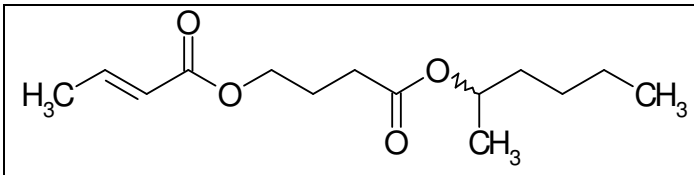
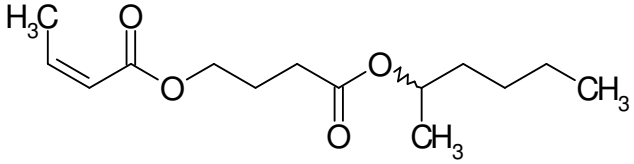
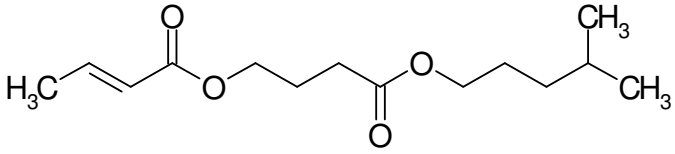
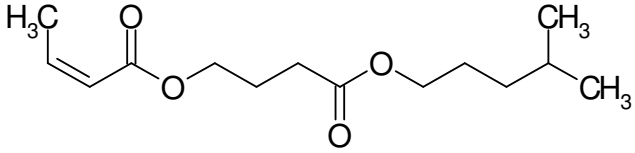
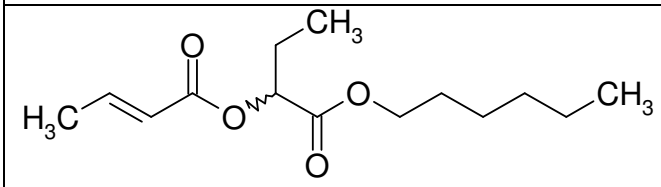
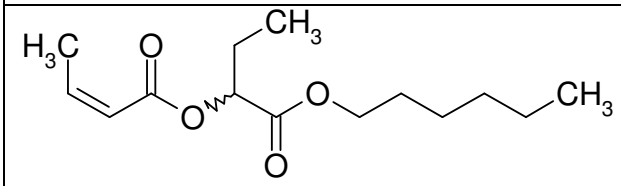
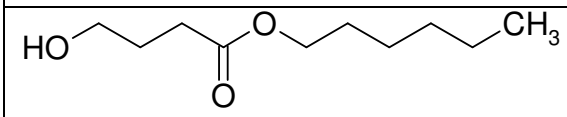
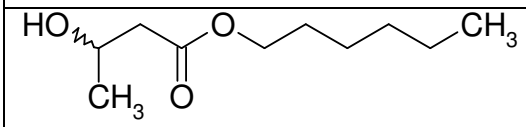
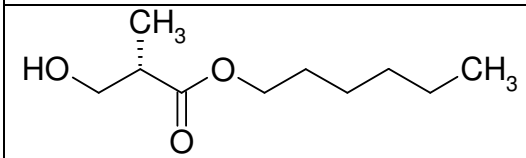
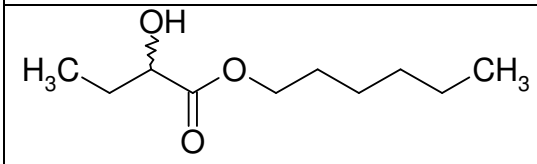
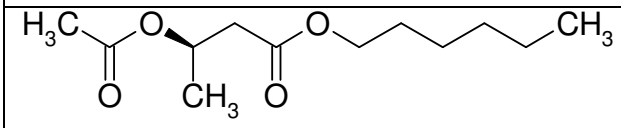
GC retention data for second series of diesters on polar (Wax) and non-polar (Sil) GC phases (Δ is difference between RI on Wax and RI on Sil).

	RI		
	Wax	Sil	Δ
D. theobroma EAG-active component	2220	1678	542
	2453	1742	711
	2360	1672	688
	2277	1609	668
	2703	1831	872
	2591	1859	732
	2582	1772	810
	2494	1710	784
	2260	1374	886

APPENDIX 4

GC retention data for third series of diesters on polar (Wax) and non-polar (Sil) GC phases (Δ is difference between RI on Wax and RI on Sil).

	RI		
	Wax	Sil	Δ
D. theobroma EAG-active component	2220	1678	542
S. singularis EAG-active component (I)	2220		
S. singularis EAG-active component (II)	1886		
	2382	1770	604
	2317	1745	572
 **(I)**	**2211**	**1676**	**535**
	2147	1650	497
	2218	1687	531
	2146	1666	480
	2314	1723	591
	2248	1698	550

	2256	1723	533
		1693	
	2335	1734	601
		1711	
	2146	1643	503
	2080	1616	464
	2149	1405	744
 **(II)**	**1899**	**1301**	**598**
	1977	1331	646
	1771	1272	499
	1938	1462	576