3.
Biological Agents for Insect Control

Chairman: DR J. J. MENN

INSECT CONTROL USING MICROBIAL TOXINS: STATUS AND PROSPECTS

A. R. JUTSUM, N. J. POOLE, K. A. POWELL

ICI Agrochemicals, Jealott's Hill Research Station,
Bracknell, Berks, RG12 6EY, UK

R. L. BERNIER

C-I-L Research Centre, 2101 Hadwen Road, Sheridan Park,
Mississauga, Ontario, L5K 2L3, Canada

ABSTRACT

Microbial toxins are biological poisons derived from micro-organisms such as bacteria and fungi. They can be exploited commercially in a number of ways:

1) Contained within the producing organism,
2) As viable products in their own right,
3) As leads for further chemical or microbial modification, or
4) Engineered genetically into symbionts, carriers or crop plants.

Two specific sources of toxin are described to exemplify the various approaches being pursued. These are the protein toxins from Bacillus thuringiensis and the avermectins produced by Streptomyces avermitilis.

INTRODUCTION

Jutsum (1988) has recently reviewed the present and future application of biological control agents ranging from parasitoids and predators through to pathogens and pheromones. The scope of this paper, though, is restricted to the use of microbial toxins for insect control. There are currently in excess of 1500 naturally occurring micro-organisms or microbial metabolites which are known to possess insecticidal properties, and further novel toxin-producing strains are being produced using genetic engineering methods and natural selection. However, in the future we will have the ability to exploit microbial toxins in a number of different ways including as

1) Dead microbes
2) Living microbes
3) Natural toxins
4) Modified toxins
5) Engineered symbionts
6) Engineered carriers, and
7) Engineered crop plants

SCOPE OF THE PAPER

A microbial toxin can be defined as a biological poison derived from a micro-organism, such as a bacterium or fungus. Pathogenesis by microbial entomopathogens occurs by invasion through the integument or gut of the insect, followed by multiplication of the pathogen resulting in the death of the host. Studies have demonstrated that the pathogens produce insecticidal toxins important in pathogenesis (Burges, 1981). Many toxic compounds, such as Beauvericin, and the destruxins of Metarhizium anisopliae, have now been isolated and characterised. Most of the toxins produced by microbial pathogens which have been identified are peptides, but they vary greatly in terms of structure, toxicity and specificity (eg, see Grove and Pople, 1980; Burges, 1981; Dunphy and Nolan, 1982). However, it is impossible to cover such a broad topic as microbial toxins in a paper of this length, so we have restricted our review to the toxins of Bacillus thuringiensis and Streptomyces avermitilis.

The toxins of B. thuringiensis are the most widely investigated example. The organism was first isolated in 1902 by Ishiwata (see Aoki and Chegasaki, 1915), but it was not until the 1950's that the diamond-shaped crystals or "parasporal bodies" in sporulating cells of B. thuringiensis were recognised as being involved in killing insect larvae (Hannay, 1953). Hannay and Fitz-James (1955) demonstrated that the crystals were mainly protein and Angus (1956a, b) showed that the crystals caused paralysis of the midgut and cessation of feeding in the silkworm, Bombyx mori, while the spores were necessary for the establishment of septicemia.

In contrast, the toxins produced by Streptomyces avermitilis - avermectins - were first described less than a decade ago (Putter et al, 1981), but many studies have been performed with these macrocyclic lactone toxins as insecticides, in addition to work performed to elucidate the acaricidal and nematicidal properties.

MARKET FOR MICROBIAL TOXINS

In 1988, the global end-user insecticide market was estimated as $6075 million (County Natwest Woodmac, 1989), with over half of the usage in cotton, fruit and vegetables. A geographical breakdown of insecticide sales by value is shown in Table 1, along with an estimate of sales of B. thuringiensis.

TABLE 1

Sales of insecticides and B. thuringiensis by geographical area, 1988

AREA	SALES IN $ MILLIONS	
	ALL INSECTICIDES	B.THURINGIENSIS
Far East	2015	5
USA	1115	6
W Europe	990	2
Latin America	705	3
Rest of the World	1250	15

Thus, sales of B. thuringiensis are believed to account for less than 1% of world insecticide sales, with application concentrated in a few specific outlets, such as forestry, vegetables, maize and public health.

The market for toxins isolated from B. thuringiensis, such as the product, DiBeta, is currently small compared to sales of the whole organism. In contrast, sales of the isolated avermectin toxin and analogues thereof, are vast, at around $200 million per annum. However, sales are primarily into animal health outlets and only limited use is made of the products for insect control in crops at the present time.

Future markets for microbial toxins will centre on the use of whole organisms, and the application of isolated or altered toxins, but the major increase will come from the introduction of toxins into plants via genetic engineering. Specific market niches can also be identified which are highly suited to the use of microbial toxins:

1. Outlets where conventional chemical agents give insufficient levels of control, for example, where there is insecticide resistance, as is the case with Plutella xylostella in parts of the Far East.

2. Outlets where conventional chemical agents are too expensive.

3. Outlets where governments restrict the application of conventional chemical agents, as practised in Canadian forestry.

4. Outlets where the environment is contained and controlled, for instance, in glasshouses (where biological control is already employed) or even in rice paddy.

Eventually, microbial toxins may be exploited in virtually any market, provided that they are as good as, or better than, existing control agents in terms of cost, efficacy and reliability, or have a significant toxicological or environmental advantage.

The major utility for microbial toxins in the future may reside in genetically manipulated microbial pest control agents. Estimation of the potential market is exceedingly difficult on a particular time scale as much of the research is performed by agrochemical and biotechnology companies 'behind closed doors'. However, market estimates for "biopesticides" range from conservative ones to $455 million of sales in the USA in 2025 (Stanford Research Institute International, 1984) to extreme estimates of 50% market penetration by the year 2000 (Klausner, 1984). Only time will tell which market projections are correct.

POTENTIAL USES OF MICROBIAL TOXINS

a) In the organism

B. thuringiensis is a gram-positive bacterium forming elliptical spores, contained in unswollen sporangia, and a parasporal body (or crystal) which appears mainly as a bipyramidal shape. Many insecticidal metabolites of *B. thuringiensis* have been identified and characterized including various enzymes and a nucleotide-like structure named beta-exotoxin that is used mainly in the USSR for improved insect control. However, the most important biologically-active metabolite produced by *B. thuringiensis* is the parasporal protein crystal formed upon sporulation and named delta-endotoxin. The crystals from various subspecies are composed of up to four proteins with molecular weights ranging from 26 to 140 kDa. In the case of the lepidopteran-specific subspecies, the major component of this crystal is a 130-140 kDa protein referred to as the protoxin. Upon dissolution at alkaline pH in the midgut of targeted larvae, the protoxin is further "activated" by specific proteolysis and the toxic moiety released (Huber and Luthy, 1981). Upon its attachment to specific receptors of the columnar cells in the midgut epithelium, the cells swell and are released from the basement membrane and finally burst. The insect stops feeding, becomes rapidly dehydrated and generally dies within the next 48 hours.

Many strains of *B. thuringiensis* have now been isolated and classified upon biochemical, enzymatic and serological criteria. The generally accepted key for the taxonomic division of the species of *B. thuringiensis* is based on the antigenic properties of the flagella as developed by de Barjac and Bonnefoi (1962). However, *B. thuringiensis* strains can also be allocated to different subspecies or varieties based on their pathotypes or insecticidal activity for different insects.

The five naturally occurring pathotypes reported by Ellar et al (1986) are described in Table 2.

TABLE 2 Pathotypes of *Bacillus thuringiensis*

PATHOTYPE	EXAMPLE	COMMERCIAL PRODUCTS AVAILABLE
1. lepidopteran-specific	*B. thuringiensis* var. *kurstaki*	Dipel (Abbott) Bactospeine (Philips Duphar) Thuricide, Javelin (Sandoz)
2. dipteran-specific	*B. thuringiensis* var. *israelensis*	Vectobac (Abbott) Bactimos (Philips Duphar) Teknar (Sandoz)
3. coleopteran-specific	*B. thuringiensis* var. *san diego*	Trident (Sandoz) M-One (Mycogen)
4. those active against both Lepidoptera and Diptera	*B. thuringiensis* var. *aizawai*	Certan (Sandoz)
5. Non-toxic to insects	*B. thuringiensis* var. *dakota*	-

B. thuringiensis var kurstaki (mainly HD1 and HD12 isolates) has been used for some years on a limited scale in both agriculture and forestry. It is well known that B. thuringiensis is very selective in its biological action, and kills only a limited range of insects; birds, mammals, and fish are not affected. This selectivity is a key feature of B. thuringiensis, since it leads to valuable properties in the marketable product; very favourable toxicology and environmental profile, zero pre-harvest interval on vegetable crops, "bio-rational" registration in North America, etc. However, B. thuringiensis var kurstaki has drawbacks including speed and mode of action, and solar radiation sensitivity which have limited its usage. Combining these problems, B. thuringiensis application on foliage remains a challenge in most agricultural situations. Anti-feeding effects observed in various B. thuringiensis formulations combined with non-optimised application rates and spray technology especially in agriculture have decreased significantly its attractiveness to crop producers. Finally, the narrow spectrum of activity of B. thuringiensis, which is an advantage with regards to its environmental impact, becomes problematic in most agricultural situations where crops are threatened by a complex of various pests.

Over the past few years, many major agrochemical companies (including ICI) have undertaken massive screening programmes to seek for natural isolates showing better intrinsic activity, improved resistance to environmental stresses and broader spectrum of activity.

An initial screening of 500 strains isolated from soil, insect and grain samples allowed ICI to isolate a novel B. thuringiensis var kurstaki strain (A20) (Bernier et al, 1986) with enhanced activity against various Lepidoptera. Comparative diet bioassay data for three lepidopterous pests are shown in Table 3.

TABLE 3

Comparative efficacy of B. thuringiensis, var kurstaki strain A20 and the standard HD1

INSECT	B. THURINGIENSIS STRAIN	LC50 (ppm)	POTENCY INCREASE
Trichoplusia ni	A20	0.9	6.3
	HD1	5.7	
Plutella xylostella	A20	0.4	10.0
	HD1	4.0	
Heliothis zea	A20	19.7	2.7
	HD1	52.9	

In addition, strain A20 exhibits good activity against the Spruce budworm Choristoneura fumiferana, the Gypsy moth, Lymantria dispar and the European corn borer, Ostrinia nubilalis. Although A20 is more

active than HD1, its intrinsic activity against other important agricultural pests is low, but recent progress in molecular biology has allowed a better understanding of B. thuringiensis crystal gene structure and opened the way to genetic manipulation of B thuringiensis strains. However, public perception and the lack of proper guidelines from governmental agencies pushed several companies to choose a more "natural" way to improve B. thuringiensis activity and spectrum. The serendipitous discovery of a natural plasmid transfer system by Gonzales and Carlton (1982) has allowed the production of new B. thuringiensis strains with improved intrinsic activity and spectrum. Such a method allowed the transfer of lepidopteran-active crystal genes into coleopteran-active B. thuringiensis strains to generate new hybrid clones active against insect species in both orders. A similar approach is presently used in our laboratories to improve the activity of our lead strains.

The first delta-endotoxin gene cloned was from B. thuringiensis var kurstaki in 1981 (Schnepf and Whiteley, 1981). Since then, many other genes from different B. thuringiensis strains have been cloned, sequenced and compared. The isolation of these genes has enabled both the analysis and manipulation necessary to improve spectrum of activity as well as intrinsic activity. Mycogen Corporation recently filed a European Patent Application (Edwards et al, 1987) describing a new process for altering the insect host range of B. thuringiensis toxins, by recombining in vitro the variable regions(s) of two or more delta-endotoxin genes.

Finally, another approach in B. thuringiensis improvement is based on formulation changes. Improved formulations could extend B. thuringiensis crystal persistence within the environment and improve its application; hence providing an optimum coverage of targeted plant tissues for a better effectiveness. Furthermore, a better understanding of spray technology and B. thuringiensis foliage deposition has been one important factor in the success of B. thuringiensis in forestry. Although B. thuringiensis utilization in agriculture remains fundamentally different from forestry application, a similar approach in B. thuringiensis application could improve its efficacy.

Regardless of the approach taken to improve the insecticidal activity of B. thuringiensis strains, the most severe limitation to B. thuringiensis improvement is the lack of a reliable analytical method for potency assessment. Until recently there were no reliable analytical methods to successfully assess B. thuringiensis potency. Although B. thuringiensis protoxin and activated toxin can be easily monitored on SDS-PAGE or by rocket immunoelectrophoresis, discrepancies between protein monitoring and biological activity were frequent. Recently, the use of insect cell lines, particularly the CF1 line isolated from Choristoneura fumiferana allowed the introduction of a fast and reliable method to assess B. thuringiensis activity.

The introduction of B. thuringiensis insecticidal genes into various microbial hosts has led to the design of new expression and delivery systems. For instance, Monsanto Company claimed the production of

genetically-engineered Pseudomonas fluorescens (a plant colonizing micro-organism) expressing insecticidal activity against lepidopterous larvae and having substantially the immunological properties of the crystal protein toxin of B. thuringiensis (Watrud and Perlak, 1986). Such an insecticidally-active, genetically-engineered, plant-colonizing micro-organism could provide a superior method of combating certain lepidopterous insects through a better delivery of the insecticidal compound. Crop Genetics International developed the InCide process in which the insecticidal B. thuringiensis activity is delivered through a bacterium capable of entering into endosymbiotic relationships with host plants.

In this particular case, a B. thuringiensis crystal gene was introduced into a strain of Clavibacter xylicynodontis, an endophytic bacterium associated with several major crops including corn (Anon, 1988). Finally, Mycogen Corporation succeeded in expressing the crystal activity into Pseudomonas fluorescens. The recombinant micro-organism is killed during production and its insecticidal protein encapsulated at the same time. The final product is a recombinant but non-viable bacterial insecticide with improved stability opposite environmental factors due to the encapsulation processes (Spear, 1987).

In contrast to Bacillus thuringiensis, little work has been performed with Streptomyces avermitilis as a whole organism producing avermectins or with the related Streptomyces bikokenkinki which produces milbemycins. Nonetheless, studies have been performed with these soil micro-organisms using whole (living or dead) Streptomycete cells or biomass to control pest species (Poole and Holden, 1982). Use of whole organisms has not, however, been commercialised.

b) Isolated and purified toxins

B. thuringiensis is used quite widely as a whole organism for pest control, but isolated toxins are needed to only a limited extent in commercial situations. The best known product is DiBeta which contains the beta exotoxin.

In the case of the avermectins, much research work has been performed with the isolated toxins, but to date, products have been commercialised for only a few insects such as leaf miners and fire ants.

S. avermitilis produces eight major fractions of macrocyclic lactones, of which Avermectin B_{1a} was generally the most active against feeding stages of Lepidoptera, Coleoptera and Hemiptera, although Avermectin B_{2a} was more active against the coleopterous pest Diabrotica undecimpunctata (Dybas and Green 1984). The toxin has been commercialised, as abamectin and contains 80% B_{1a} and 20% of the minor homologue B_{1b} (Merck, Sharpe and Dohme).

Generally the avermectins are viewed as acaricides and nematicides, but useful insecticidal efficacy can be achieved. The insecticidal activity of the avermectins has been reviewed recently by Strong and Brown (1987) who described tests with avermectins for some 84 species

of insects in ten orders. They concluded that naturally occurring avermectins (and synthetic analogues) are toxic to many species, although tolerance varies and death can be very slow, taking from 1-30 days. At high doses, treated insects are progressively immobilised, some show a disturbed water balance and become distended with fluid, while others show disruption of moulting and metamorphosis. Avermectins also affect mating behaviour, egg development, oviposition and egg hatching, as well as inhibiting feeding at sub-lethal doses. Nonetheless, adoption of the naturally occurring avermectin toxins in pest control has been very limited to date.

c) Chemically modified toxins

Attempts to synthesise new avermectin-like compounds from scratch have proved unsuccessful as no molecules with more than one thousandth of the activity of the naturally occurring toxin have been produced (Kay & Turnbull, 1985). Nonetheless, chemical modification of the natural toxins has met with much success. For example, reduction of Avermectin B_1 mixture yields 22, 23 dihydroavermectin B_1 which is used extensively in the animal health sector (Campbell, 1985). In addition, further promising semi-synthetic avermectins are being produced with reported activity of 1200 times that of Avermectin B_{1a} on lepidopterous pests.

d) Expression in plants

Recently, important progress in the field of plant genetic engineering has allowed the introduction of foreign DNA material into plant tissue and its expression using Agrobacterium-mediated transformation. For instance, Plant Genetic Systems reported the expression of a Bacillus thuringiensis crystal protein gene in tobacco plants (Von Montagu et al, 1986). Since then, many plant constructs have been obtained in other crops including potato and tomato plants (Fishhoff et al, 1987).

This area has been reviewed recently by Leemans (1989).

COMMERCIALISATION OF MICROBIAL TOXINS AND THE FUTURE

Patentability

In order to exploit profitable discoveries, agrochemical and biotechnology companies try to obtain industrial property rights to allow them exclusivity for selling the product for a fixed number of years. This is relatively straightforward for identified toxins, but it is difficult to obtain protection for natural strains of toxin-producing pathogens. Nonetheless, patents have been filed on certain novel, naturally occurring, micro-organisms, such as B. thuringiensis var kurstaki and tenebrionis. Production methods and formulations can also be patented, but competitors can still attempt to circumvent the patents in order to develop other products based on the same agent. Genetic manipulation, however, offers the potential for increased scope opposite patent protection.

Registration/Environmental Acceptability

It is entirely appropriate that micro-organisms producing toxins, as well as isolated toxins, should undergo extensive safety testing before widespread use. Current evidence does, however, suggest that the responsible use of toxin-producing pathogens does not adversely affect vertebrates, beneficial invertebrates or plants. Initially, the registration of natural occurring pathogens was less stringent than for toxins, but with the trend towards more effective pathogens, undesirable pathogenicity may become a problem. The stance of registration authorities on this issue is still evolving, but the Environmental Protection Agency in the USA has stated that it does not intend to restrict progress in the area of biotechnology by over-regulation.

With purified toxins, restrictions on registration do occur. DiBeta, containing the metabolite thuringiensin, is sold in Latin America, but it is subject to further regulatory questions in the USA which may delay its introduction. Incidentally, anxiety has always been high in the USA regarding B. thuringiensis products that contain toxins other than the crystalline endotoxins. There have also been difficulties in obtaining registration for avermectins in food crops, while the semi-synthetic avermectin, Ivermectin exhibits undesirable environmental attributes when expelled from the bodies of livestock in the faeces (Wall and Strong, 1987).

Reliability

Isolated toxins usually give reliable control, but with toxin producing micro-organisms, efficacy can be markedly affected by environmental variables such as moisture, temperature, sunlight and pH. Such failings are being overcome through improvements in formulation and application. These include improved product stability achieved through the use of better stabilisers and gelling agents, better adhesion or spread on targets and/or substrates, and improved persistence which is attained through the use of ultraviolet stabilisers. The sensitivity of these micro-organisms to ultraviolet light and humidity can be overcome by producing more resistant mutants through strain selection or by genetic engineering of the toxin coding genes into, for example, the plant genome or a bacterial epiphyte.

Cost-Effectiveness

The cost-effectiveness of isolated or synthetic toxins is a function of the activity of the toxophore against a particular insect species. However, toxin-producing micro-organisms are generally believed to be more expensive to produce than agrochemicals, but costs could fall markedly as demand increases. Currently, such micro-organisms are sold at a premium in a limited number of outlets, but in most sectors globally, such agents will have to provide the user with cost-efficacy similar to that of conventional agrochemicals. This will only be achieved by improving fermentation processes, reducing media costs, increasing yield, modifying culture systems, or by inserting genes coding for toxins into other organisms or plants.

Benefits of Genetic Engineering

The potential applications, benefits and problems of genetic engineering of micro-organisms such as B. thuringiensis are shown in Table 4.

It is evident from the five applications listed that a range of benefits may be attained, and much exciting progress should be made with genetically engineered micro-organisms in the coming decade.

Resistance

The impression is often given that insects will develop resistance to toxins and other chemical toxophores, but not to toxin-producing micro-organisms. However, Briese (1986) reported that theoretical studies indicated that intensively applied control measures of a chemical or microbial nature will invariably select for resistance. Thus, insects resistant to B. thuringiensis can be selected when continuous B. thuringiensis exposure is provided. For example Plodia interpunctella obtained from grain storage bins routinely treated with B. thuringiensis var kurstaki become less susceptible (McGaughey, 1985). This can be attributed to successive generations of P. interpunctella breeding in contact with the spores and crystals of B. thuringiensis. Similarly, a 24-fold increase in resistance was observed in seven generations of continuous selection using Heliothis virescens fed on P.fluorescens containing B. thuringiensis (Stone et al, 1989).

Engineering of B. thuringiensis resistance into plants may also result in resistance to the toxic protein. The hypothesis for this is that the B. thuringiensis gene will be expressed constitutively in plant tissues. Thus it will be present throughout the growing period of the plant and allows for multigenerational exposure of target pests. However, the use of specific tissue expressing promotors could allow for the production of the B. thuringiensis toxin in targeted tissues, hence reducing the appearance of resistance.

Engineered plants, unlike the use of chemical toxins and toxin-producing micro-organisms, will not provide temporal refugia from insecticide pressure. If B. thuringiensis resistance follows the classical parameters of chemical resistance, then the continuous selective pressure may accelerate the rate at which resistance can develop. This problem can, however, be significantly delayed or even prevented by mixing recombinant and wild type seeds to provide spatial refugia or by routinely using conventional agrochemicals on the resistant plants to provide broader control of pests. It is also possible to use a multiple gene system permitting expression of a variety of different toxophores to prevent or reduce the development of insect resistance. Nonetheless, this whole area is one requiring fundamental examination if successful long-term strategies are to be developed. In fact, a body similar to the Insecticide Resistance Action Committee (IRAC) has been formed by the agrochemical and seeds producers, in order to ensure these novel approaches can be fully exploited over a long time period.

TABLE 4 Potential applications, benefits and problems of genetic engineering of micro-organisms (after Andrews et al, 1987)

APPLICATION	DESIRED BENEFIT	PROBLEMS
Transforming/ transducing/ mating systems	Broaden host range Increase toxicity Expand potential markets Natural, and hence safe, product Possible patent protection	Essentially a random process. Requires many assays.
Recombinant DNA manipulation/ mutagenesis	Enhance specific toxin activity Produce new forms of toxin protein Increase toxin yield/cell Decrease unit cost of toxin production Expand susceptible species range Increase numbers and kinds of toxin producing strains Development of strains capable of producing toxin during the vegetative stage Consistent yields of entomocidal product Improved fermentation yield Improved marketability Increased activation of the toxin Patent Protection	Requires intricate knowledge of toxin; mode of action, structure: activity relationships etc.
Molecular cloning of toxic genes. Two or more genes for distinct insecticide activity in the same host cell, or entomocidal toxin gene into other prokaryotes (eg. Cyanobacterium sp., Nostoc, Pseudomonas sp.)	Patent protection Creation of a multipurpose biocide possessing activities against two or more different target insects Overcoming insect resistance Increased host spectrum Recyclibility Increased persistence	Requires understanding of the biology of the new host system.
Entomocidal toxin gene into insect viral genomes	Elimination of need for intermediate vector Inhibition of feeding behaviour at onset of viral infection (toxin-mediated)	Consideration required of host range, production, efficacy, etc.
Entomocidal toxin gene into the insect's food source (eg. tobacco, soyabean, cotton)	Increased plant tolerance and resistance to insect pests Eliminate the need for repeated sprayings Increase environmental longevity and field persistence Recyclibility Eliminate processing, manufacturing transportation and farmer application of toxin	Requires careful consideration of potential resistance. May need to consider tissue specificity etc.

CONCLUSIONS

Microbial toxins can be exploited commercially in a range of ways. They can be applied within the producing organism as living or dead microbes; they can be used as commercially viable products in their own right; they can be employed as building blocks for further chemical or microbial modification; or they can be engineered into symbionts, carriers or crop plants. These approaches offer significant opportunities for the future, but intelligent development and careful monitoring of use will be essential in order to add these new approaches to the armoury of insect pest control agents, and to maintain their effectiveness.

REFERENCES

Andrews, R. E. Jr; Faust, R. M.; Wabiko, H.; Raymond, K. C.; Bulla, L. A. Jr. (1987). The biotechnology of Bacillus thuringiensis. CRC Critical Reviews in Biotechnology, 6, 2, 163-232

Angus, T. A. (1956a) Association of toxicity with protein-crystalline inclusions of Bacillus sotto Ishiwata. Canadian Journal of Microbiology, 2, 122

Argus, T. A. (1956b) Extraction, purification and properties of Bacillus sotto toxin. Canadian Journal of Microbiology 2, 416

Anon (1988) Engineered biopesticide to start field tests. Chemical & Engineering News, May 30, p12

Aoki, K.; Chegasaki, Y. (1915) Uber die pathogenitat der sog Sotto Bacillen (Ishiwata) bei Seidenrauven. Mitt. Med. Fak. Kais, 13, 419

Bernier, R. L.; Collins, M. D.; Gray, A. L. (1986). Bacterial strains. UK Patent Application, Application number 8828374.2.

Briese, D. T. (1986) Host resistance to microbial control agents. In "Biological plant and health protection", edited by J. M. Franz, Fortschr. Zool. 32, 233-256

Burges, H. D. (1981) Microbial control of pests and plant diseases. Academic Press, London and New York

Campbell, W. C. (1985) Ivermectin : an update. Parasitology Today, 1, 10-16

County Natwest Woodmac (1989) Agrochemical Service, May 1989

De Barjac, H.; Bonnefoi, A. (1962) Essai de classification biologique et sérologique de 24 souches de Bacillus de type B. thuringiensis. Entomophaga 8 : 223-229

Dunphy, G. B.; Nolan, R. A. (1982) Mycotoxin production by the protoplast stage of Entomopthora egressa. Journal of Invertebrate Pathology 39, 261-263

Dybas, R. A.; Green, A. St. J. (1984) Avermectins : their chemistry and pesticidal activity. Proceedings of the 1984 British Crop Protection Conference, 3, 947-954

Edwards, D. L.; Herrnstadt, C.; Wilcox, E. R.; Wong, S. Y. (1987) Bacillus thuringiensis toxins. European Patent Application. Application number : 86309588.1

Ellar, D. J.; Knowles, B. H.; Drobniewski, F. A.; Haider, M. Z. (1986) The insecticidal specificity and toxicity of Bacillus thuringiensis delta-endotoxin may be determined respectively by an initial binding to membrane-specific receptors followed by a common mechanism of cytolysis. In : Proceedings of the Fourth International Colloquium of Invertebrate Pathololgy, Veldhoven, The Netherlands.

Fishhoff, D. A.; Bowdish, K. S.; Perlak, F. J.; Marrone, P. G.; McCormick, S. M.; Neidermeyer, J. G.; Dean, D. A.; Kusano-Kretzmer, K.; Mayer, E. J.; Rochester, D. E.; Rogers, S. G.; Fraley, R. T. (1987) Insect tolerant transgenic tomato plants. Biotechnology Vol 5, p807-813

Gonzales, J. M. Jr.; Carlton, B. C. (1982) Plasmid transfer in Bacillus thuringiensis, p 85-95. In : Streips, U.N.; Goodgal, S.H.; Guild, W.R.; and Wilson, G.A.; (eds), Genetic Exchange : a celebration and a new generation. Marcel Dekker, New York

Grove, J. F.; Pople, M. (1980) The insecticidal activity of Beauvericin and enniatin complex. Mycopathologia 70, 103-105

Hannay, C. L. (1953) Crystalline inclusions in aerobic sporeforming bacteria. Nature (London) 172, 1004.

Hannay, C. L.; Fitz-James, P. C. (1955) The protein crystals of Bacillus thuringiensis Berliner. Canadian Journal of Microbiology, 1, 694

Huber, H. E.; Luthy, P. (1981) Bacillus thuringiensis delta-endotoxin: composition and activation. In : E. W. Davidson (ed.), Pathogenesis of invertebrate microbial diseases. Allenheld, Osmun Publishers, Totowa, N.J.

Jutsum, A. R. (1988) Commercial application of biological control : status and prospects. Philosophical Transactions of the Royal Society, London, Series B, 318, 357-373

Kay, I. T.; Turnbull, M. D. (1985) Synthetic approaches to the avermectin toxophore. In : "Recent advances in the chemistry of insect control", Janes, N.F. (ed.). Royal Society of Chemistry Special Publication, Number 53, 229-244.

Klausner, A. (1984) Microbial insect control using bugs to kill bugs. Biotechnology 2, 408-419

Leemans, J. (1989) Insect control via the crop. British Crop Protection Council Symposium on "Progress and Prospects in Insect Control" (in press)

Von Montagu, M. C. E.; Vaeck, M. A.; Zabeau, M. F. O.; Leemans, J. J-A.; Hofte, H. F. P. (1986) Modifying plants by genetic engineering to combat or control insects. European Patent Application. Application number : 86300291.1

McGaughey, W. H. (1985) Insect resistance to the biological insecticide Bacillus thuringiensis. Science, Washington, 229, 193-195.

Poole, N. J.; Holden, J. S., (1983). Method and compositions for combatting pests. European Patent Application. Application Number 102702.

Putter, I.; MacConnell, J. G.; Preiser; F. A;, Haidri, A. A.; Ristich, S. S.; Dybas, R. A. (1981) Avermectins : novel insecticides, acaricides and nematicides from a soil microorganism. Experientia 37, 963-964.

Schnepf, H., Whitely, H. (1981) Cloning and Expression of the Bacillus thuringiensis crystal protein gene in Escherichia coli. Proceedings of the National Academy of Science. 78 : 2893-2897.

Spear, B. B. (1987) Genetic Engineering of Bacterial Insecticides. In : Biotechnology in Agricultual Chemistry, Chapter 17, pp204-239

Stanford Research Institute International (1984) Agricultural biotechnology - its potential impacts on : producers of agricultural chemicals, fertilizers and seeds; genetic engineering companies. Biotechnology Program - 1984, Menlo Park, California : SRI International.

Stone, T. B.; Sims, S. R.; Marrone, P. G. (1989) Selection of tobacco budworm for resistance to a genetically engineered Pseudomonas fluorescens containing the delta endotoxin of Bacillus thuringiensis sub-species kurstaki. Journal of Invertebrate Pathology 53, 2, 228-234.

Strong, L., Brown, T. A. (1987) Avermectins in insect control and biology : a review. Bulletin of entomological Research, 77, 357-389

Wall, R.; Strong, L. (1987) Environmental consequences of treating cattle with the anti-parasitic drug ivermectin. Nature (London) 327, 418-421

Watrud, L. S.; Perlak, F. J. (1986) Insertion of the Bacillus thuringiensis crystal protein gene into plant-colonising microorganisms and their use. European Patent Application. Application number : 85870174.1

THE CONTROL OF INSECT PESTS BY VIRUSES; OPPORTUNITIES FOR THE FUTURE USING GENETICALLY ENGINEERED VIRUS INSECTICIDES

D.H.L. BISHOP, M.P.G. HARRIS, M. HIRST, A. T. MERRYWEATHER and R.D. POSSEE

Institute of Virology & Environmental Microbiology, Mansfield Road, Oxford OX1 3SR

ABSTRACT

The objectives of the genetic engineering of baculovirus insecticides is to improve their speed of action while maintaining their host specificity and other attributes that make them desirable alternatives to chemical pesticides. Four field releases of engineered insecticides have been undertaken. The first release (1986-1987) used a genetically marked *Autographa californica* nuclear polyhedrosis virus (AcNPV). The results demonstrated that an innocuous piece of DNA, appropriately positioned in the AcNPV genome, was an effective means to tag the virus without affecting its phenotype. It allowed the virus to be identified in bioassays of plant and soil samples. The second and third field trials (1987-1988) demonstrated that a genetically crippled ("self-destructive") virus, also appropriately marked, could be made. This virus was unable to persist in the environment. Such engineered viruses constitute "fail-safe" substrates for field studies of engineered viruses. The field data obtained with this virus showed that it did not persist in the environment, neither in soil, on vegetation, nor within the corpses of caterpillars. The fourth release (1988) involved a polyhedrin-negative virus that contained a "junk" gene (the *Escherischia coli* β-galactosidase) as a phenotypic marker. This study was undertaken in order to measure the expression level of a foreign gene in infected caterpillars in a field situation. Engineered viruses that express the *Bacillus thuringiensis* δ toxin and other potentially active products are now under investigation in the laboratory. The overall goal is the development of new generations of custom-designed virus insecticides that will be environmentally safe to deploy for pest control and for crop protection. The research includes not only the development of such viruses, but also laboratory risk assessment and field environmental impact analyses.

INTRODUCTION

Since the last century certain naturally occurring baculoviruses have been used to control insect pests. Unlike most chemical insecticides, they affect only a few species of insect. Such baculoviruses have no effect on other insects, or other invertebrates, or plants, or vertebrates; nor do they pollute the

environment or cause adverse reactions in soil or water (farmland, rivers, lakes, etc.). Members of the Baculoviridae family of viruses are unique in that they apparently only infect arthropods. Because they are slow to exert an effect, the use of baculovirus insecticides has been superseded by chemical insecticides. However in some situations viruses and other biological control agents, are still preferred to chemicals. Examples include (1) areas that are environmentally sensitive (e.g., in forests and water catchment areas), or (2) where the cost of chemical insecticides is prohibitive, or (3) where the pest species has developed resistance to the available chemical insecticides.

A joint WHO-FAO meeting in 1973 on insect viruses endorsed the use of baculoviruses as pest control agents. The report noted that, in addition to their specific host ranges, baculoviruses exhibit good storage properties, are safe to handle, are relatively easy to produce and are widely distributed in nature, particularly among insects. More than a dozen baculoviruses have been employed commercially to control insect pests. Reports by Podgewaite (1985) and Entwistle & Evans (1985) list some of the baculoviruses which have been used. The environmental safety of baculoviruses is a matter of record and a major factor when considering their further development by genetic engineering. This record of safety has been confirmed by experience gained over many decades involving the use of naturally occurring baculovirus insecticides in agriculture and forestry. Studies of normal epizootics of insect disease have also shown that baculoviruses are safe to man and other fauna, and that their effect is limited to certain insect species.

The objective of the programme of genetic engineering of baculovirus insecticides is to improve their speed of action. This is desirable since during the normal infection process a baculovirus undergoes several cycles of replication (Fig. 1). These cycles take time - up to several days, or weeks, depending on the virus, the host and the environmental conditions (e.g., temperature). By contrast, most chemical insecticides act quickly, killing the target insect (and often other, beneficial insects) in a matter of hours. Using genetic engineering procedures it should be possible to minimize the time taken for a viral insecticide to act by incorporating other genes (e.g., toxins, insect hormone genes, etc.) into the viral genome.

The principal target of a baculovirus, such as an NPV, is the larval (caterpillar) stage of the host. Viruses are ingested together with food. The polyhedrin protein is removed by proteolytic digestion when the virus reaches the alkaline environment of the caterpillar midgut (Fig. 1). Infectious virus particles infect cells in the epithelium of the caterpillar's midgut. Virus DNA enters the cell nucleus and replication ensues. In Lepidoptera (e.g., for AcNPV) non-occluded virus particles are released from these cells to spread the infection via the haemocoel to cells in other tissues of the larva. Late in the infection course, inclusion bodies (polyhedra) are produced. The virulence and extent of the virus infection induces death of the host species. The replication process can be prolific with up to 10^9 progeny polyhedra present in the corpse of an infected insect. Infected insects commonly exhibit behavioural abnormalities which may be of benefit to the subsequent spread of virus to other larvae. For example, infected larvae may ascend to the tips of plants prior to death, thereby facilitating the distribution of virus over the foliage below. Virus is released from decaying larval corpses and can be spread by physical forces (e.g., rain splash). Viruses may also be distributed passively by animals that eat the remains of the insect and subsequently defaecate at other sites. Virus ingestion by birds, rodents and beneficial insects such as carabids does not lead to infection of those species, instead the baculovirus passes

directly through their intestines without inactivation and is deposited with their faeces.

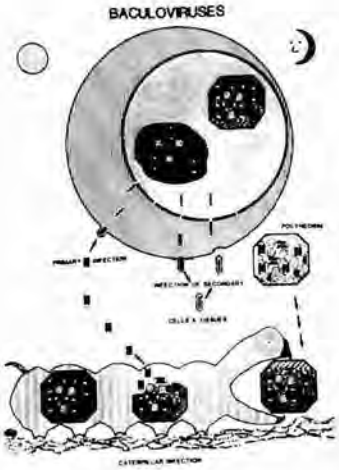


FIGURE 1 Schematic course of an infection by an occluded baculovirus such as AcNPV. Polyhedrin inclusion bodies (PIBS) are ingested with food (foliage) and the viruses liberated to infect midgut cells. After replication, non-occluded progeny are released to infect other cells and organs. Cell and larval death results in the release of PIBS to the environment.

RISK ASSESSMENT AND ENVIRONMENTAL IMPACT

Baculoviruses, like all viruses, are obligate parasites and have to be ingested by a *permissive* host and gain entry to a cell before they can replicate. If there is no available host, then the virus remains inert. In this regard baculoviruses differ from most bacteria or other free-living micro-organisms. In the absence of a permissive host, the inability of viruses to replicate *per se* inhibits them from escaping and multiplying *ad libitum*. In view of these characteristics, viruses are not models for assessing the risks involved with genetically engineered, free-living organisms, such as bacteria. However, due to their host preferences, baculoviruses are excellent subjects with which to conduct a risk assessment programme since their infection requirements (presence of a permissive host species) allows restrictions to be imposed on the study that limit risk. To inhibit an engineered virus from replicating *in natura*, the field trials that were undertaken at Oxford between 1986 and 1989 were performed at a time of year (the autumn/winter) when the natural permissive insect host was not present in the environment (infected caterpillars were supplied from the laboratory). In addition, the field facility used for the studies was designed to restrict egress of infected hosts and to limit entry to the site of other insects, or rodents, moles, or larger animals that might be involved in the dissemination of virus (Fig. 2). By such physical and temporal restraints, any risk to the environment due to the introduction of a genetically engineered virus (or its spread) was minimised. Finally, after each

study, the field site was disinfected and demonstrated to be free from virus before new insect species emerged in the following Spring.

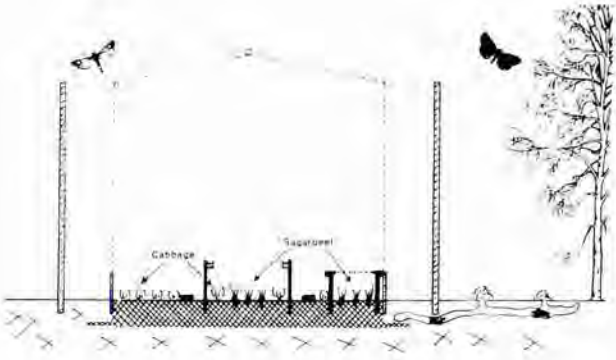


FIGURE 2 Schematic of the netted facility used for the field testing of genetically engineered AcNPV. As illustrated, the facility was designed to restrict the entry and egress of arthropods and other animals.

The research programme at Oxford involving genetically engineered baculoviruses is primarily concerned with the assessment of risks associated with the introduction of an engineered virus into a natural habitat. It would be irresponsible to construct and release into the environment a genetically engineered baculovirus insecticide that expresses a new (foreign) gene without a thorough understanding of the possible consequences. A major issue in terms of risk is whether the host range of an engineered virus differs from that of the parent virus. To investigate this question extensive host range analyses have been undertaken in the laboratory for each of the engineered viruses we have prepared prior to seeking permission for field trials. A second consideration of risk concerns the possible spread of virus from the field site. Spread could be mediated by environmental conditions such as rain, by the movement of the host species, or by other animals (birds, rodents, predatory insects, etc.). By studying natural epizootics of viral infection, as well as those induced when viruses are applied to caterpillar infestations in the wild, Entwistle, Adams, Evans & Rivers (1983) demonstrated that such spread occurs. To limit spread of the engineered viruses, a netted field facility was used (Fig. 2) which restricted entry or egress of insects and other animals from the site.

In the investigations reported by Entwistle and associates (1983) it was never entirely possible to rigorously determine if the virus recovered from the environment originated from the applied virus, or whether it represented a naturally occurring isolate of the same virus. In order to address this issue a genetically marked virus was developed for the first (1986-1987) study. The marker was an innocuous piece of non-coding DNA inserted into an intergenic, non-regulatory position in the genome. This allowed the recovered virus to be differentiated from other viruses or from other DNA samples.

Another consideration of potential risk is the question of the exchange of genetic information. Although DNA exchange is frequent and well documented among bacteria (involving plasmids, conjugation, etc.), such exchange is probably a low frequency event for baculoviruses, except between closely related baculoviruses in ecologically intimate circumstances. The transfer of a foreign gene *from* a virus into a dead cell, or to non-germline cells would be an event without consequence. DNA transfer involving the acquisition by a baculovirus of genetic information *from* cells of a host species could occur (albeit infrequently) and produce an altered phenotype. If this occurs naturally then the question with an engineered virus is whether the frequency of it occurring is increased, or whether particular DNA sequences are transferred due to the presence of the engineered DNA. The occurrence and frequency of such events can be sought by studying virus passaged in the laboratory in permissive cells, or in the permissive hosts (caterpillars). To date, no such transfer has been documented in our studies with any of the engineered insecticides.

Gene exchange between genetically compatible baculoviruses may occur when two closely related baculoviruses co-infect the same host. Marker rescue studies indicate that such rates could be >1% for closely related viruses (unpublished data). The likelihood of gene exchange occurring between such viruses will depend on whether the target host is already infected with a related virus, or whether another virus co-infects the same target host *at the same time*. The transfer of foreign DNA between baculoviruses can be analysed by studying the progeny virus recovered from dually infected cells (or by appropriately designed marker rescue studies). Data we have obtained from such studies indicate that gene transfer from a distantly related baculovirus only occurs at very low frequencies ($<10^{-7}$), if at all. So far in our studies, we have not been able to identify a U.K. baculovirus that is closely related to AcNPV, or that is genetically compatible with AcNPV.

Another consideration of risk associated with an engineered organism is the question of its genetic stability, i.e., whether the introduced gene induces genetic instability. This can be investigated by genome analyses of virus passaged in the laboratory. No genetic instability has yet been identified with any of the virus constructions we have made or field tested.

In summary, each genetically engineered baculovirus that was prepared was subjected to a series of laboratory analyses to address the questions listed above. The laboratory investigations involved the following: the construction of the candidate virus insecticide, verification of the precise genetic change by DNA sequence analyses, laboratory studies of the phenotype and genetic stability (mutability) of the viruses, host range and genetic compatibility determinations, and analyses of the physical stability of the virus in simulated systems (plant surfaces and soil). Other than the expected genetic alteration designed to be incorporated into the viral genome, no unexpected change in the genome was detected and no genetic instability. The viral phenotypes (e.g., host ranges, physical stabilities) have also been as expected.

In each study the laboratory data were submitted to all the relevant regulatory authorities for independent evaluation, especially in relation to the characteristics of the site proposed for the release, the insect fauna of the area, the possibility for spread beyond the immediate ecosystem and the proposed physical constraints imposed at the release site (etc.). Permission to undertake the releases described below were given in the form of licences to conduct the studies under certain prescribed conditions (site arrangements, procedures to

be followed for the introduction of the engineered virus, disinfection of the site after use, etc.). Since baculoviruses are insecticides, the licences were issued by the U.K. Ministry of Agriculture, Fisheries and Food (MAFF) under the Food and Environment Protection Act 1985 and Regulation of the Control of Pesticides Regulations 1986. Primary evaluation of the proposals was made by the Health and Safety Executive (HSE) Advisory Committee on Genetic Manipulation (ACGM), in consultation with MAFF, the Department of the Environment (DOE) and the Nature Conservancy Council (NCC). In addition, other interested parties were informed, including the Natural Environment Research Council's senior management, the owners of the field site (Oxford University), senior University authorities, the University Safety Officer, the Oxford HSE factory inspector, the Vale of the White Horse Environmental Health Officer, the Environmental Services Committee of the Vale of the White Horse District Council and agencies such as "Friends of the Earth". Press coverage was also sought (national and local papers, including, for the 1988 releases, a "Public Notice" placed in local papers). Radio and television interviews aided the process of informing the general public of the proposed studies.

FIELD STUDIES USING A GENETICALLY MARKED AcNPV

The first release involved a genetically marked AcNPV that was otherwise identical in every way to the parent AcNPV (Bishop 1986). The virus was designed so that it could be distinguished from all other baculoviruses as well as from genome sequences of the target, or another, host. The marker was a synthetic oligonucleotide, 80 base-pairs in length, inserted into the viral genome. The oligonucleotide was placed **downstream** of the AcNPV polyhedrin coding region.

The synthetic oligonucleotide was introduced into the AcNPV genome so that it was located just after the end of the polyhedrin gene coding sequence, but within the immediate 3' non-translated sequences (Bishop *et al.* 1988). It served only as a marker, or flag. The position of the marker was verified by restriction enzyme and by sequence analyses. The marker contained translation stop codons in all six reading frames, no ATG codon and no other genetic signal likely to affect the replication, or gene product expression, of the virus. Due to the precise location of the insertion (viz: a dispensible, intergenic, non-regulatory position in the AcNPV genome), the oligonucleotide neither added to, nor detracted from the constitution, or synthesis, of any of the natural AcNPV gene products. This was verified by analyses of the viral phenotype (infectivity, proteins synthesized) by comparison to that of the unmodified virus.

For the field studies it was decided to use caterpillars of the Small mottled willow moth (*Spodoptera exigua*) as the target pest. This is a noctuid moth with practically a world-wide distribution; it is a seasonal immigrant to the U.K. It eats a range of herbaceous dicotyledons, including sugar beet, and has a wide distribution in communities of low growing plants, both wild and cultivated. Larvae of this moth pass through 5 instars. The site chosen and used for the release of the marked virus (and the other genetically engineered derivatives in 1987 and 1988) was situated in a field of light loam soil bounded by agricultural land at the Oxford University field station at Wytham, Oxfordshire. The area has been extensively studied with regard to fauna and flora for many decades. Moth species native to the region were collected at

night using two ultraviolet light traps. The moths were trapped throughout the late spring, summer and autumn of 1986 (and 1987).

After obtaining the requisite permissions (see above) the first field trials with the marked AcNPV were undertaken (Bishop *et al.*, 1988). In September of 1986, early third instar *S. exigua* larvae were fed overnight in the laboratory with diet containing 10 LD_{50} PIB equivalents of the marked AcNPV incorporated into an artificial diet plug. The infected larvae (*ca* 200) were re-fed with diet containing similar quantities of virus and, after they had eaten all the diet, taken in sealed containers to the site and placed on the sugar beet plants in the central, plexiglass enclosed, area of the containment facility (Fig. 3, top). Uninfected larvae were released onto nearby plants in an adjacent, totally netted enclosure. After one week none of the infected caterpillars remained alive (Fig. 3, bottom). Dead caterpillars (estimated to be *ca* 55) were evident on many of the plant surfaces. Other dead caterpillars were observed in or on the surface of the soil. Samples of soil and foliage (cabbages, sugar beet, and chickweeds that grew within the facility) were returned to the laboratory at 1-2 wk intervals for analysis for virus. These analyses were continued over a 6-month period (until February 1987).

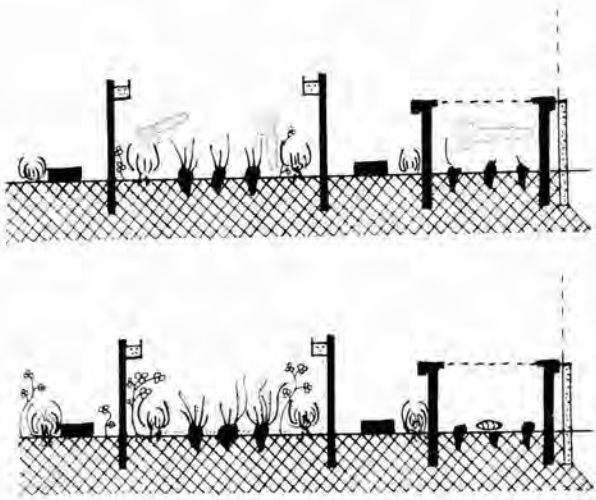


FIGURE 3 Infection protocols in the netted facility used for the field trials of genetically engineered baculoviruses. In the top panel, infected (centre) and uninfected (right side) caterpillars were placed on sugar beet and allowed to feed *ad libitum*. By the end of the study viruses released from the infected caterpillars were recovered on foliage and in soil. The uninfected caterpillars caused substantial damage to the plants and pupated. At the end of the study plants and pupae were removed (and autoclaved) and the site disinfected.

Virus was assayed on the recovered plants by allowing groups of permissive *T. ni* larvae (second instar) to completely eat the foliage. The larvae

were then reared on artificial diet until pupation or death, then assayed for virus (polyhedra, DNA probing). As expected for plants that originally had only a limited number of infected *S. exigua* caterpillars on them (and consequently a limited amount of residual virus), not all the *T. ni* larvae became infected. However, virus was identified at every sampling period throughout the entire 6 month period. Virus was identified on plants that were present in the site at the initiation of the experiment (cabbages, sugar beet), as well as those (e.g., chickweed) that grew up in the facility after the caterpillars had died. The amounts of virus on the infected plants or in the soil were not quantified. Virus identified after replication in *T. ni* larvae retained the marker element in its original form. Chickweed recovered from outside the facility, or from plants collected outside the enclosed area did not yield virus.

The presence of virus in soil was assayed by planting cabbage seeds in soil samples brought back to the laboratory. The derived seedlings were then fed to *T. ni* larvae and the insects reared until pupation, or death, and similarly analysed for the marked virus. Again, although only *ca* 12% of the caterpillars died of virus infections, marked virus was identified by this procedure at each sampling period. In summary and as expected, the study demonstrated the persistence of the marked virus in the field site.

In the completely netted enclosure where uninfected caterpillars were placed, the larvae consumed substantial quantities of foliage for three weeks after the release. By the end of that period caterpillars were removed, all the plants were destroyed by autoclaving and the area flooded with water to retrieve pupae. Virus was never isolated from these insects.

At the completion of the study in February 1987, the field site was cleared and foliage removed and sterilized to destroy any remaining virus. The site was treated with 5% formalin on three separate occasions, twice in February and again in March, 1987. Soil samples were taken before and after each treatment and used in the laboratory to propagate 200-400 cabbage seedlings. The seedlings were fed to *T. ni* larvae to act as a biological indicator for the presence of virus. The larvae were reared until pupation, or death. All insects, infected or healthy, were processed to detect the presence of the marked virus as described above. The results showed that infectious virus was present in the soil before the first formalin treatment, in amounts sufficient to infect most of the *T. ni* larvae. No attempt was made to quantify the numbers of infectious virus. After the third formalin treatment no virus was detected in the soil samples; all the insects that fed on the cabbage remained healthy, pupated and developed normally. None contained any detectable quantity of the marked virus. These tests demonstrated that virus had survived in the soil throughout the 6-month period and that the site had been successfully disinfected by formalin treatment.

STUDIES WITH MARKED, GENETICALLY CRIPPLED AcNPV

A second release of a genetically engineered baculovirus insecticide was undertaken in 1987. This time, the AcNPV polyhedrin gene and its transcription promoter were removed in their entirety from the virus and replaced by another (different) synthetic oligonucleotide (Bishop *et al.*, 1988) The oligonucleotide sequence was positioned so that it would not add to, nor detract from, the expression of any AcNPV gene product. This time no attempt was made to exclude ATG codons from the sequence. The size and constitution of the oligonucleotide were such that it would serve only as a unique marker for

the recombinant virus. The sequence and position of the oligonucleotide in the viral genome was verified by sequence analyses. No alteration to the phenotype of the virus was detected by protein analyses, other than that predicted by the loss of the polyhedrin protein and the inability of the virus to form occlusion bodies. The host range, genetic and physical stability of the recombinant virus was determined as before. The data indicated that the virus was more restricted in host range than the parent virus and that although genetically stable it was physically unstable (i.e., it degraded rapidly in soil or on foliage).

After review of the laboratory data, a licence was issued by MAFF to undertake a release under the same conditions and using the same protocols and facility that were employed for the occluded, marked recombinant AcNPV (Fig. 3). The objective was to determine if the polyhedrin-negative virus released from dead caterpillars persisted in the environment in soil and on plant surfaces. Caterpillars were infected in the laboratory and taken to the field site and released in the central enclosed area on to sugar beet. By one week all the caterpillars died. No virus capable of infecting second instar *T. ni* larvae were subsequently identified either on the plant surfaces, or in soil samples. Even the corpses of dead caterpillars did not yield infectious virus. In summary, the data obtained with the marked, so-called "self-destructive" virus, demonstrated that it did not persist in the environment.

To determine whether the virus could be used as a biological control agent *per se*, a third release using the same virus was undertaken in 1988 to measure virus efficacy. Virus was placed on sugar beet leaves on which adult *S. exigua* had laid eggs. The plants were then transferred from the laboratory to the field facility to monitor infection and survival rates. The dose response data indicated that at high inoculum doses all the emerging larvae died from virus infection and that no infectious virus remained after 1-2 weeks.

FIELD TRIALS USING A "FAIL SAFE" AcNPV CONTAINING A "JUNK" GENE

Since the polyhedrin-negative virus did not persist in the environment it is a suitable vehicle for the expression of a foreign gene since it has a "fail-safe" genotype (i.e., the virus will degrade in the environment after expression of a foreign gene). To investigate the expression of a foreign gene by such a recombinant baculovirus a genetically crippled AcNPV was prepared with the bacterial enzyme β-galactosidase substituted *in lieu* of the coding region of the AcNPV polyhedrin gene. The enzyme was included as a "junk" gene to allow its level of expression to be quantitated in infected caterpillars. Details of the construction will be given elsewhere. Laboratory analyses established that the recombinant virus exhibited the same host range phenotype as the marked and genetically crippled virus described above. Also as described above studies on the stability of the virus following successive passage in tissue culture, or in permissive hosts, demonstrated that the virus was genetically stable and did not lose the inserted gene upon passage. Physically, however, the virus exhibited the same susceptibilty to degradation in soil or on leaf surfaces as the self-destructive virus described above. Following permission to perform field tests infected caterpillars were placed on leaves of sugar beet plants in the field facility at Wytham and the infections and recovery of virus monitored as a function of time. The caterpillars died from virus infection and the β-galactosidase enzyme was expressed.

PHENOTYPE ANALYSES OF A "FAIL SAFE" AcNPV CONTAINING *B. THURINGIENSIS* δ TOXIN

A recombinant baculovirus has been prepared in which the AcNPV polyhedrin gene has been replaced with the δ toxin of *B. thuringiensis* (B.T., subspecies *Kurstaki*, isolate HD-73). Although details of the construction and properties of the virus will be reported elsewhere (A.T. Merryweather, M.P.G. Harris, M. Hirst and R.D.Possee, submitted for publication) the virus expresses the toxin in a form that is lethal to feeding insects that are susceptible to the toxin (Table 1). Host range, genetic stability and physical stability analyses are in progress to asses the value of this recombinant for pest control.

TABLE 1

Effect of expression of B.T. toxin on feeding of *T. ni* caterpillars*

Sample	Refused diet	Fate of insects that consumed diet		
		Viral deaths	Non-viral deaths	Pupated
No virus	0	0	0	48
Fail-safe AcNPV	0	25	0	24
Fail-safe AcNPV with BT toxin	50	0	0	0

Groups of *ca* 50 caterpillars were fed diet containing the extract of 5000 infected cells and their feeding and subsequent fate monitored.

DISCUSSION

In considering the results of the studies that are reported here, it is important to emphasize that the objectives were to assess and minimise any risk that might be involved in the release to the environment of a genetically engineered virus insecticide. The study is part of a programme of scientific research, rather than the commercial development of a product. As such, the results and information derived from the studies *only apply to baculovirus insecticides*, and only to the particular virus that was used. Having said that in order to be scientifically accurate, there is good reason to believe that other baculoviruses, similarly marked or manipulated will behave in a like manner.

Various factors were considered in order to minimize risk to the environment of the proposed releases. These included the choice of virus, the choice of the target pest, the design of the engineered viruses, the design of the field site and the procedures employed to conduct the experiment (including the final disinfection of the site). After preparing the engineered viruses, they were subjected to extensive laboratory analyses. From the data obtained we could not identify any *measurable* risk associated with the proposed experiments. This does not exclude the possibility of an unforeseen risk; however none was identified in the laboratory studies undertaken prior to the field trials. With the results that were obtained from the laboratory analyses, we therefore applied to the appropriate authorities for permission to proceed with the field trials. The data were independently evaluated and, after obtaining the requisite licences, the field trials were instigated and the predictions of the laboratory studies were verified.

The results from the field studies established that an innocuous genetic marker is a suitable tool for the identification of an engineered virus released into the environment. They also showed that a genetically crippled virus can be prepared that is rapidly degraded in the environment after killing its caterpillar host. Such a "fail-safe" virus is a suitable candidate for further engineering, and for the preparation of viruses which act more quickly than the natural virus (through the incorporation of genes which determine insect specific toxins, or hormones, or other genes).

ACKNOWLEDGEMENTS

We thank Mr C.F. Rivers, Mr P.H. Sterling, Mrs M.E.K. Tinson, Mr T. Carty and Mr M.L. Hirst for their assistance in collecting and/or rearing the insects for the host range studies. The genetic manipulations were undertaken in part by Dr I. Cameron of this Institute, host range tests by Ms Cynthia Allen. In 1986 moth collections and data analyses were undertaken by Mr S.M Eley. In 1987 similar collections were undertaken by Ms C. Doyle and Mr A.C. Forkner of the Institute. Carabid surveys and analyses were undertaken by Dr. J.S. Cory. Ultraviolet light measurements in the facility were conducted by Mr H.J. Killick. The support of these and other members of the Institute's staff, particularly Mr P.F. Entwistle, and Mr. J.S. Robertson, are gratefully acknowledged. Dr J.I. Cooper and his staff kindly provided the plants. We are indebted to Mr R. Broadbent and Mr R.M. MacKenzie who constructed the field facility.

REFRENCES

Bishop, D.H.L. (1986) UK release of genetically marked virus. Nature 323, 496.

Bishop, D.H.L.; Entwistle, P.F.; Cameron, I.R.; Allen, C.J.; Possee, R.D. (1988) Field trials of genetically engineered baculovirus insecticides. In: The Release of Genetically Engineered Microorganisms M.Sussman, G.H.Collins, F.A.Skinner, D.E.Stewart-Tull (Eds), London: Academic Press, pp .143-179.

Entwistle, P.F.; Evans, H.F. (1985) In: Comprehensive Insect Physiology, Biochemistry and Pharmacology L.I.Gilbert, G.A.Kerkut (Eds), Oxford UK: Pergamon Press, Vol. 12, pp. 347-412.

Entwistle, P.F.; Adams, P.H.W.; Evans, H.F.; Rivers, C.F. (1983) Epizootiology of a nuclear polyhedrosis virus (Baculoviridae) in European Spruce Sawfly (*Gilpinia hercyniae*): spread of disease from small epicentres in comparison with spread of baculovirus diseases on other hosts. Journal of Applied Ecology 20, 473-487.

Podgewaite, J.D. (1985) Strategies for field use of baculoviruses. In: Viral Insecticides for Biological Control K.Maramorosch, K.E.Sherman (Eds), New York: Academic Press, pp. 775-797.

WHO/FAO (1973) Expert Group (GTRES): The use of viruses for the control of insect pests. World Health Organization Technical Report Series 531.

INSECT CONTROL VIA THE CROP

H. VAN MELLAERT, H. JOOS and J. LEEMANS

Plant Genetic Systems, J. Plateaustraat 22, B - 9000 Gent, Belgium

ABSTRACT

Plant molecular biology allows to introduce genes from foreign organisms into crop plants. Through these techniques of "molecular breeding", crop varieties with introduced insect resistance have been obtained. Insect resistance is realized through plant expression of insecticidal crystal protein genes from Bacillus thuringiensis.

INTRODUCTION

In the last decade, remarkably fast progress in plant molecular biology has taken place. The first transgenic tobacco plants were developed in 1983. Only 6 years later, the capacity to apply cell and tissue culture and gene transfer techniques to important crops has grown dramatically. At present, the transformation and regeneration of more than twenty different plant species, including several important field crops, has been achieved. At the same time, dramatic progress has also been made in the identification and improvement of genes encoding valuable agronomic traits, such as insect resistance, virus resistance and herbicide resistance. Transfer of these genes has led to the creation of crop varieties with properties which were unachievable by classical breeding approaches. Basic knowledge on plant molecular biology can now be applied in a "molecular breeding" approach. This constitutes a novel tool for varietal improvement.

This paper focusses on the introduction of insect resistance into crops. Recent field data are presented and the scientific principles underlying insect resistant crops are reviewed. Expected future developments are discussed.

FIELD AND LABORATORY DATA ON INSECT RESISTANT PLANTS OBTAINED THROUGH MOLECULAR BREEDING

The first field trials with insect resistant plants were performed in 1986 in the U.S.A. by Rohm and Haas Co. with tobacco (dwarf variety SR1) transformed by Plant Genetic Systems N.V. First evaluations indicated good control of natural infestations of tobacco hornworm (Manduca sexta) and tobacco budworm (Heliothis virescens). Field trials with transgenic commercial varieties of tobacco were initiated in 1988.

Again control of natural infestations of tobacco hornworm and budworm was entirely satisfactory. Foliar damage and number of larvae per plant were scored and highly significant effects were observed. At the end of the season damage in the transgenic plants was of no economic significance whereas control plants were severely defoliated. Larvae surviving on the transgenic plants were highly parasitized. It is presumed that the total absence of chemical insecticides favoured the development and survival of a dense population of beneficial insects. These results indicate that insect resistant transgenic plants may become an essential element in integrated pest management.

Other crops with introduced insect resistance include potato and tomato. Laboratory data obtained on potato tubers confirm resistance to potato tuber moth (Phtorimaea opercullella). A first field trial has been performed but the level of natural infestation with this insect was too low to assess its effect. Additional field trials will be performed in 1990. Laboratory data on tomato indicate resistance to tomato fruitworm (Heliothis armigera). A field trial is ongoing.

THE BASIS OF INTRODUCED INSECT RESISTANCE

Gene transfer in "molecular breeding"

Whereas traditional breeding can only transfer genetic information among individuals from the same or closely related species, molecular breeding allows to bring genes into a plant from any organism (be it a micro-organism, a plant or an animal).

The most widely used system to transfer foreign DNA into plants is the Ti-plasmid of Agrobacterium tumefaciens.

Vectors for molecular breeding have been constructed which lack the information for tumor formation (a so-called disarmed Ti-plasmid) but which can be used to transfer foreign genes into plant cells. A complete plant can be regenerated from transformed plant cells through appropriate methods of plant tissue culture. In principle the foreign gene can be expressed in the transformed plant and the trait encoded by this gene will be inherited as a stable Mendelian trait.

An alternative system consists in the direct transfer of DNA through cell electroporation, microinjection or particle gun bombardment. These methods are mainly developed for cereal transformation but they still lack the precision and convenience of Agrobacterium mediated transformation.

At present molecular breeding is applicable to a wide range of crop plants, many dicotyledonous plants can today be transformed with relative ease. Transformation/regeneration methodology for others is continuously

progressing. Transformation of cereals has only been documented in a few cases, but it can be expected that important progress will be made in the near future.

In molecular breeding only well characterised (sequenced) genes are transferred, of which the gene product is known and also characterised. In contrast traditional plant breeding may transfer traits from wild varieties into a crop plant, without knowing the genes responsible for this trait. Hence, today's scope for molecular breeding is limited to traits which can be encoded by a well defined protein.
For general reviews see: Tempé and Schell, 1987; Grasser and Fraley, 1989.

Insecticidal crystal proteins from Bacillus thuringiensis

Bacillus thuringiensis is the most successful microbial insecticide. Its success resides in the high insecticidal activity towards target species but equally in its complete inocuity for higher animals and even for non target insects (Krieg, 1986). Most Bacillus thuringiensis strains are toxic to Lepidopteran insect larvae only. However, strains with activity against Coleoptera (Krieg et al., 1983) and Diptera (Goldberg and Margalit, 1977) have been described. The latter ones are nowadays intensively used for control of blackflies and mosquitoes, both important vectors of human disease (Hudson, 1985).
Field applications of Bacillus thuringiensis, however, suffer from a very short field stability of the insecticidal protein.

Insecticidal activity in B. thuringiensis preparations is mainly associated with insecticidal crystal proteins (ICP's) called delta-endotoxins. These are specifically synthesized upon sporulation and deposited in protein crystals.

Crystals, when ingested by the insect, dissolve in the alkaline environment of the midgut. The delta-endotoxins are then attacked by midgut proteases. Part of the delta-endotoxin molecules (most often having a molecular weight around 130 kDa) is proteolytically degraded. However, a protease resistant core fragment of around 60 kDa remains (Lilley et al., 1980). This is the actual toxic moiety. It binds to specific receptors in the midgut epithelium and disturbs the integrity of the brush border membrane (Hofmann et al., 1988a, 1988b). Although this causes no immediate knock-down, it upsets the insect's balance in such a way that it stops feeding and slowly dies.

A remarkable variation exists in the insecticidal activity of the different ICP's. Thus insecticidal activity against Lepidoptera, Diptera or Coleoptera is associated with marked differences in the ICP primary sequence. ICP's with different spectra within the order Lepidoptera have also been discovered (Hofte et al., 1988). The various delta-endotoxins can be recognized as members of a same family of proteins on the basis of certain sequence homologies and conserved features. Still the difference in primary sequence, even among Lepidopteran ICP's is very pronounced.

EXPRESSION OF AN ICP IN DIFFERENT CROPS

The ICP gene bt2 was cloned from B.thuringiensis subsp. berliner strain 1715 (Höfte et al., 1986). Bt2 is a 130 kDa delta-endotoxin, with activity against Lepidopteran larvae. The bt2 gene was cloned into Ti-plasmid plant expression vector. As a plant specific promoter sequence we used the Agrobacterium derived TR promoter. Plants were transformed using Agrobacterium mediated DNA transfer. Different constructions were evaluated (see figure 2) (Vaeck et al., 1987).

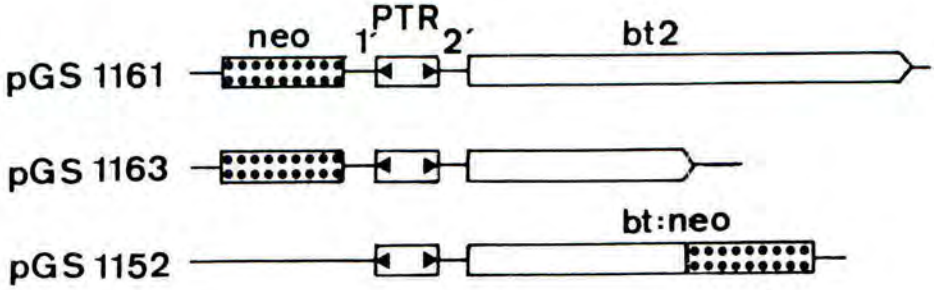


FIGURE 1

Scheme of some constructs used in plant transformation experiments. PGS 1161 contains the full length bt2 gene behind the TR2' promoter. The antibiotic resistance marker gene neo is expressed from the TR1' promoter. pGS 1163 has a truncated bt2 gene fragment behind TR2' and neo behind TR1'. In pGS 1152 the bt2 gene fragment is directly fused to the neo gene and the fusion product is expressed from the TR2' promoter.

With a full length bt2 gene behind the TR promoter we did not obtain any significant expression (Vaeck et al., 1987). This was also found by other workers using the 35S promoter from cauliflower mosaic virus (Fischhoff et al., 1987). We identified two clues which enabled us to markedly increase expression levels. First of all, the 3' end of the bt2 gene, encoding the part of the delta-endotoxin which is proteolytically digested in the insect midgut, was removed and a truncated gene construct was used. Expression was strongly increased. Secondly, a fusion was made between the bt2 gene fragment and the antibiotic resistance marker neo, such as to encode a Bt2-NPTII fusion protein. Selection for strong antibiotic resistance then directly correlates with high ICP gene expression.

Bt2 expression in tobacco plants, transformed with these constructs, was measured with ELISA: expression levels in the best individual transformants were around 100ng/mg total leaf protein (Vaeck et al., 1987). The insecticidal activity of these plants on first instar tobacco hornworm (Manduca sexta) larvae was demonstrated in leaf disc assays and subsequently in greenhouse and field tests.

We have also transformed potatoes and tomatoes with the same constructs. In both cases we obtained plants with Bt2 expression levels comparable to those in the best tobacco plants. Laboratory tests with Manduca sexta confirmed insect resistance.

FUTURE PROSPECTS FOR ICP-TRANSFORMED PLANTS IN AGRICULTURE

In three crops we have demonstrated the practical feasibility of transformation with B.thuringiensis ICP genes for engineering of insect resistance. Field tests support our expectations that these plants will be developed as first generation commercial products, probably from the early 1990's on. Such pioneer products will be essential to gain acceptance by the farmers, the breeders and the public as well as to clear the way for registration of these novel products.

However, we expect a further breakthrough for a second generation of B.thuringiensis ICP engineered plants, along with further progress along the following lines:

1. Engineering plants with different B.thuringiensis ICP genes
2. Transformation methods for other crops
3. Enhanced and regulated ICP gene expression.

At the present the largest set of B.thuringiensis ICP genes is available for Lepidopteran larvae. Lepidopteran pests alone are responsible for about 40 % of the insecticide use worldwide. Further screening activities for novel ICP's also concentrate on finding novel genes with activities against Coleoptera. Coleoptera account for another 10 % of insecticide consumption.
Furthermore it may be possible to identify novel ICP's with activity against other insect orders. In future efforts on engineered insect resistance it should be possible to control the entire complex of pest species on a given crop by optimally selecting one or several ICP genes, thus maximally reducing the need for insecticide treatments. In addition we have shown that in some cases different ICP's bind to different receptor sites in the same insect species (Hofmann et al., 1988b). When different target sites are involved in the action of different ICP's it may be useful to combine ICP's for prevention of development of insect resistance.

Routine transformation methods are not yet available for some of the world's most important crops, e.g. cereals. In the U.S., corn is among the crops where insecticides are most intensively used (27 % of total US

insecticide sales) (Agrow, world agrochemical news, 1988). Another crop with intense use of insecticides is cotton. Transformation of cotton has been reported recently (Umbeck et al., 1987).

Expression of B.thuringiensis ICP may not be needed in all tissues and at all stages of plant development. Thus regulatory agencies may require ICP levels to be quite low in edible plant parts. Gould (1988) suggested that, in order to retard development of resistance, it should be favourable to keep selection pressure on target insect species low by leaving some plant organs without ICP. Plant promoter sequences which direct expression to specific organs (root, stem, leaf, ...) are already available (Kuhlemeier et al., 1987). As research in this field continues a more extensive set of tissue- and stage-specific promoters will certainly become available. A very attractive example of regulated expression is found with the TR promoter which we have used in our constructs. Expression from the TR promoter is strongly stimulated by wounding. Thus it could be shown that ICP expression will actually be strongly stimulated by insect feeding damage on the leaf, whereas ICP expression in non-affected leaves remains very low.

CONCLUSION

In view of their efficiency and non-toxicity to non-target organisms, ICP's from B.thuringiensis offer an attractive approach for engineering insect resistance into plants.
Several crops have already been transformed with B.thuringiensis ICP genes. Excellent field control of some lepidopteran pest insects was obtained. The first generation of ICP engineered plants is now in line for product development. Further progress along three lines 1. Engineering plants with different B.thuringiensis ICP genes, 2. Transformation methods for other corps, and 3. Enhanced and regulated ICP gene expression, will broaden the scope for application of this technology.

REFERENCES

AGROW, World Agrochemical News, facts and figures, George street publications Ltd, Richmond, UK.

Fishhoff D.A., Bowdish K.S., Perlak, F.J., Marrone, P.G., McCormick, S.H., Niedermeyer, J.G., Dean, D.A., Kusano-Kretzmer, K., Mayer, E.J., Rochester, D.E., Rogers, S.G. and Fraley, R.T., 1987, Insect tolerant transgenic tomato plants, Biotechnology, 5: 087-813.

Gasser C.S. and Fraley R.T., 1989, Genetically engineering plants for crop improvement, Science, 244: 1293 - 1299.

Goldberg, L.J. and Margalit, J., 1977, A bacterial spore demonstrating rapid larvicidal activity against Anapheles sergentii, Urotaenia unquiculata, Culex univitattus, Aedes aegypti, Culex pipiens, Mosquito News, 37: 355-358.

Gould, F., 1988, Genetic engineering, integrated pest management and the evolution of pests, Trends in biotechnology, 6: S15-S18.

Höfte, H., De Greve, H., Seurinck, J., Jansens, S., Mahillon, J., Ampe, C., Vandekerckhove, J., Vanderbruggen, H., Van Montagu, M., Zabeau, M. and Vaeck, M., 1986, Structural and functional analysis of a cloned delta-endotoxin of Baciluss thuringiensis berliner 1715, Eur. J. Biochem., 161: 273-280.

Höfte, H., Van Rie, J., Jansens, S., Van Houtven, A., Vanderbruggen, H. and Vaeck, M., 1988, Monoclonal antibody analysis and insecticidal spectrum of three types of Lepidopteran-specific insecticidal crystal proteins of Bacilllus thuringiensis, Appl. Environm. Microbiol., 54: 2010-2017.

Hofmann, C., Lüthy, P., Hutter, P. and Pliska, V., 1988a, Binding of the delta-endotoxin from Bacillus thuringiensis to brush-border membrane vesicles of the cabbage butterfly (Pieris brassicae), Eur. J. Biochem., 173: 85-91.

Hofmann, C., Vanderbruggen, H., Höfte, H., Van Rie, J., Jansens, S. and Van Mellaert, H., 1988b, Specificity of Bacillus thuringiensis delta-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts, Proc. Natl. Acad. Sci. U.S.A., in press.

Hudson, J.E., 1985, The development of Bacillus thuringiensis H-14 for vector control, Tropical Diseases bulletin, 82: R1-10.

Krieg, A., Huger, A.M., Langebruch, G.A., Schnetter, W., 1983, Bacillus thuringiensis var. tenebrionis: ein neuer gegenuber Larven von Coleopteren wirksamer Pathotyp., Zschr. angew. Entom., 96: 500-508.

Krieg, A., 1986, Bacillus thuringiensis, ein mikrobielles Insektizid, Acta phytomedia nr. 10, Paul Parey scientific publishers, Berlin and Hamburg, 191 pp.

Kuhlemeier, C., Green, P.J. and Chua, N.H., 1987, Regulation of gene expression in higher plants, Ann. Rev. Plant Physiol., 38: 221-257.

Lilley, M., Ruffel, R.N. and Sommerville, H.J., 1980, Purification of the insecticidal toxin in crystals of Bacillus thuringiensis, J. Gen. Microbiol., 118: 1-11.

Tempé J. and Schell J., 1987, La manipulation des plantes, La Recherche, 18: 696 - 709.

Umbeck, P., Johnsosn, G., Barton, K. and Swain, W., 1987, Genetically transformed cotton (Gossypium hirsutum L.) plants, Biotechnology, 5: 263-266.

Vaeck, M., Reynaerts, A., Höfte, H., Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M. and Leemans, J., 1987, Transgenic plants protected from insect attack, Nature, 327: 33-37.

MYCOINSECTICIDES: PRESENT USE AND FUTURE PROSPECTS

A.K. CHARNLEY

School of Biological Sciences, University of Bath, Claverton Down, Bath, Avon, BA2 7AY, UK

ABSTRACT

Entomopathogenic fungi were among the first successful agents of biological control but they have yet to realise their potential. Although large scale production of mycoinsecticides occurs in Brazil, China and the USSR, there is no commercial product widely available at the present time. The prospects for mycoinsecticides are assessed. It is suggested that essential development of more virulent, fast-killing strains will depend on investment in studies on the mechanisms of fungal pathogenesis.

INTRODUCTION

Entomopathogenic fungi were among the first organisms to be used for the biological control of pests eg Metchnikoff's (1879) and Krassilstschik's (1888) seminal attempts to control wheat cockchafer, Anisoplia austrica, and the sugarbeet curculonid, Cleonus punctiventris, in Russia using mass produced Metarhizium anisopliae. Despite early promise disillusionment in the use of fungi for insect control became widespread following their poor showing in trials against a range of species around the world. Interest in entomopathogenic fungi as with other forms of biological and cultural control of pests wained with the initial overwhelming success of chemical insecticides.

In recent years the advent of insecticide resistance, increasing development costs and fears about the non-target effects of chemical pesticides have renewed interest in biological forms of pest control including pathogenic fungi.

More than 700 species of fungi from ca. 90 genera are pathogenic to insects. Most are found within the Deuteromycetes and Entomophthorales. In common with plant and vertebrate pathogenic fungi, insect pathogenic fungi invade primarily through the host integument (exoskeleton, Fig. 1). Since, unlike entomopathogenic bacteria and viruses, ingestion is not a pre-requisite for fungal infection, members of the order Hemiptera, whose sucking mouth parts usually preclude pathogen uptake, may be parasitized as well as the non-feeding stages of many insects. Some fungi have restricted host ranges eg. Aschersonia aleyrodis infects only scale insects and whitefly, whereas others are much less specific eg. M. anisopliae has been identified from ca 300 species from Lepidoptera, Coleoptera, Orthoptera and Hemiptera, though individual strains are selective (Samson et al., 1988).

Biological control agents including fungi can be utilized inoculatively, viz. small scale release to promote establishment in the population, or inundatively, viz. single or multiple applications of large doses to promote the rapid development of an epizootic. The most successful examples of the use of entomopathogenic fungi for pest control have employed inundation, often termed the "mycoinsecticide" approach. The object of this contribution is to review the current status of mycoinsecticides and the prospects for this form of pest control.

LARGE SCALE USE OF MYCOINSECTICIDES

Metarhizium anisopliae ("Metaqino") is produced in Brazil by some 5 commercial companies as well as grower cooperatives and individual plantation owners. It is used for control of the sugarcane spittle bug, Mahanarva posticata, in the NE of the country. M. anisopliae var majus is also used in parts of the Pacific and SE-Asia to augment baculovirus control of the coconut pest Oryctes rhinoceros at high larval densities (see Gillespie, 1988).

Beauveria bassiana is employed on a large scale (>1m ha) in the People's Republic of China to control pine caterpillars (primarily Dendrolimus punctatus), green leafhoppers (Nephotettix spp.) and the corn borer (Ostrinia nubilalis) (Franz and Krieg, 1980). In the USSR B. bassiana ("Boverin") is produced for the control of the Colorado beetle, Leptinotarsa decemlineata and the codling moth Laspeyresia pomonella. Boverin was used in conjunction with a low dose of insecticide (eg trichlorphon) over some 8000ha in 1977 (Lipa, 1985).

In a large scale trial in Switzerland, treatment of swarming cockchafers, Melolontha melolontha, at their feeding sites with Beauveria brogniartii over an area of 80ha (corresponding to a breeding area of about 4000ha) appears to have brought about long lasting control (Keller, this volume)

Verticillium lecanii strains were first introduced commercially in the UK for the control of aphids ("Vertalec") and whitefly ("Mycotal") on protected ornamental and vegetable crops. The products were discontinued in 1986. Factors contributing to their withdrawal included the small market potential and the erratic performance of the mycoinsecticide.

Hirsutella thompsonii was registered in 1981 in the USA by Abbott as "Mycar" for control of the citrus rust mite, Phyllocoptruta oleivora. Production was halted in 1985. McCoy (1986) attributed commercial failure of "Mycar" to the requirement for cold storage needed to retain viability of the inoculum, lack of a good bioassay to ensure quality control, difficulty in producing infective spores and inconsistent mite control in the field (probably the result of inadequate RH after application).

COMMERCIAL INTEREST IN MYCOINSECTICIDES

The total value of pesticide (insecticides, acaricides and nematicides) sales world wide in 1985 was 5,000m US $ (Jutsum, 1988). World sales of biological pesticides made up less than 1% of this figure with Bacillus thuringiensis alone accounting for 0.6%. Clearly to date the impact of entomopathogenic fungi on the crop protection scene has been minimal. An assessment supported by the meagre list of "successes" described in the previous section. A move to "safer" forms of pest control and the use of integrated pest control world wide has been slow, primarily because chemicals have been comparatively cheap and effective.

There are a number of constraints on the use of fungi as insecticides (Quinlan, 1986; Jaronski, 1986):
1. Fungi can be relatively expensive to produce. However, the long term control exerted by the spread of the disease may make multiple applications unnecessary, removing the cost differential (if any) between mycoinsecticide and comparable chemical insecticide.
2. The short shelf life of spores necessitates cold storage.
3. They can be highly specific, thus additional control measures may be required for other members of a pest complex.
4. Application needs to coincide with high RH, relatively low pest numbers and a fungicide free period.
5. Kill takes up to 2-3 weeks compared with 2-3h for some chemical insecticides. For V. lecanii this made prophylactic use essential in order to avoid plant damage.

The glasshouse experience with V. lecanii has shown that the above technical constraints necessitate a more educated grower with faith in the product despite absence of immediate effect and the need for prophylactic treatment.

The view of multinational chemical companies has been that the expense of research and development of new chemical agents is only justified for pests of world crops (eg. rice, maize and cotton). The large overheads of such companies and in-house competition for project-support tends to militate against investment in specialised, small market products including mycoinsecticides. At present there appears to be 3 situations in which biological control agents including fungi would provide a viable commercial option: where conventional chemical control gives insufficent control or where there is insecticide resistance; where conventional chemicals are too expensive; or where government restricts application of a chemical.

Despite these problems many of the large multinationals are now pursuing projects on biological control. However, perhaps predictably most interest appears to be focussed on microbial toxins, with a view to expressing pathogen toxin genes in plants, or to using the toxins themselves as starter molecules for developing new chemical insecticides. The impetus for the latter has come from the discovery of a group of pesticidal macrolide-like antibiotics in culture filtrates of the soil actinomycete Streptomyces avermitilis (Jutsum, this

volume)

FUTURE MYCOINSECTICIDE DEVELOPMENT

Widespread use of mycoinsecticides in China, Brazil and the USSR may be repeated in other low-input labour-intensive agricultures around the world. But the apparently low efficiency (eg 40-70% by Metaquino) and/or unreliability of control achieved with these particular products would not be acceptable in the harsh economic climate of the developed world. In the short term with our present level of knowledge it seems likely that commercial production of mycoinsecticides is going to be restricted to small commercial companies operating in specialist areas (Jutsum, 1988).

It is clear that we should no longer rely on single weapons to combat pests. Insect Pest Management (IPM) should proceed on a broad front, embracing selective pesticides, pheromones, resistant plants, insect-avoiding cultural practice etc.. Entomopathogenic fungi have no non-target effects and are compatible with other crop protection measures; thus they have a place in IPM schemes. This ensures that development work will continue particularly in the following areas.

Soil-borne pests

Among the most serious insect pests are those that inhabit soil, particularly in the larval stage. Many of the synthetic pesticides used to control them in the past are excessively persistent and dangerous to the environment and thus in the process of being withdrawn (Keller and Zimmerman, 1989). The vine weevil, Otiorhynchus sulcatus, a widespread serious pest of ornamental plants and soft fruit around the world, is a prime example. Aldrin, which provided the most efficient control of this insect, has just been banned in the UK . Soil normally provides a high moisture content and suitable temperatures for fungi, though microbial antagonism can be restricting and physical interactions between fungal propagules and soil can limit movement. A number of studies have demonstrated the potential, particularly of M. anisopliae, for control of the vine weevil under glass (see Keller and Zimmerman, 1989; Soares et al, 1983; Moorhouse et al., this volume). The pasture cockchafer, Aphodius tasmaniae, in southern Australia (Coles and Pinnock, 1982) and porina caterpillars, Wiseana spp, (Latch and Kain, 1983) are two other soil borne insects which are targets for control with M. anisopliae.

Glasshouse pests

The protected environment afforded by the glass house can provide the sustained period of high RH and adequate temperatures necessary for spore germination and fungal growth. Widespread problems with pesticide-resistant aphids, whitefly and thrips (including the newly endemic western flower thrips, Frankiniella occidentalis) in greenhouses in North Western

Europe ensures considerable interest in the return of "mycotal" and "vertalec" to the market. A V. lecanii product has recently been made commercially available in Scandinavia and others may follow soon (Payne, 1989).

Rice pests

Fungi may be particularly suited for use in the tropics where humidities are much higher than in temperate regions and weather patterns more predictable (Gillespie, 1988). A number of research gropups around the world are investigating the use of entomopathogenic fungi for the control of the brown plant hopper, Nilaparvata lugens, and the green leafhopper, Nephotettix spp, on rice. Rombach et al. (1986) and Gillespie et al. (1986, unpubl.) have shown population reductions with M. anisopliae and B. bassiana, but control has proved inferior to that achieved with chemical insecticides.

Stem borers

Work in France (Riba, 1984) and USA (Feng, 1985) has shown that B. bassiana can provide control of the European corn borer Ostrinia nubilalis comparable to that with chemical insecticides.

Parasite vectors

The oomycete Lagenidium giganteum is being investigated for the control of mosquito populations in rice and flood pasture in California. Kerwin and Washino (1984) have developed a technique for the production of oospores in liquid media and field trials of this inoculum have shown much potential.

IMPROVING MYCOINSECTICIDES

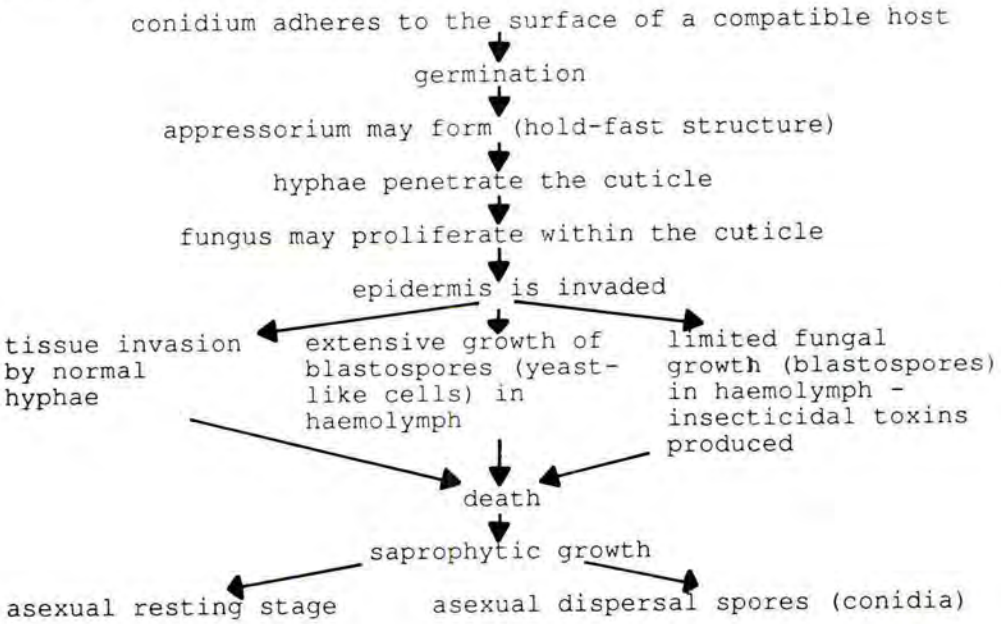
The more widespread acceptance and use of mycoinsecticides will depend on improvements in a number of key areas.
1. Production methods need to be cheaper. This will require greater yield over a reduced time scale. The preferred method, liquid fermentation, is at present not available for production of the most infective and/or stable spore types (Bartlett and Jaronski, 1988). This is particularly a problem for Entomophthoralean fungi. McCabe and Soper (1985) have described a system for the production of dry viable preparations of mycelium. The draw back with this method is that mycelial fragments are not infective; contact with water in the field stimulates required conidiogenesis. The extra step prolongs the response time. Semi-solid fermentation of Deuteromycete fungi using cereal grains holds much promise (Gillespie, pers. comm.)
2. Develop new formulations that will extend the shelf life, improve efficiency of application and field persistence. A water retaining formulation would be particularly helpful.
3. Produce stress tolerant strains which are less affected by low RH, uv, high temperatures and fungicides.
4. Produce more virulent strains with faster kill, such that they could be used against larger pest populations and obviate the need for prophylactic use.
5. Investigate the possibility of integrating mycoinsecticides

with existing control measures eg application with low doses of insecticide has received little attention. Chitin synthesis inhibitors by weakening insect cuticle can facilitate entry of entomopathogenic fungi (see next section).

PRODUCING MORE VIRULENT STRAINS

Determinants of pathogenicity

Understanding the mechanisms of fungal pathogenicity is critical to a rational attempt at producing more virulent and also possibly less specific mycoinsecticides. Unfortunately the attributes that make for a good insect-killing fungus are imperfectly understood. However, progress is being made (see reviews by Samson et al., 1988; Charnley, 1989; Charnley and St. Leger, 1989). Three approaches have been taken to unravel the determinants of pathogenicity: examine isolates of a species for correlations between particular traits and pathogenicity; determine the pathogenicity of mutants hyper or hypoproductive for a particular trait; study the biochemistry of the host-parasite interaction making use of specific inhibitors. Comparisons between isolates for pathogenicity and a particular attribute may only reveal the great variability within a species for numerous factors, many of which may influence but be unrelated to the attribute in question. Induction of mutants in a common genetic background may reduce variability but mutagenesis can affect several loci resulting in pleiotropic effects. Thus there is no guaranteed short-cut to the experimental approach for revealing the determinants of pathogenicity but, as will be shown, there is a place for all three approaches. Disease development of a Deuteromycete entomopathogen is outlined below.



Adhesion of the spore to the cuticle

Adhesion appears to be a prerequisite for successful invasion as noted for hypovirulent strains of M. anisopliae, the conidia of which failed to attach to the larval syphon of Culex pipiens (Al-Aidroos and Roberts, 1978). Spore adhesion has frequently been correlated with virulence or host specificity eg Coelomoyces psorphorae on mosquitoes (Zeobold et al, 1979). However, conidia of Nomuraea rileyi adhere indiscriminately to host and non-host cuticle, while aggressive and non-aggressive isolates of Conidiobolus obscurus adhere to pea aphid cuticle (Boucias and Latge, 1986). It would appear that for some fungi adhesion is a non-specific event while for others it involves mutual recognition. Carbohydrate binding proteins (lectins) have been implicated in host recognition and attachment of L. giganteum and C. psorophorae, but competitive studies failed to show that lectins associated with conidia of B. bassiana, M. anisopliae and N. rileyi are important in attachment. For the latter three species adhesion appears to be due to hydrophobic forces exerted by the rodlets covering the spores (see Charnley, 1989).

Spore germination

Dillon and Charnley (1989) showed that germination of M. anisopliae is initiated by water but progress to the first overt stage of germination (swelling) is dependent on an exogenous nutrient. Prior exposure to water ("soaking") synchronised and accelerated swelling, germ tube and appressorial formation when a nutrient was finally provided. Soaked spores were significantly more pathogenic than the controls (Hassan et al., 1989). The importance of the speed of germination to pathogenicity has been noted also in comparative studies on isolates eg. B. bassiana against Heliothis zea (Pekrul and Grula, 1979, V. lecanii against Macrosiphoniella sanborni (Jackson et al., 1985). Most Deuteromycete fungi have simple non-specific requirements for germination. The interaction between available nutrients , potentially inhibitory compounds eg lipids and phenols, and competing microorganisms on the cuticle surface has yet to be looked at in detail.

Differentiation of penetrant structures

Strain aggressiveness has been correlated with direct rather than delayed penetration of the cuticle by the fungus (Pekrul and Grula, 1979; Jackson et al., 1985). Studies on the molecular events that control differentiation of penetrant structures are in their early stages. Current evidence for M. anisopliae suggests that differentiation of appressoria is strictly governed by the concentration of low molecular weight nitrogen compounds on a conducive (=hard) surface (St. Leger et al., 1989). Thigmotropic and chemical stimuli for the production of appressoria appear to initiate translation primarily during the second round of nuclear division because differentiation is blocked if DNA and RNA inhibitors are applied before this time.

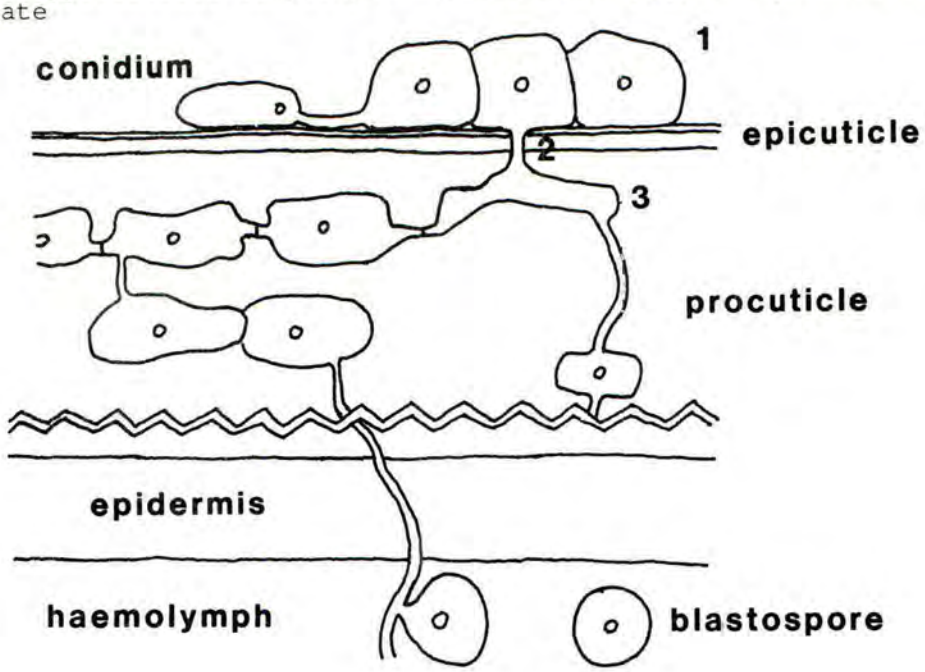
Penetration of host cuticle

Penetration of host exoskeleton appears to involve both mechanical and enzymic components (Fig. 1) (for review of the evidence see Charnley, 1984, 1989; Charnley and St. Leger, 1989). The outermost layer of the cuticle, epicuticle has a highly complex, ill-defined structure and it is difficult to assign a role for specific enzymes in the penetration of this layer. Ultrastructural studies which have demonstrated the early histolysis of the "wax" layer on the cuticle beneath infection structures might implicate lipase/esterase (Zacharuk, 1970). However, on most insects "extractable" cuticular lipids are composed mainly of a complex mixture of alkenes and alkanes with triacyglycerols and wax esters (potential substrates for lipases or esterases) comparatively minor components. Too little is known of the "bound" and "non-extractable" lipid fraction to know if they contain esters (Blomquist, 1984). Lipoprotein lipases are secreted by M. anisopliae and B. bassiana (St. Leger, Charnley and Cooper, unpubl) and this enzyme perhaps in conjunction with the chymoelastase (Pr1, see later) could aid the penetration of the inner epicuticle (polymerized lipoprotein).

Figure 1 Penetration of host exoskeleton (cuticle) by a Deuteromycete entomopathogen

In soft cuticles eg caterpillars growth across the cuticle is more or less direct, in hard cuticles eg wireworms the fungus proceeds in a step-wise fashion.

1 = appressorial complex, 2 = penetration peg, 3 = penetration plate



The main body of the cuticle, the procuticle, comprises primarily chitin fibrils embedded in a protein matrix. Enzymes capable of degrading both these polymers are produced *in vitro* by entomopathogenic fungi (St. Leger *et al*., 1986a). However, chitinase is not detectable during penetration by *M. anisopliae* of insect cuticle *in vivo* (St. Leger *et al*., 1987a). This is because production is controlled by both induction and catabolite repression mechanisms (St. Leger *et al*., 1986b), and chitin is masked by protein and therefore not available for hydrolysis until prior action by endoprotease (St. Leger *et al*., 1986c). Studies which have apparently established a correlation between chitinase and virulence amongst mutants of *B. brogniartii* (Paris and Ferron, 1979) and isolates of *V. lecanii* (Jackson *et al*., 1985) must in the light of the above be treated with caution. The importance of chitin as a mechanical barrier to penetration and as a stabiliser of the cuticular protein matrix in the absence of *M. anisopliae* chitinase, is evident from a study using the insecticide Dimilin, a specific inhibitor of chitin synthesis in insects. Hassan and Charnley (1983) showed that dual applications of Dimilin and *M. anisopliae* had a synergistic effect against larvae of *Manduca sexta*. Ultrastructural observations demonstrated that fungal penetration through Dimilin-affected cuticle was dramatically enhanced (Hassan and Charnley, 1989). Dimilin-affected cuticle (without chitin) was almost completely destroyed compared with pre-ecdysial cuticle (laid down prior to insecticide treatment) where hydrolysis is apparently selective (presumably protein only) and restricted to the vicinity of the fungal hyphae. Consistent with these ultrastructural observations, pharate 5th instar *Manduca sexta* cuticle, produced during treatment with Dimilin and thus completely disrupted by the insecticide (Hassan and Charnley, 1987) was considerably more susceptible to the pathogen protease than the control (St Leger, Charnley and Cooper, unpubl).

In contrast to endochitinase there is considerable experimental evidence consistent with a role for protease in penetration. Chymoelastase (Pr1) is produced *in vitro* at high levels by all virulent isolates of Deuteromucete entomopathogens studied, it has extensive cuticle-degrading ability (St. Leger *et al*., 1987b) and it is produced by the pathogen *in situ* during infection (St. Leger *et al*., 1987a). In addition treatment of *M. sexta* larvae with a specific inhibitor of Pr1, turkey egg white inhibitor, during infection significantly delayed mortality (St. Leger *et al*., 1988a). The inhibitor also reduced melanization of the cuticle (a defence reaction on the part of the host), and invasion of the haemolymph as well as maintaining the host's growth rate. The inhibitor or antibodies raised against Pr1 delayed penetration of the cuticle but did not affect spore viability or prevent growth and formation of appressoria on the cuticle surface. This suggests that inhibition of Pr1 reduced infection by limiting fungal penetration of the insect cuticle. *In vitro* studies using the inhibitor showed that the accumulation of protein degradation products from cuticle, was dependent on

active Pr1. This confirms its major part in solubilizing cuticle proteins and making them available for nutrition.

The production of cuticle-degrading endoproteases with similar modes of action by all Deuteromycetes studied, suggests that it is unlikely that they contribute to host specificity or virulence, though the common occurence implies an indispensible function for these enzymes. The Pr1-type enzymes produced by fungi differ only in charge (IEF). However, this does have practical significance as binding to cuticle is a pre-requisite for activity. Regions of the cuticle with different charge may be favourable or unfavourable to binding (and thus degradation) by individual enzymes, with consequences for the parts of the body which can be invaded by enzymic action. This may influence speed of penetration and thus virulence.

Future work on the role of enzymes in cuticle penetration should establish the importance of the trypsin-like proteases (Pr2) produced by M. anisopliae and other Deuteromycetes (St. Leger et al., 1987b). These enzymes have little cuticle-degrading activity and may have a regulatory function. Many studies comparing isolates have used non-specific substrates such as casein which would not differentiate between Pr1 and Pr2. Since both enzymes are produced constitutively but subject to carbon and nitrogen de-repression (St. Leger et al., 1988b), careful attention should be given to the composition of growth media to ensure a true estimate of protease production.

Exoproteases have received comparatively little attention, though St. Leger et al., (1987a) have shown the production of aminopeptidase during penetration of blowfly wings by M. anisopliae. Post proline dipeptidyl aminopeptidases have also been identified in culture filtrates of M. anisopliae. Both aminopeptidases when combined with Pr1 will enhance release of amino acids from cuticle (St. Leger, Charnley and Cooper, unpubl.). Provision of nutrients during penetration of the relatively massive barrier presented by insect cuticle may be as much an important a function of cuticle-degrading enzymes as their contribution to the penetration process. The former may be more important than the latter during invasion of highly sclerotized cuticle (the protein is hardened by phenolic cross-linkages, and thus comparatively refractory to enzymolysis) where mechanical pressure appears to play a greater role in penetration. The extent of growth on desert locust exuviae (non-digested remains of old cuticle shed at the moult; sclerotized cuticle only) in vitro confirms the potential of enzymes for obtaining nutrients during penetration of sclerotized sclerites (St. Leger et al., 1986c).

In contrast to the profuse in vitro production of extracellular enzymes on cuticle by entomopathogenic fungi, light histochemical and electron microscope studies indicate that in vivo enzyme activity is restricted until infection is well advanced and the cuticle breached (Charnley, 1984). However, ultrastructural localization using an immunogold technique of Pr1 during penetration of M.sexta by M. anisopliae

shows that activity diffuses into the cuticle surrounding penetrant hyphae and is not restricted to the hyphal wall (Goettel, St. Leger, Rizzo, Staples and Roberts, unpubl.)

Host defence

Deposition of oxidized phenols (melanin) in cuticle by host phenoloxidase (po) is the first overt response to infection. Antimicrobial effects of phenols are well established, but in insect cuticles melanization appears to be primarily an effective defense against weak or slow growing pathogens and is ineffective against more virulent pathogens. Protease inhibitors within the cuticle may serve to restrict pathogen enzyme activity. Within the haemocoel the main cellular response of the insect is a multihaemocytic encapsulation of the fungal element following initial recognition of the fungus by the haemocytes. Some evidence implicates lectins in the recognition process but activation of the prophenoloxidase (ppo) system has been more widely implicated in non-self recognition to date (see Charnley, 1989). B 1,3 glucans in the fungal cell wall trigger the cascade which is responsible for ppo activation. Deposition of po (which is sticky) on the fungus attracts haemocytes which accumulate to form the capsule. In some small insects eg mosquitoes and chironomids, melanin deposition occurs directly on the fungus (humoral encapsulation) without the participation of blood cells.

Fungi attempt to evade host defences by a number of ruses. Some species of Entomophthorales form protoplasts in the insect haemocoel. The absence of B 1,3 glucans from the membrane of the protoplast may allow the fungus to invade the insect without a host-defense reaction. The yeast-like blastospores, produced by Deuteromycetes in the insect haemolymph, reduce the effectiveness of the cellular defences by sheer weight of numbers and not being as antigenic as the mycelium (see Charnley, 1989). Finally the cyclodepsipeptide toxins, destruxins (dtx) produced by M. anisopliae appear to interfer with haemocyte function, specifically by suppressing ppo activation (Huxham et al., 1989).

Entomopathogenic Deuteromycetes in comparison with most Entomophthorales, have a short parasitic phase and a prolonged saprotrophic existance on the cadaver. In the former, early host death is attributed to the action of insecticidal toxins (Evans, 1989). For most Deuteromycetes evidence for toxin production is circumstantial. However, recent evidence is consistent with dtx being a determinant of virulence for M. anisopliae. Samuels et al. (1988a) have investigated the virulence of 4 isolates of M. anisopliae towards Manduca sexta, and used HPLC to isolate and quantitate dtx. They found a correlation between symptoms in mycosed insects (paralysis), limited fungal growth in the haemocoel prior to death, short time to death, high virulence, and high dtx production in vitro and in vivo. In Manduca dtx injections caused initial tetany followed by flaccid paralysis (Samuel et al., 1988b). This effect was direct on the muscle and was specific to Lepidoptera

and adult Diptera. Data were consistent with dtx acting to open endogenous Ca channels in the muscle. Thus in Manduca, and probably other Lepidopteran hosts of M.anisopliae, dtx is active in causing symptoms, principally by paralyzing muscle. However, in other insect hosts of M. anisopliae such as Orthoptera, whose muscles are not susceptible to dtx, and in susceptible insects infected with low dtx-producing strains, the toxin may act indirectly to assist the pathogen overcome host defences perhaps as stated earlier by interfering with haemocyte activity (Samuels et al., 1988b).

Strain improvement

It is clear from the previous section that we do not yet know enough about the molecular basis of pathogenicity to carry out programmes of rational strain selection and improvement. However, significant progress has been made in the last few years particularly in our understanding of the role of cuticle-degrading enzymes, sufficient to give hope for the future; as long as research funding in this area is maintained.

Since it is likely that optimal characteristics will reside in different wild-type isolates or mutants, it will be necessary to recombine the traits, what ever they prove to be, in one strain by some form of genetic manipulation to produce a super pathogen.

Deuteromycetes do not have a sexual cycle and thus recombination can only be achieved either by use of the parasexual cycle or by direct genetic manipulation (Heale, 1988; Heale et al., 1989). Recombinants from heterozygous diploids produced by hyphal anastomosis or protoplast fusion have been identified from M. ansiopliae and V. lecanii (Heale, 1988; Heale et al., 1989). These procedures generally rely on the availability of complementary drug-resistant or nutritional auxotrophic markers in the parental strains or mutants, and haploid recombinants are identified as fast growing sectors on selective media. Isaac, Gillspie and Heale (unpubl, reported in Heale et al., 1989) combined high pathogenicity and enhanced sporulation in a cross between 2 strains of M. anisopliae. However, this result appears to be the exception rather than the rule. Frequently parasexual recombinants exhibit reduced pathogenesis in comparison with the wild-type parents due possibly to disruption of clusters of pathogenicity genes.

The recent demonstrations of transformation in a number of filamentous fungi (eg Fincham, 1989) have indicated that molecular cloning techniques could be used to investigate pathogenicity determinants of entomopathogenic fungi, isolate genes coding for specific pathogenicity determinants and produce organisms with enhanced virulence. At the present time M. anisopliae seems to be the most appropriate fungus for this approach as there are two putative pathogenicity/ virulence determinants viz endoprotease Pr1 and dtx. The development of a transformation system and cloning vectors for M. anisopliae is an essential prerequisite for such an approach. Restoration of

pathogenicity to an endoprotease deficient mutant by transformation with a cloned wild type gene would confirm the status of the enzyme as a pathogenicity determinant. For the future once recombinant plasmids containing genes coding for virulence factors have been identified it may be possible to use them in a programme of strain improvement, particularly as transformation in filamentous fungi is frequently accompanied by gene amplification.

Recently the group at Bath University have demonstrated the DNA mediated transformation of M. anisopliae to benomyl resistance using a cloned beta-tubulin gene from Neurospora crassa (Bernier et al., 1989; see elsewhere in this volume), paving the way for molecular analysis of pathogenesis in this fungus.

ACKNOWLEDGEMENTS

I would like to thank Drs J. Clarkson, R.J. Dillon and A. Gillespie for constructive criticism of the draft of this paper.

REFERENCES

Al-Aidroos, K.; Roberts, D.W. (1978) Mutants of Metarhizium anisopliae with increased virulence towards mosquito larvae. Canadian Journal of Genetics and Cytology 20, 211-219.

Bartlett, M.C.; Jaronski, S.T. (1988) Mass production of entomogenous fungi for biological control systems. In: Fungi in Biological Control Systems M.N.Burge (Ed), Manchester: University Press, pp. 61-85.

Bernier, L.; Cooper, R.M.; Charnley, A.K.; Clarkson, J (1989) Transformation of the entomopathogenic fungus Metarhizium anisopliae to benomyl resistance. FEMS Microbiology Letters (In Press).

Blomquist, G.J. (1984) Cuticular lipids of insects. In Infection Processes in Insects D.W.Roberts and J.R.Aist (Eds), New York: Rockefeller Foundation, pp. 54-60.

Boucias, D.G.; Latge, J.P. (1986) Adhesion of entomopathogenic fungi on their host cuticle. In Fundamental and Applied Aspects of Invertebrate Pathology R.A.Samson, J.M.Clark and D.Peters (Eds), Wageningen: Foundation of the 1Vth International Colloquium on Invertebrate Pathology, pp. 432-433.

Charnley, A.K. (1984) Physiological aspects of destructive pathogenesis in insects by fungi: a speculative review. In Invertebrate-Microbial interactions J.M.Anderson, A.D.M. Rayner and D.W.H.Walton (Eds), Cambridge: University Press, pp. 229-271.

Charnley, A.K. (1989) Mechanisms of fungal pathogenesis in insects. In The Biotechnology of Fungi for Improving Plant Growth J.M.Whipps and R.D.Lumsden (Eds), Cambridge: University Press (In Press).

Charnley, A.K.; St. Leger, R.J. (1989) The role of cuticle-degrading enzymes in fungal pathogenesis in insects. In

The Fungal Spore and Disease Initiation in Plants and Animals G.T.Cole and H.C.Hoch (Eds), New York: Plenum Press (In Press).

Coles, R.; Pinnock, D.E. (1984) Current status of the production and use of Metarhizium anisopliae for control of Aphodius tasmaniae in South Australia. In Proceedings of the 1Vth Australian Applied Entomology Research Conference P.Bailey and D.Swincer (Eds), Adelaide: South Australia Government, pp. 357-361.

Dillon, R.J.; Charnley, A.K. (1989) Initiation of germination in conidia of the entomopathogenic fungus, Metarhizium anisopliae. Mycological Research (In Press).

Evans, H.C. (1989) Mycopathogens of insects of epigeal and aerial habitats. In Insect-Fungus Interactions N.Wilding, P.M.Hammond and J.F.Weber (Eds), London: Academic Press, pp. 205-237.

Fenz, Z. (1985) A phenological model for mycosis of the European corn borer, Ostrinia nubilalis caused by Beauveria bassiana, M.Sc. Thesis, Cornell University, USA.

Fincham, J.R.S. (1989) Transformation in fungi. Microbiology Reviews 53, 148-170.

Franz, J.M. (1980) Mikrobiologische Schadlingsbekampfung in China. Ein Reisenbert Forum Mikrobiologie 3, 173-176.

Gillespie, A.T. (1988) Use of fungi to control pests of agricultural importance. In Fungi in Biological Control Systems M.N.Burge (Ed), Manchester: University Press, pp. 37-60.

Gillespie, A.T.; Collins, M.D.; Atienza, A. (1986) Control of Nilaparvata lugens with entomogenous fungi. In Fundamental and Applied Aspects of Invertebrate Pathology R.A.Samson, J.M.Vlak and D.Peters (Eds), Wageningen: Foundation of the 1Vth International Colloquium of Invertebrate Pathology, p. 244.

Hassan, A.E.M.; Charnley, A.K. (1983) Combined effects of diflubenzuron and the entomopathogenic fungus Metarhizium anisopliae on the tobacco hornworm Manduca sexta. Proceedings 10th International Congress of Plant Protection 3, p790.

Hassan, A.E.M.; Charnley, A.K. (1987) The effect of Dimilin on the ultrastructure of the integument of Manduca sexta. Journal of Insect Physiology 33, 669-676.

Hassan, A.E.M.; Charnley, A.K. (1989) Ultrastructural study of the penetration by Metarhizium anisopliae through Dimilin-affected cuticle of Manduca sexta. Journal of Invertebrate Pathology. (In Press).

Hassan, A.E.M.; Dillon, R.J.; Charnley, A.K. (1989) Influence of accelerated germination of conidia on the pathogenicity of Metarhizium anisopliae for Manduca sexta. Journal of Invertebrate Pathology (In Press).

Heale, J.B. (1988) The potential impact of fungal genetics and molecular biology on biological control, with particular reference to entomopathogens. In Fungi in Biological Control Systems M.N.Burge (Ed), Manchester: University Press, pp 211-235.

Heale, J.B.; Isaac J.E; Chandler, D. (1989) Prospects for strain improvement in entomopathogenic fungi. Pesticide

Science 26, 79-92.
Huxham, I.M.; Lackie A.M.; McCorkindale, N.J. (1989) Inhibitory effects of cyclodepsipeptides, destruxins, from the fungus Metarhizium anisopliae, on cellular immunity in insects. Journal of Insect Physiology 35, 97-107.
Jackson, C.W.; Heale, J.B.; Hall, R.A. (1985) Traits associated with virulence to the aphid Macrosiphoniella sanborni in eighteen isolates of Verticillium lecanii. Annals of Applied Biology 106, 39-48.
Jaronski, S.T. (1986) Commercial development of Deuteromycetous fungi of arthropods: a critical appraisal. In Fundamental and Applied Aspects of Invertebrate Pathology R.A.Samson, J.M.Vlak and D.Peters (Eds), Wageningen: Foundation of the 1Vth International Colloquium of Invertebrate Pathology, pp. 653-656.
Jutsum, A.R. (1988) Commercial application of biological control: status and prospects. Philosophical Transactions of the Royal Society B 318, 357-373.
Keller, S.; Zimmermann, G. (1989) Mycopathogens of soil insects. In Insect-Fungus Interactions N.Wilding, N.M. Collins, P.M.Hammond and J.F.Weber (Eds), London: Academic Press, pp. 239-270.
Kerwin, J.L.; Washino, R.K. (1984) Efficacy of Romanomermis culicivorax and Lagenidium giganteum for mosquito control: Strategies for use of biological control agents in rice fields of the Central Valley of California. Proceedings of the California Mosquito Vector Control Association 52, 86-92.
Krassilstschik, J. (1888) La production industrielle des parasites vegetaux pour la destruction des insectes nuisible. Bulletin Science France, Belgique. 19 461-472.
Latch, G.C.M.; Kain, W.M. (1983) Control of porina caterpillar (Wiseana spp.) in pasture by the fungus Metarhizium anisopliae. New Zealand Journal of Experimental Agriculture 11, 351-354.
Lipa, J.J. (1985) Progress in biological control of the Colorado beetle (Leptinotarsa decemlineata) in Eastern Europe. Bulletin E.P.P.O. 15, 207-211.
McCabe, D.; Soper, R.S. (1985) Preparation of an entomopathogenic fungal insect control agent. United States Patent 4,530,834, July 23, 1985, pp. 1-4.
McCoy, C.W. (1986) Factors governing the efficacy of Hirsutella thompsonii in the field. In Fundamental and Applied Aspects of Invertebrate Pathology R.A.Samson, J.M.Vlak and D.Peters (Eds), Wageningen: Foundation of the 1Vth International Colloquium of Invertebrate Pathology, pp. 171-174.
Metchnikoff, E. (1879) Maladies des hannetons duble. Zapiski imperatorskogo obshcestaa sel'skago Khozyaistra yuzhnoi rosii 17-50.
Paris, S.; Ferron, P. (1979) Study of the virulence of some mutants of Beauveria brogniartii. Journal of Invertebrate Pathology 34, 71-77.
Payne, C.C. (1989) Microbial control of insect pests: current and potential uses. Chemistry and Industry 6 182-186.
Pekrul, S.; Grula, E.A. (1979) Mode of infection of the corn

earworm (Heliothis zea) by Beauveria bassiana as revealed by scanning electron microscopy. Journal of Invertebrate Pathology 34, 238-247.
Quinlan, R.J. (1986) Biotechnology - the last hope for entomopathogenic fungi. In Fundamental and Applied Aspects of Invertebrate Pathology R.ASamson, J,M. Vlak and D. Peters (Eds), Wageningen: Foundation of the 1Vth International Colloquium of Invertebrate Pathology, pp. 607-610.
Riba, G. (1984) Application en essais parcellaires de plein champ d'un mutant artificiel du champignon entomopathogene Beauveria bassiana (Hyphopmycete) contre la pyrale du mais, Ostrinia nubilalis (Lep.: Pyralidae). Entomophaga 29, 41-48.
Rombach, M.C.; Aguda, R.M.; Shepard R.M.; Roberts D.W. (1986) Infection of rice brown planthopper, Nilaparvata lugens (Homoptera: Delphacidae), by field application of entomopathogenic hyphomycetes (Deuteromycotina). Environmental Entomology 15, 1070-1073.
Samson, R.A.; Evans, H.C.; Latge, J.-P. (1988) Atlas of entomopathogenic fungi. New York: Springer Verlag.
Samuels, R.I.; Charnley, A.K.; Reynolds. S.E. (1988a) The role of destruxins in the pathogenicity of 3 strains of Metarhizium anisopliae for the tobacco hornworm Manduca sexta. Mycopathologia 104, 51-58.
Samuels, R.I.; Reynolds, S.E.; Charnley, A.K. (1988b)Calcium channel activation of insect muscle by destruxins, insecticidal compounds produced by the entomopathogenic fungus Metarhizium anisopliae. Comparative Biochemistry and Physiology, 90C, 403-412.
Soares, G.G.; Marchal, M.; Ferron, P. (1983) Susceptibility of Otiorhynchus sulcatus (Coleoptera, Curculionidae) larvae to Metarhizium anisopliae and Metarhizium flavoviride (Deuteromycotina, Hyphomycetes) at two different temperatures. Environmental Entomology 12, 1886-1890.
St. Leger, R.J.; Charnley, A.K.; Cooper, R.M. (1986a) Cuticle-degrading enzymes of entomopathogenic fungi: synthesis in culture on cuticle. Journal of Invertebrate Pathology 48, 85-95.
St. Leger, R.J.; Cooper, R.M.; Charnley, A.K. (1986b) Cuticle-degrading enzymes of entomopathogenic fungi: regulation of production of chitinolytic enzymes. Journal of General Microbiology 132 1509-1517.
St. Leger, R.J.; Cooper, R.M.; Charnley, A.K. (1986c) Cuticle-degrading enzymes of entomopathogenic fungi: cuticle degradation in vitro by enzymes from entomopathogens. Journal of Invertebrate Pathology 47, 167-177.
St. Leger, R.J.; Cooper R.M.; Charnley, A.K. (1987a) Production of cuticle-degrading enzymes by the entomopathogen Metarhizium anisopliae during infection of cuticles from Calliphora vomitoria and Manduca sexta. Journal of General Microbiology 133, 1371-1382.
St. Leger, R.J.; Cooper, R.M.; Charnley, A.K. (1987b) Distribution of chymoelastases and trypsin-like enzymes in five species of entomopathogenic Deuteromycetes. Archives of Biochemistry and Biophysics 258, 123-131.

St. Leger, R.J.; Durrands, P.K.; Charnley, A.K.; Cooper, R.M. (1988a) The role of extracellular chymoelastase in the virulence of Metarhizium anisopliae for Manduca sexta. Journal of Invertebrate Pathology 52, 285-294.

St. Leger, R.J.; Durrands, P.K.; Cooper, R.M.; Charnley, A.K. (1988b) Regulation of production of proteolytic enzymes by the entomopathogenic fungus Metarhizium anisopliae. Archives of Microbiology 150, 413-416.

St. Leger, R.J.; Butt, T.M.; Goettel, M.S.; Roberts, D.W.; Staples, R.C. (1989) In vitro studies on the production of infection structures by the entomopathogenic fungus Metarhizium anisopliae. Experimental Mycology (In Press).

Zacharuk, R.Y. (1970) Fine structure of the fungus Metarhizium anisopliae infecting three species of Larval Elateridae. II Conidial germ tubes and appressoria. Journal of Invertebrate Pathology 15, 81-91.

Zeobold, S.L.; Whistler, H.C.; Shemanchuk, J.A.; Travland, J.B. (1979) Host specificity and penetration in the mosquito pathogen Coelomomyces psorophorae. Canadian Journal of Botany 57 2766-2770.

TWO LARGE FIELD TRIALS TO CONTROL THE COCKCHAFER MELOLONTHA MELOLONTHA L. WITH THE FUNGUS BEAUVERIA BRONGNIARTII (SACC.) PETCH

S. KELLER[1], E. KELLER[2], C. SCHWEIZER[1], J.A.L. AUDEN[3], A. SMITH[3]

[1]Federal Research Station for Agronomy, Reckenholzstr. 191, CH-8046 Zürich
[2]Plant Protection Service, CH-8268 Arenenberg
[3]Biotechnology, Ciba-Geigy AG, CH-4002 Basel

ABSTRACT

Having developed a method to specifically contaminate the breeding sites of the cockchafer with the aid of infected females, two large field trials were carried out in 1985 and 1988 respectively. Twenty six sites along woodland borders, where the swarming beetles aggregate, were treated with blastospores. The mean dose amounted to 2.0 - 3.7 x 10^{14} spores/ha. A time-consuming but precise tree by tree-treatment resulted in high infection rates among the adults, whereas a more time-saving but less precise treatment lead to lower infection rates. Nevertheless even this treatment gave a markedly reduced reproduction rate.
In the 1985 trials the fungus was successfully transmitted in all the breeding sites examined. However only at those sites with a relatively high larval density, the disease developed strongly. A generation after the treatment, the proportion of heavy feeding damage by the adults at woodland borders was significantly reduced.

INTRODUCTION

In the region of our experiments (north eastern Switzerland, south of Lake Constance) the development of the cockchafer is synchronous and lasts 3 years. The adults swarm between the end of April/beginning of June. During this period they concentrate mainly at the borders of forests. After a feeding/egg maturation period of about 7-10 days the females fly back to the breeding sites burrow into the soil, and deposit their eggs at a depth of about 10-20 cm. Some of them leave the soil after about 4-6 days and develop a second egg batch. The larvae hatch after 4-6 weeks. They have 3 instars and their development lasts 2 years. They feed on the roots of almost all plant species including trees; all kinds of crops can therefore be damaged.

Beauveria brongniartii is a naturally occurring fungus repeatedly observed to cause epizootics among cockchafer populations. The suggestion to use it as a microbial insecticide was first made about 100 years age, but control trials have so far failed. The main problem lies in getting the inoculum into the soil, the habitat of the pest insect. Most of the previous workers poured or sprayed spore suspensions or dusted the spores on the surface where they remained, not penetrating deeply enough into the soil. The observation period was also too short to reveal significant effects. In principal there are two ways to contaminate the breeding sites: (1) Introduction of infective material by mechanical methods and (2) Introduction using the egg depositing females as vectors. The first method is suitable for protection of specific crops (e.g. orchards, strawberry fields etc.), the second one to regulate populations or parts of them.

In Switzerland priority has been given to the second method. Preliminary trials under controlled conditions demonstrated that the fungus can be transmitted successfully to the soil where a certain proportion of the young larvae succumbed to the disease. A relatively high unspecific mortality among the progeny of treated females was also observed (KELLER, 1978).

The first field trial was started in 1976. Using a mist blower we treated swarming beetles with blastospores at the edge of a forest. The fungus was successfully introduced into the population but the collapse of the population occurred only during the second generation after the treatment. In the following 3 generations the density remained stable at a low level and the fungus induced mortality was still relatively high. In 1982 two further sites were treated in the same manner. The densities were lower and the population decrease induced by the fungus treatment was less pronounced indicating a density dependent effect (KELLER, 1986). Based on these results we started two large field trials in 1985 (KELLER et al., 1986) and in 1988 respectively the results of which are presented here.

MATERIAL AND METHODS

Area of experiments

The experiments are being done in the eastern part of the canton Thurgau (north eastern Switzerland), delimited by the towns of Kreuzlingen, Romanshorn, Bischofszell and Weinfelden. The experimental sites are at an altitude of between 440 and 550 m. The mean precipitation is about 1100 mm, the mean temperature in January -4°-0°C, that in July 20-24°C. The soils are predominantly brown earth or gley-like brown earth. The area is slightly hilly and interspersed with smaller and greater areas of woodland. Cattle farming and arboriculture dominate.

The forest sites to be treated were selected on the basis of the regularly monitored feeding damage caused by the adults, on larval damage and on the results of soil samples. They correspond largely to the aggregation sites of the adults, partly in the traditional breeding area and partly in areas recently colonised by the beetles.

Usually only borders of woodland were treated, rarely whole sections of woodland. To standardise the area to be treated a kilometer of a woodland border was equivalent to two hectares. In 1985 we treated 14 sites in the northern part of the Uranean flight area with a total surface of 70.1 ha, 18.9 ha of which were treated twice, resulting effectively in a treated surface of 89 ha. In 1988 we treated 12 sites totalling 73 ha in the south-western part of the same area. 20 ha of these were treated once, 32 ha twice and 21 ha three times, a total, effectively, of 147 ha.

Fungus material

Prior to the trials we carried out virulence tests on larvae and adult beetles with a selection of fungal isolates. Those listed in table 1 proved to be the most virulent ones. Re-isolates of these from larvae and adults provided the material for the trials.

Table 1: The fungus strains used for the trials and their origins

Year of trial	strains used		original strain		
	Nr	reisolated from	Nr	origin	date of isolation
1985	165	L2	119	trial 1982	mixture of 3 isolates
	168	adult	do		
	166	L2	129	Ried-Brig. L3	6.1.1983
	167	L2	132	Istighofen L3	6.1.1983
	169	adult	do		
1988	166R	L3 (preselection on adults)	166	trial 1985	(see above)
	255/256R	L3 (preselection on adults, then mixed	255	Engishofen L3	19.11.1986
			256	Buhwil L3	19.11.1986

Conidia from tube cultures of each strain were suspended in 10ml of culture medium and stored at 4°C to form the source for the production. The fungus was produced for field use in the liquid culture medium used by BLACHERE et al (1973). Initially each strain was cultured separately in 500ml Erlenmeyer flasks in the laboratory. The resulting broths were then united and used to inoculate 2 l flasks. The contents of these were used to inoculate 30 l of medium in a 50 l fermenter at the production plant and this was scaled up to 300 l in a 500 l fermenter. At each of these production steps, the medium was inoculated with 2.5 % of its volume. For the final production, 4'000 l of medium in a 5'500 l fermenter were inoculated with 5 % of the volume. The spore content of the inocula was between 1×10^8 and 4×10^8 spores/ml. The fermentation from the first flask culture until the end of the production stage lasted 16 days.

During the fermentation the most important parameters were continuously registered and dry weights and spore concentrations determined, using a THOMA 0.01 mm haemocytometer.

The spore suspension with a total volume of 24000 l was delivered in 800 l containers immediately after the end of the fermentation and stored at 1°C in an apple store house located in the region of the trials. All the material was used within four weeks. No significant loss of virulence could be detected during this storage period. The spore content at the end of the fermentation amounted to $1.1 - 2.1 \times 10^9$ blastospores/ml. The germination rate was 99 % after 4 days and 95 % after 9 days.

Application of the spores

With exception of one site, which was treated in 1985 with a mist-blower, all treatments were carried out using a "Aerospatiale SA 315 Lama" helicopter. Its carrying capacity was 500 l and the spray pressure was 2.7 bar. In 1985 the formulation usually comprised of 250 l spore suspension, 250 l water and 5 kg skimmed milk powder. The latter served as sticker and UV-protectant. With each flight about the same surface of deciduous forest was treated, that is about 1.3 ha. The treatments were carried out very precisely, tree by tree, but were rather time consuming.

In 1988 the formulation comprised 250 l spore suspension, 200 l water and 50 l skimmed milk. It was planned that each site should receive the same dose of spores. At sites with repeated treatments the flight speed was adjusted according to the number of treatments. By this means we expected to reduce the time taken to apply the treatments and to be able to respond more flexible to changes in the course of the cockchafer flight.

To reduce possible deletorious effects of sunlight on the spores the treatments were usually carried out between 5 and 9 p.m.

Monitoring the trials

To determine the effects of the spore treatments we collected 140-170 beetles from each site usually 1-4 hours before and 1-2 hours after each treatment, placed them individually in plastic boxes with wet peat and stored them at 20°C. After all the individuals had died, we determined the infection rate based on the symptoms. The reproduction rate as further indicator of a direct influence of the treatment is expressed as the ratio of the density of the larvae in the autumn after the swarming to the density of the adults before swarming.

The long term effects of the treatments on the population are monitored by soil samples from meadows in selected areas. Each year, usually in late autumn, we examine 20 samples (50 x 50 x 35 cm) per site to determine the density. The living individuals are reared in the laboratory to determine the infection rate. The feeding damage caused by the adults along woodland borders was used as a further parameter to study the long term effects of the treatments. The feeding intensity was divided into 3 classes (light, medium, heavy - SCHNEIDER, 1954) and regularly mapped on a 1:25000 map.

RESULTS

Application of the spores

In 1985 a total of 32500 l spore suspension was applied corresponding to 373 l/ha or 500 m of forest border respectively. The dose varied between 2.0 and 3.7×10^{14} spores/ha. In 1988 a total of 33500 l spore suspension with a mean content of 7.7×10^{8} spores/ml was applied to 73 ha resulting in a dose of 3.5×10^{14} spores/ha.

Infection rates of the beetles

At all sites treated in 1985 and 1988 between 3 and 57 % of cockchafers were naturally infected (table 2). In the 1985 trial the first treatment increased the infection rate from an average of 21 % to 86.5 %; the second

one from 79.1 % to 95.5 %. At a single site it was only 40 % after the first treatment whereas at all other sites it varied between 78 and 99 %. Infection levels following treatment in 1988 were lower, perhaps because of the changed application method (table 2).

Table 2: Infection rates (%) of cockchafers before and after the treatments, mean and extreme values (in brackets)

Year	1st treatment			2nd treatment			3rd treatment		
	Nr. of sites	before	after	Nr. of sites	before	after	Nr. of sites	before	after
1985	14	21 (3-57)	87 (40-99)	5	79 (38-96)	96 (91-98)			
1988	12	18 (8-33)	63 (30-89)	10	45 (26-65)	60 (43-89)	3	39 (30-47)	86 (77-90)

Development of the populations treated in 1985

Before the treatment there were no naturally infected third instar larvae (L3) and no infected adults at 5 and 3 sites, respectively, of 8 (table 3). After the treatment a consistent increase in the mean infection rate was noticed. At two sites it was conspicuously high among the adults. Of particular interest is the fact that the fungus became established at all sites demonstrating the widespread suitability of the conditions for a fungus-induced population decrease.

Table 3: Development of the population density (D, nos/m2) and the infection rate (IR, %) at 8 sites treated 1985.
(L2, L3: larvae of the 2nd and 3rd instar respectively, ad: adults).

Site	Parameter	Before treatment L3/1983	ad./1984	After treatment L2/1985	L3/1986	ad/1987	L2/1988
Sommeri	D	14.4	9.0	22.0	10.2	3.4	18.2
	IR	4.3	11.1	4.3	22.0	8.3	a*
Dünnershaus	D	34.6	11.2	50.2	15.2	6.6	29.4
	IR	0	0	5.6	28.3	36.0	a*
Engishofen A	D	18.8	6.2	30.2	11.0	2.4	6.0
	IR	4.3	16.0	12.7	38.1	12.5	a*
Engishofen K	D	29.8	21.2	53.2	9.4	7.0	14.8
	IR	4.7	10.7	9.7	8.6	21.4	a*
Happerswil	D	24.6	15.0	52.6	17.4	5.2	46.2
	IR	0	0	10.1	5.4	52.2	a*
Lenzwil	D	19.6	9.4	11.0	10.0	7.0	43.6
	IR	0	0	0	13.5	6.9	a*
Zuben	D	13.0	6.0	7.8	3.8	3.6	23.0
	IR	0	3.8	0	0	61.5	a*
Schönenbaum-	D	8.4	7.2	27.4	11.8	12.0	56.2
baumgarten	IR	0	3.6	1.1	20.0	16.3	a*
Mean density		20.4	10.6	31.8	11.1	5.9	29.7
Mean infection rate		1.7	5.7	5.4	17.0	26.9	a*

a*: not yet determined

The effect of treatment on the reproduction rate

By comparing the densities of the adults determined in 1987 and that of the second instar larvae (L2) determined in 1988 the reproduction rates (L2/ad) for each site were estimated. Table 4 compares the reproductive rate over this period at eight sites not treated since 1985 with that at six sites treated in 1988 and clearly demonstrates that the 1988 treatment markedly reduced the reproductive rates. Since other influences (soil, climatic conditions etc.) are very improbable, this effect must be attributed to the treatment.

Table 4: Influence of the spore treatment of the adults on the reproduction rate RR (density L2/density parents)

Populations	RR at site no. 1	2	3	4	5	6	7	8	RR average
Test region 1985 (untreated 1988)	5.4	4.5	2.5	2.1	8.9	6.2	6.4	4.7	5.09
Test region 1988 (treated 1988)	3.5	2.2	2.0	1.4	2.3	1.5			2.15

Development of the feeding damage by cockchafers in the test region of 1985

The lengths of the woodland borders damaged by the cockchafer in the years 1982, 1985 and 1988 are listed in table 5. The total lengths of damaged borders remained more or less constant during this period whereas those with heavy damage decreased markedly and those with medium and low damages increased slightly after the treatment.

Table 5: The lengths of damaged woodland borders (in km) in the region of the 1985 trials

Year	Damaged woodland borders heavy	medium	low	total
1982	14.6	17.6	23.1	55.3
1985	17.8	17.5	18.4	53.7
1988	9.3	20.4	25.8	55.5

DISCUSSION

The results of preliminary trials revealed that populations decrease no earlier than in the second generation after the treatment. The two large field trials described here are therefore too recent to draw conclusions about success or failure. The results so far obtained, however, demonstrate that it is possible to contaminate the breeding area with the fungus by spraying the swarming adults with blastospores. Also previous results (KELLER, 1978) showing an immediate effect of the treatment on the reproduction rate (reduced fertility of the treated/infected females or reduced fitness of their progeny) were confirmed. This effect appears desirable, but it may be disadvantage. The existing results indicate that the larval density after treatment is important for the development of the disease. For example the first 5 sites listed in table 3, where the disease developed strongly, had a density of more than 20 larvae/m2. The two sites, however, where no infected larvae were found after treatment had densities of 11 larvae/m2 and below. The high infection rate among adults at Zuben can not be explained. The low initial infection rate at the last site can be

interpreted as result of the poor inoculation of the adults which led to an infection rate of only 40 %. Yet the fungus established in the meantime.

The density dependent effect of this control method is also indicated by the feeding damage caused by the adults (table 5). Only those sites with heavy damage revealed a significant decrease (almost 50 %) of the length of damaged forest border.

A disadvantage of this method is the fact that blastospores in suspension should not be stored for more than 4 weeks. Ideally the end of the production process should coincide with the cockchafer flight. This is difficult to achieve since the cockchafer flight and therefore the application time varies within about 4 weeks. Also the production of Beauveria blastospores interrupts the routine production of other microorganisms of the plant. Consequently if this control method is to be used on a regular basis, a spore preparation with a good shelf-live must be developed.

ACKNOWLEDGEMENT

The authors are greatly indebted to Dr. N. Wilding, Rothamsted Experimental Station, for critically reviewing the manuscript and correcting the English phraseology.

REFERENCES

BLACHERE, H.; CALVEZ, J.; FERRON, P.; CORRIEU, G.; PERINGER, P. (1973). Etude de la formation et de la conservation d'une préparation entomophathogène à base de blastospores de Beauveria tenella (Delacr.) Siemaszko. Ann. Zool. écol. anim. 5, 69-79.

KELLER, S. (1978). Infektionsversuche mit dem Pilz Beauveria tenella an adulten Maikäfern Melolontha melolontha L. Mitt. Schweiz. Ent. Ges., 51, 13-19.

KELLER, S. 1986). Control of May beetle grubs (Melolontha melolontha L.)with the fungus Beauveria brongniartii (SACC.) PETCH. Proc. 4th Int. Coll. Invertebr. Pathol. Veldhofen (The Netherlands), 525-528.

KELLER, S.; KELLER, E.; AUDEN, J.A.L. (1986). Ein Grossversuch zur Bekämpfung des Maikäfers (Melolontha melolontha L.) mit dem Pilz Beauveria brongniartii (SACC.) PETCH. Mitt. Schweiz. Ent. Ges. 59, 47-56.

SCHNEIDER, F. (1954). Planung in der Maikäferbekämpfung aufgrund einer Befallskartierung in den einzelnen Gemeinden. Mitt. Schweiz. Landw. 2, 17-34.

4.
Insect Control Systems and Modelling

Chairman: DR N. R. McFARLANE

INSECTICIDE RESISTANCE MANAGEMENT REVISITED

R.M. SAWICKI and I. DENHOLM

AFRC Institute for Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts., AL5 2JQ

ABSTRACT

Recent developments in existing and new Insecticide Resistance Management (IRM) to control resistance to insecticides in cotton pests are reviewed. Considerable progress has been made in identifying the most suitable monitoring techniques, and research into the mechanisms of resistance to the synthetic pyrethroids (SPs) has led, especially in Australia, to the examination of piperonyl butoxide to overcome SP resistance in bollworms. The review also highlights major areas where research is needed to make IRM more effective.

INTRODUCTION

In recent years, nationwide insecticide resistance management (IRM) strategies in Zimbabwe, Egypt and Australia have successfully dealt with existing or potential problems of resistance to insecticides in key pests of cotton, the crop most threatened by insecticide resistance. These strategies, whose aim is conserving susceptibility, are based on the assumption that the frequency of the S and R genotypes can be manipulated by regulating operational factors such as the frequency and rate of applications, by exploiting the supposed poorer fitness of R individuals, and by encouraging the dilution of the selected gene pool through immigration of S individuals from untreated areas. IRM is achieved though large-scale temporal and spatial restrictions in the number of treatments, including where possible standard integrated pest management (IPM) practices. IRM strategies were with one exception (the acaricide rotation in Zimbabwe) introduced to contain or prevent resistance to the synthetic pyrethroids (SPs) (Elliott et al. 1978) in key Lepidopteran pests, Heliothis armigera, H. virescens, and Spodoptera littoralis. These voluntary or mandatory strategies limit either the number of sprays or the period during which SPs can be used, and recommend alternating compounds of other chemical groups outside the SP window to minimize the development of resistance to non SPs.

There have been very significant changes in many aspects of IRM since we reviewed this topic (Sawicki and Denholm 1987) and the following is a brief account of the most recent developments in this rapidly changing field.

EXISTING STRATEGIES

Thailand

As expected, the efforts to stem SP R in Heliothis armigera in Thailand have not succeeded. The continued heavy larval pressures and the very strong larval resistance to SPs forced growers to abandon cotton and switch to soya as the main between-rice season crop. As a result, the cotton acreage has shrunk to a tenth of what it was in 1981 (152,000 ha), and it is no longer rated by growers as a valuable crop even in the eastern areas

where pesticide usage has been less than in tracitional cotton growing areas of central Thailand.

It can be assumed that the considerable loss of confidence in SPs and the consequent large reduction in their use have been responsible for the very significant (almost five-fold) drop in SP resistance recorded recently by A.R. McCaffery (M. Fua and M.D. Collins 1989, pers. comm.). Bollworm control has now improved, but if and when cotton growing regains momentum extensive control failures will re-occur (Morton and Collins 1989).

Egypt

In Egypt, Spodoptera littoralis has decreased very much in importance in recent years, and has been replaced by Pectinophora gossypiella which is now regarded as the major threat to the Egyptian cotton crop. Since P. gossypiella is also well controlled by SPs and its attack and generation times coincide with those of S. littoralis, there has been no need to either switch to different insecticides or to alter the timing of the second yearly spray (SPs only) to obtain good control of both pests.

In 1987, the authorities, very perturbed by the surprisingly strong levels of resistance to some of the SPs recorded in S. littoralis during both the early and late season monitoring, and by the considerable number of reported SP field failures, feared that the strategy adopted 10 years earlier might have to be abandoned or significantly altered (Mohib Zaki 1988, pers. comm.). However in 1988, minimal lepidopteran pest pressure necessitated little spraying and SPs gave again good control.

With such dramatic changes in pest pressure and pesticide usage it is impossible to predict what is likely to happen even in the near future, but in view of recent events the long-term effectiveness of the strategy is uncertain.

Zimbabwe

In Zimbabwe things have not altered. SPs continue giving satisfactory control of bollworms, and the acaricidal rotation (Duncombe 1975) maintains good protection of cotton with existing acaricides. The screening of Tetranychus evansi for cross-resistance to identify the compounds suitable for setting up an acaricide rotation in tobacco has been completed (Blair 1989), and the acaricides to be introduced in the proposed rotation are now being tested for residue and taint determination.

Australia

Last season's low pest pressure resulted in satisfactory control of H. armigera in Eastern Australia (Forrester, 1989). However in spite of the greatly reduced use of SPs at stage 2, the SP R frequency in the Namoi/Gwydir area of N.S.W. rose unexpectedly to c. 61%, a figure much higher than the 35-45% recorded during the previous three seasons. Forrester's results (Fig. 1) indicate that there has been a relentless increase in SP R frequency at almost every stage of each season since the introduction of the Strategy, and last season he also recorded a sharp rise in the frequency of SP R in larvae from Inverell, an unsprayed area of N.S.W. His results are in reasonable agreement with those of Gunning who, through monitoring mainly unsprayed areas, found that resistance frequencies mirrored closely those she obtained in the cotton growing areas.

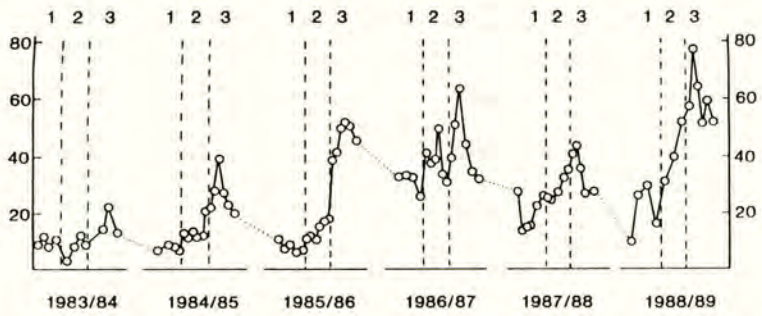


Fig. 1. Frequency of pyrethroid-resistant H. armigera at Namoi/Gwydir (Forrester 1989)

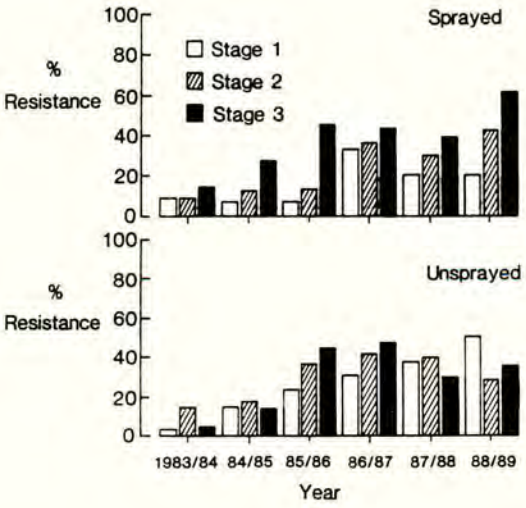


Fig 2. Frequency of pyrethroid-resistant H. armigera in sprayed (Forrester 1989) and unsprayed (Gunning, in preparation) crops

Gunning's data (pers. comm.) on SP R frequencies (Fig. 2) are somewhat less disturbing even though they clearly show a very heavy and extensive contamination of the untreated populations, reaching frequencies as high as 50%. Her figures are encouraging in that they indicate that over the last 3-4 seasons there has been no consistent or dramatic increase in SP R frequencies, at least in the unsprayed areas, and this suggests that the Strategy stabilized SP R frequency during the last 3 or 4 seasons. It is however doubtful whether the high SP R frequencies recorded by the two Australians in the treated and untreated areas can ensure efficient control since when there is a "return to seasons with heavy or even average

pressure there can be a return of control problems with pyrethroids in H. armigera-prone eastern cropping areas" (Forrester 1989).

In contrast to this rather bleak picture the frequency of endosulfan resistance continues to be low and stable. Whether or not this is due to the restrictions on the use of endosulfan within the Strategy is not known. The methomyl resistance frequency has hovered between 20 and 40% for the last three seasons with no significant change between the stages of the season or the year. The eggs are still susceptible to methomyl, and Gunning has recorded no resistance to thiodicarb .

NEW STRATEGIES

Two new strategies have been set up recently. In Columbia, following complaints of poor SP control, monitoring by the agrochemical industry confirmed strong and widespread resistance to SPs in Heliothis virescens, the tobacco budworm (TBW). These tests were subsequently corroborated by the two Columbian Cotton Federations. Thereafter the agrochemical industry proposed to the relevant administrative and cotton growers authorities the settting up of a nationwide SP strategy, modelled on the Australian Strategy, to combat resistance to SPs in TBW. In this TBW control plan there was to be a SP spray window during the period of maximum flowering, and SPs, already banned during the early part of the season, were not to be used after the SP window. This has now been officially adopted with minor modifications for all the cotton growing regions. The SP spray window has been set on a calendar basis as in Australia although most of the cotton is rain fed (Collins 1989, pers. comm.).

The strategy was introduced last season, but according to Collins (pers. comm.) its success will probably only partly depend on implementation. Major factors outside the strategy will be of significant importance; in particular the change in the pest status of TBW. There has been in recent years a sharp increase in populations of the boll weevil Anthonomus grandis, and a corresponding drop in pressure from TBW. As a result endosulfan sprays, used mainly against boll weevils have increased considerably (OPs are progressively less used) while the number of SP sprays has been halved, to an average of 2.5 per season because of the lesser pressure from TBW. In addition TBW is now increasingly controlled successfully with IGRs and formulations containing Baccillus thuringiensis (B.t.). The widespread use of these products, coupled with the continued drop in TBW pressure and the increasing importance of sucking pests, will almost certainly marginalize the problem of SP R in TBW and the importance of strict adherence to the SP strategy.

In the U.S.A. the detection of a progressive decrease in tolerance in TBW to SPs in the US cotton belt by both the public (Leonard et al. 1987, Luttrell et al. 1987, Plapp et al. 1987, 1988, Roush and Luttrell 1987) and private (Staetz 1985, Riley, 1989; Riley et al. 1989) sectors in the mid-1980s led the US companies involved in SPs to form an inter-company technical group (PEG-US) to evaluate the threat of SP resistance in TBW, and the relevance of the different monitoring techniques for assessing the significance of SP R frequencies for control quality. This initiative coincided with parallel extensive monitoring done by F.W. Plapp in Texas (Plapp et al. 1989), and by university workers from Mississippi (Kitten et al. 1989; Stadelbacher et al.,1989), Louisiana (Graves et al. 1989), and Arkansas, (J. Phillips 1989 pers. comm.) who all used Plapp's Adult Vial Test (AVT) (Plapp et al. 1987). These extensive surveys showed: a) the

widespread presence throughout the cotton belt of TBW surviving doses of cypermethrin discriminating between S and R male moths or larvae, b) a significant increase in the frequency of survivors in AVT tests over the season c) control failures in 1987 and /or 1988 e.g. in the Brazos Valley of Texas, and Louisiana that could be attributed to SP R in TBW.

In 1988 PEG and PEG-US agreed to work with the National Cotton Council of America and leading academic and government groups to develop Cotton Insecticide Resistance Management Plans for the USA. (Jackson 1989). More recently the IRAC insecticide manufacturers not belonging to PEG agreed to participate in the Insecticide Resistance Management Plans to co-ordinate the use of non-pyrethroids in TBW control .

According to these Plans SPs should be applied only during mid-season, repetitive treatments with the same insecticide should be avoided, SPs should not be used to treat SP control failures, and SP treatments should be timed to treat early larval instars. The timing of treatments and scouting are considered vital. These Resistance Management Plans, though voluntary, are followed by most growers but not equally in all cotton growing States, and it has been claimed, without formal justification, that last season's decrease in SP R was due to the decrease in the use of SPs in the Resistance Management Plans.

A considerable amount of the US effort has gone into determining the accuracy and relevance of the three monitoring techniques: AVT, a foliar spray for 1st instars developed by ICI, and the topical test for 3rd instars used by the Australians. According to PEG- US (Riley *et al*. 1989) AVT is very suitable for field monitoring to target problem areas when combined with field efficacy information. Once these areas are identified, the foliar and/or topical tests on field collected samples can more precisely define tolerance levels and confirm the presence of resistant populations.

SP RESISTANCE IN OTHER COTTON GROWING AREAS

There is now very strong resistance to SPs in *H. armigera* in Indonesia (Topper C.T. 1989, pers. comm.) which has been confirmed by bioassays (McCaffery *et al*.1988), and in the State of Andhra Pradesh in India (Mehrotra K.N. 1988, Reddy A.S.1988, King A.B.S. 1988, Sawicki R.M.1989, all unpublished documents; McCaffery *et al*. 1988). Strong SP resistance was detected two years ago in the main cotton growing area of the State, an area 75km wide and 200 km long where growers had used SPs almost exclusively since the 1982-83 season. In other, even neighbouring cotton growing areas which rely less on SPs, *H. armigera* populations are still susceptible to pyrethroids (Mehrotra 1989, unpublished document). During the 1987-88 season exceptionally large populations of *H. armigera*, strongly resistant to SPs and most other widely used insecticides, devastated the cotton crop. Last season (1988-89) the pest pressure was equally heavy and the damage as intense but the bollworms were far less resistant to SPs than during the previous season (McCaffery; Mehrotra 1989, pers. comm.). The rapid and sharp drop in SP R was probably caused by a very marked decrease in the use of SPs during the 1988-89 season because most grower following official advice, either used little or no SPs. It is ironic that the highest cotton yields were obtained last season by growers who had disregarded the official advice and had used multiple treatments of SP to control the bollworms.

Exceptional whitefly (Bemisia tabaci) infestations and unusually heavy and uncontrollable attacks by H. armigera in areas using SPs excessively have led to the setting up of discussions between the Central and State governments and the Pyrethroid Management Group (PMG) of the Indian agrochemical industry to examine how to prevent the recurrence of widespread control failures through the excessive use of SPs.

ROLE OF BIOASSAYS IN RESISTANCE MONITORING

Bioassays have played a vital role in detecting resistance and in evaluating changes in resistance frequencies of field populations subjected to different treatment regimes. Considerable work has now been done to determine which of the bioassays discriminate best between S and R adults or larvae.

Discrimination between S and R insects

McCaffery et al (1988) who examined discrimination between S and SP R larvae and adults of H. armigera and H. virescens found that the vial test discriminated worst between S and R adults (RF males 21) and third instar larvae (RF 26). The dip test was better (RF first instar 178; third instar 120); while discriminations by topical application of first and third instars (RF 1125 and 1472 respectively) were only exceeded by the foliar residue assay (first instar RF 1455: third instar RF 3430). With H. armigera, topical application of larvae gave consistently better discrimation than foliar residue bioassays.

Leaf residue and topical application tests are obviously best for detecting resistance frequencies, but are also the most labour-intensive. The reasons that U.S. workers have adopted the vial test so extensively despite its many shortcomings and poorer discrimination are that this test is cheap, straightforward, and takes advantage of an abundant supply of males caught in pheromone traps.

Relevance of bioassays to field control

The level of resistance disclosed by bioassays does not necessarily reflect the response of larvae to treatments applied in the field. For example, the dose of cypermethrin used in the USA to discriminate between S and R TBW males in glass vials (10 mg/vial) is 16-22 times less than the recommended foliar rate in the field (Gage & Hatfield 1989). This discrepancy between resistance levels in bioassays and the field is well illustrated by work done by Daly (1988) and Daly et al. (1988) who showed that the standard rate of fenvalerate applied from the air is lethal to S and R neonates alike and becomes selective only when larvae are more than 3 days old. To overcome this difficulty, I.C.I. used two types of bioassay in its monitoring programme in Columbia; a standard topical application technique against 3rd instars, and the leaf dip test at two rates: a discriminating rate which kills field susceptibles, and the full field-recommended rate which identifies the fraction of the population that is likely to remain uncontrollable by properly applied field rate. For the last 2-3 years the proportion of survivors of the discriminating dose has hovered around 60-80%, whilst a maximum of 20% first instars survived 48h. contact with the full recommended rate. The fraction of uncontrollable larvae varied during the season and within and between the different cotton growing areas (Collins M.D. 1989, pers. comm.).

The quality of field control depends not only on the frequency of R insects and their level of resistance, but also on the number of insects in the crop. From all accounts the good control obtained in Egypt and Australia during the last season in spite of high SP R frequencies is directly attributable to relatively slight pest pressures. This idea is built into the Australian Strategy, which recommends amongst others avoiding use of SPs when H. armigera pressure is high (Davidson 1989).

RESEARCH ON RESISTANCE MECHANISMS

In 1983, R Gunning (1989 pers. comm.) identified three mechanisms of pyrethroid resistance in H. armigera: detoxication inhibited by piperonyl butoxide (PBO), probably via a microsomal oxidase system (mfo), delayed penetration,and massive target-site insensitivity confirmed independently by S.N. Irving. Of these three mechanisms, nerve insensitivity appeared to be by far the most frequent and important in SP R populations collected at the time.

More recently, J.C. Daly (1989 pers. comm.) isolated a strain with relatively strong SP resistance that was totally suppressible by PBO. Both Forrester and Gunning have used PBO to demonstrate the importance of this mfo mechanism in contemporary field populations of H. armigera. Last season, according to Forrester (1989), pre-treatment with 50 ug PBO/larva killed up to 90% of R individuals. However, Gunning, who used 10 ug PBO in her monitoring of unsprayed populations in New South Wales, killed only 50-60% of resistant individuals with PBO synergised fenvalerate. In 1983 this dose had killed a mere 10%.

Gunning, who monitors electrophysiologically for the presence and frequency of nerve insensitivity in populations of H. armigera in the major cotton-growing areas and at unsprayed sites, observed no major within season differences during 1986/87 and 1987/88, but noted a drop in frequencies (50-70%) as compared with 1983. However she found that last season (1988/89) the frequency of nerve insensitivity had dwindled to 10-30%.

Her recent experiments done with J.C. Daly to isolate and characterise this mechanism showed that by itself nerve insensitivity contributes now very little to SP resistance. Hence between 1983 and 1989 there appears to have been a marked qualitative and quantitative change in the nature of SP resistance mechanisms, especially nerve insensitivity in H. armigera. This last factor, apparently the principal cause of resistance in 1983, has totally disappeared and been replaced by the PBO-suppressible mfo resistance. Delayed penetration is still present in 70-90% of individuals.

On the basis of these results, the Australians are testing the efficacy and potential of mixtures containing pyrethroids and photostabilised PBO for controlling SP R insects in the field. This potentially promising development clearly shows the value of resistance genetics for resistance management.

SP resistance mechanisms similar to those in H. armigera have been reported by Little et al. (1988) for the U.S. PEG87 SP R strain of H. virescens, in which trans-permethrin was absorbed slower but eliminated faster than in an S strain. The major route of detoxication via a monoxygenaset led to the formation of hydroxylated cypermethrin, but the products of hydrolysis suggested additional esterase activity which may be

a secondary effect. Electrophysiological studies indicated the presence of nerve insensitivity. In a different study Dowd et al. (1987) found enhanced pyrethroid hydrolysis as being important in pyrethroid resistance in TBW.

RESEARCH ON POPULATION BIOLOGY

The work on factors influencing the dynamics of resistance genes done in Australia by G.P. Fitt, J. Daly and N.W. Forrester (in preparation) has cast considerable light on the importance of agricultural practices within the Strategy.

Extensive laboratory and field experiments have failed to demonstrate significant differences in the fitness of S and SP R H. armigera. This strongly suggests that decreased fitness of R individuals does not reduce resistance frequencies within the Strategy. Moreover in field experiments SPs not only select directly for resistance, but also indirectly by reducing pupal parasitism in cotton, thereby increasing pupal survival (particularly of SP Rs) in this as compared to other crops.

It is also becoming clear that summer cropping patterns in eastern Australia exert a profound influence on the seasonal distribution and dispersal of H. armigera. Although the vast areas of dry land crops support much higher populations of H. armigera than cotton, in hot dry seasons much of the Heliothis population concentrates on cotton, the only attractive crop available, which therefore acts as a sink for immigrants from very large areas. These selected insects then disperse and increase the SP R frequency on surrounding crops. This bottleneck in the life-cycle explains the rapid and ever-increasing contamination of unsprayed areas with SP R individuals (Fig. 2) and a similar bottleneck may have resulted in the strong and widespread SP R of H. armigera in Thailand (Collins 1986).

PROSPECTS FOR IRM

Although research done into the various aspects of SP R of H. armigera, especially by the very small Australian team, has undoubtedly led to improvements in fine tuning of the Strategy, prospects for the long term success of this and other resistance management strategies are uncertain. The consistent increase in SP R frequency within refugia indicates that dilution of resistance through immigration was never achieved. Therefore the primary objective of the Strategy, maintaining dynamic equilibrium in the overall frequency of S and R insects, for which restrictions in SP use at Stage II had been introduced has not worked. This failure has been the penalty paid for having to set up a pragmatic Strategy without initial scientific appraisal or precedent.

Even though events have gone too far to stem resistance to SPs in Australia, it is still most desirable to determine whether such an equilibrium is possible because this information is needed in the other strategies for which the Australian example is the templet.

It has been argued (Morton and Collins 1989) that the management of SP resistance will become less important with the increase in difficulties in control of secondary pests, the decrease in importance of Lepidopterans on cotton, and the introduction of alternative chemicals to SPs. They forsee that a 'holistic' approach to pest management involving amongst others wide-ranging technical support and service by chemical manufacturers to

assist growers in establishing cultural and biological control techniques which will reduce dependence on SPs. This may well happen whilst there is no new class of chemistry to rival the pyrethroids, but as soon as an SP replacement is found it will most likely be as heavily exploited and abused as the SPs.

Since alternatives to conventional insecticides such as transgenic plants which express B.t. or other toxins (Jenkins 1989) are also likely to create resistance problems, IRM strategies will retain their importance even if and when SPs are no longer the sine qua non of IRM.

Indeed, the importance and applicability of IRM has been highlighted by the recent recommendations of the Fruit Crops Working Group of IRAC (Lemmon 1988). The Group recommends: not more than one compound from any one acaricide group should be applied to the same crop in the same season, any one compound should be used only once per season on any one crop, compounds from the same group must not be mixed, compounds having an adverse effect on predatory insects and mites should be avoided.

These recommendations, to be implemented on a voluntary basis in Belgium and elswhere (Lemmon R.W. 1989 pers. comm.), point to new and more extensive applications of IRM. Insecticide Resistance Management will thus be very important both in existing and future pest control programmes. However, to be truly effective, the new IRM schemes will have to incorporate lessons learnt from the pioneering SP strategies on cotton.

ACKNOWLEDGEMENTS

We are most grateful for the many researchers, especially M.D. Collins, J.C. Daly, R.V. Gunning, G.P. Fitt and N.W. Forrester who allowed us to quote from their unpublished results or from papers in preparation.

REFERENCES

Blair, B.W. (1989) Laboratory screening of acaricides against Tetranychus evansi Baker & Pritchard. Crop Protection 8, 212-216

Collins, M.D. (1986) Pyrethroid resistance in the cotton bollworm, Heliothis armigera - a case history from Thailand. Proceedings 1986 British Crop Protection Conference - Pests & Diseases 2, 583-590

Daly, J.C. (1988) Insecticide resistance in Heliothis armigera in Australia. Pesticide Science 23, 165-176

Daly, J.C.; Fisk, J.H.; Forrester, N.W. (1988) Selective mortality in field trials between strains of Heliothis armigera (Hübner) (Lepidoptera: Noctuidae) resistant and susceptible to synthetic pyrethroids: functional dominance of resistance and age-class. Journal of Economic Entomology 81, 1000-1007

Davidson, S. (1989) Pyrethroid resistance in Heliothis: a genetic puzzle. Rural Research 142, 11-17

Dowd, P.F.; Gagne, C.C.; Sparks, T.C. (1987) Enhanced pyrethroid hydrolysis in pyrethroid-resistant larvae of the tobacco budworm, Heliothis virescens (F). Pesticide Biochemistry and Physiology 28, 9-16

Duncombe, W.G. (1975) The development and application of the acaricide rotation scheme in Rhodesia. Proceedings of the 1st Congress of the Entomological Society of South Africa, pp 109-118

Elliott, M.; Janes, N.F.; Potter, C. (1978) The future of pyrethroids in insect control, Annual Review of Entomology 23, 443-469

Forrester, N.W. (1989) Pyrethroid and Endosulfan Resistance in Heliothis armigera - an update. Cotton Newsletter (in the press)

Gage, E.V.: Hatfield, L.D. (1989) Efficacy relationships of pyrethroid field use rates and vial test rates for Heliothis virescens. In: Proceedings. Beltwide Cotton Production Research Conference, Nashville, Tennessee, (in the press)

Graves J.B.; Leonard, B.R.; Pavloff, A.M. (1989) An update on pyrethroid resistance in Tabacco Budworm in Louisiana. In: Proceedings. Beltwide Cotton Production Research Conference, Nashville, Tennessee (in the press)

Jackson, G.J. (1989) Insecticide resistance - the challenge of the decade. In: Pest management in cotton. M.B. Green and D.J. de B. Lyon (Eds), Chichester: Ellis Horwood, pp.27-3)

Jenkins, J.N. (1989) State of the art in host resistance in cotton. In: Pest mangement in cotton. M.B. Green & D.J. de B. Lyon (Eds), Chichester; Ellis Horwood, pp.53-69

Kitten, W.F.; Luttrell, R.G.; Andrews, G.L. (1989) Heliothis spp. resistance to pyrethroid insecticides in Mississippi. In: Proceedings. Beltwide Cotton Production Research Conference, Nashville, Tennessee (in the press)

Lemon, R.W. (1988) Resistance monitoring methods and strategies for resistance management in insect and mite pests of fruit crops. Proceedings 1988 Brighton Crop Protection Conference. Pests and Diseases 3, 1089-1096

Leonard, B.R.; Graves, J.B.; Sparks, T.C.; Pavloff, A.M. (1987) Susceptibility of bollworm and tobacco budworm larvae to pyrethoid and organophosphate insecticides. In: Proceedings. Beltwide Cotton Production Research Conference, Dallas, Texas, 320-324

Little, E.J.; Walker, A.J.; MCCaffery, A.R. (1988) A comparison of the pharmacokinetics of 14C trans-cypermethrin in a resistant and susceptible strain of Heliothis virescens. Proceedings 1988 Brighton Crop Protection Conference. Pests and Diseases 1, 427-432

Luttrell, R.G.; Roush, R.T.; Ali, J.; Mink, J.S.; Reid, M.R.; Snodgrass, G.L. (1987) Pyrethroid resistance in field populations of Heliothis virescens (Lepidoptera: Noctuidae) in Mississippi in 1986. Journal of Economic Entomology 80, 985-989

McCaffery, A.R.; Walker, A.J.; Styles, K.; Maruf, G.M. (1988) Resistance to pyrethroids in Heliothis spp.: bioassay methods and incidence in a range of populations from India and S.E. Asia. Proceedings 1988 Brighton Crop Protection Conference. Pests and Diseases, 1, 433-436

Morton, M.; Collins, M.D. (1989) Managing the pyrethroid revolution in cotton. In: Pest Management in cotton. M.B. Green & D.J. de B. Lyon Eds), Chichester: Ellis Horwood, pp 155-165

Plapp,F.W.Jr.; McWhorter,G.M.; Vance,W.H. (1987) Monitoring for pyrethroid resistance in the tobacco budworm in Texas - 1986. In: Proceedings. Beltwide Cotton Production Research Conference, Dallas, Texas, 324-326

Plapp,F.W.Jr.; Frisbie,R.E.; Jackman, J.A. (1988) Monitoring for pyrethroid resistance in the tobacco budworm - 1987. In: Proceedings. Beltwide Cotton Production Research Conference, Memphis, Tennessy, 237-239

Plapp, F.W.Jr.; Frisbie, R.E.; Jackman, J.A. (1989) Monitoring for pyrethroid resistance in Heliothis spp. in Texas in 1988 . In: Proceedings. Beltwide Cotton Production Research Conference, Nashville, Tennessy, (in the press)

Riley, S.L.(1989) Update on PEG-US/University pyrethroid monitoring program on Heliothis virescens in the U.S. cotton belt. Pesticide Resistance Management 1, 5-7

Riley, S.L.; Watkinson, I.A.; Staetz, C.A.; Simonet, D.E.; Whitehead, J.R.; Blenk, R.; Gouger, R.J.; Collins, M.D. (1989) Pyrethroid Efficagy Group (PEG-US) update on PEG-US/University pyrethroid monitoring program on Heliothis virescens in the U.S. cotton belt. Pesticide Resistance Managemen 1, 20-21

Roush, R.T. and Luttrell, R.G. (1987) The phenotypic expression of pyrethroid resistance in Heliothis and implications for resistance management. In: Proceedings. Beltwide Cotton Production Research Conference, Dallas, Texas, 220-224

Sawicki, R.M.; Denholm, I (1987) Management of resistance to pesticides in cotton pests. Tropical Pest Management 33, 262-272]

Stadelbacher, E.A.; Snodgrass, G.L.; Elzen, G.W. (1989) Resistance to cypermethrin in adult Heliothis populations collected as larvae on wild Geranium spp. and in the F1 progeny of the resistant parents In: Proceedings. Beltwide Cotton Production Research Conference, Nashville, Tennessy (in the press)

Staetz, C.A. (1985) Susceptibility of Heliothis virescens (F)(Lepidoptera:Noctuidae) to permethrin from across the cotton belt; a five-year study. Journal of Economic Entomology 78, 505-510.

MODELLING AND ECONOMICS

J. M. MCKINION

Crop Simulation Research Unit, Crop Science Research Laboratory, Agricultural Research Service, United States Department Of Agriculture, Mississippi State, MS, USA, 39762

ABSTRACT

The use of modelling in the agricultural community has been increasing since the mid-1960's as the power and availability of computers and appropriate software have increased. The scope of the systems modelled has been from simple chemical and biological processes and physical processes to whole plant and animal systems and ecosystems. As these simulations have become more realistic and accurate, their utility in research planning, serving as a communication media, and facilitating animal and field crop production have correspondingly increased. The focus of this manuscript will be upon the economic and scientific utility of the cotton crop model GOSSYM and the corresponding expert system COMAX, which uses GOSSYM as an aid in decision support, as examples in this expanding field.

INTRODUCTION

The use of simulation methodology to address complex system problems and understanding of the dynamics of these systems has become more widespread in the scientific community as computer technology has improved. As the speed of computers has increased and their cost has decreased, simulations of increasingly complex systems have become feasible. Recently, the availability of software tools from the science of artificial intelligence, expert systems methodology in particular, have made simulation of systems even more definitive.

A model is a representation of a real system. Models are always less than the real system. How much simplification is allowed is an important topic for each modelling project, but if a simpler model will suffice, simpler is preferred (Phillips et al, 1976). During recent years there has been a large increase in the application of systems analysis and mathematical modelling to biological and agricultural systems. The most important feature of this approach in analyzing a biological system is that all factors affecting the system are considered simultaneously. The exercise of assembling and formulating all of the known information about the system in a simulation model forces clear and concise thinking. It may also emphasize the potential importance of elements of the system and

their interconnection which, without the modelling process, may have seemed to be of little importance. Untested assumptions are often highlighted, which in many instances reveal the need for improved experimental design and additional experiments to further delineate the system. The appropriate use of mathematical models offers many potential benefits. They provide a concise description of complex processes, indicate ways in which experimental design can be improved and enable hypothesis testing.

PHILOSOPHY OF SIMULATION MODELLING

Simulation as used here is a numerical technique for conducting experiments on a digital computer which involves certain types of mathematical and logical models. These describe the behavior of business, economic, social, biological, physical, or chemical systems (or some component thereof) over periods of time (Maisel and Gnugnoli, 1972). A simulation model is a particular kind of model that permits study of the real system without actual modification of that system in any way. In dealing with a real system, how can one know which changes will result in the desired modified performance of the system? One can use past experience; one can call upon expert opinion (either from a real expert or an expert system implemented upon a computer); one can make changes on a limited basis. But if the system is simulated on a computer, a range of alternative modifications can be tried and their consequences studied in a systematic and controlled manner. Of course, other kinds of models can be used to make predictions about the behavior of real systems. But for complex systems, the use of simulation methodology on a digital computer is unequalled in its ability to provide realistic models of system behavior at a reasonable investment of time and money.

Simulation has other advantages. As a process is studied in preparation for a simulation, previously unrecognized relationships of deficiencies often are revealed. These discoveries may lead to immediate alterations and improvements in the process. Simulations are useful as training devices in teaching about the dynamics of a particular system. Simulation brings the knowledge of scientists from different disciplines into a holistic description of the system, providing a common communication medium for the different disciplines. Finally, simulation permits the study of a broad range of problems and the asking of extremely complex questions. The computer can manipulate elaborate descriptive and mathematical models that consider a great number of factors, provide for complex relationships, and deal simultaneously with a large number of individual units.

Of course, the powerful advantages of computer simulations are offset by certain disadvantages. These include dollar cost, the use of scarce resources, and the possible long wait before an operational

model becomes available. Before the advent of the supermicrocomputer, one of the primary disadvantages was that modeling required the resources of mainframe computers and the associated high costs thereof. However, the continuing progress of computer technology has greatly reduced that cost factor and this trend is likely to continue. An inspection of technology trends over the last 30 years indicates that von Neuman computers (single data, single instruction execution) will double in performance at the same level of cost every two years (McKinion, 1982; McKinion, 1987). The most promising software technology comes from the field of artificial intelligence and expert systems techniques. The object oriented programming paradigm also promises to be an increasingly important programming methodology as larger and larger systems are simulated which require very large programs (in excess of 100,000 lines of code). Object oriented programming systems (OOPS) have the characteristics of combining procedural code with the data pertinent to that code. A more subtle disadvantage of simulation is that the model exists as only a series of computer programs. As a result, management cannot "see" how the simulation actually operates. Thus unless management insists that a model is extensively validated, they cannot tell a good model from a bad model or a superior model from an inferior model. It is also easy to overlook the assumptions that were made either explicitly, or more subtly, implicitly during model development and the compromises that had to be made to get the model operating on the computer.

VALIDATION

Before models can be widely used, they should be tested as completely as possible. This testing procedure is called validation. The validation of system models consists of verifying experimentally whether a particular model or type of model postulated for the system represents an adequate description of the system. Basically, models cannot be verified. Models are hypotheses, which are tested by subjecting them to crucial experiments designed to falsify them, and they are accepted to the extent to which they are not falsified. Model validation is an integral component of the modelling process, being firmly imbedded within all stages rather than being an activity that is carried out once the model has been developed. Validity is a multi-dimensional concept reflecting model purpose, current theories, and experimental test data relating to the particular system of interest, together with other relevant knowledge (Finkelstein and Carson, 1985).

GOSSYM/COMAX: A MODELLING SYSTEM WITH ECONOMIC IMPACT

The GOSSYM/COMAX model-based-reasoning system, a decision aid for cotton crop management, has been extensively tested over the last five years on commercial and research farms across the Cotton Belt in the southern United States. In 1984, with support from the Cotton

Foundations and Union Carbide[1], tests were begun to determine the usefulness of the cotton simulation model, GOSSYM (Baker et al, 1983), as a tool in the management of commercial cotton crops. Two commercial farms, one in Mississippi and one in South Carolina were used in a pilot test in 1984 and 1985. In 1986, the test was expanded to 19 locations, mostly in the mid-south area. By 1987, there were approximately 70 locations involved across the Cotton Belt from California to North Carolina with all 14 cotton growing states participating. In 1988 the pilot test program was expanded again with approximately 150 to 170 locations involved. In 1989 over 210 locations are involved with approximately 300 users trained in the use of the system. Two questions were to be answered with the pilot test: first, the validity and value of GOSSYM in the farm environment, and, second, the nature and configuration of the technical support group required to make the system available to growers across the Cotton Belt. The cotton model as delivered to the pilot test users was in the form of a model-based reasoning system. The GOSSYM model is written in FORTRAN. The expert system COMAX is written in Common LISP.

The GOSSYM MODEL

GOSSYM is a dynamic, process-level simulation model of cotton growth and yield. GOSSYM essentially is a materials balance model which keeps track of carbon and nitrogen in the plant and water and nitrogen in the soil root zone. A flow diagram of the model structure is shown in Figure 1. GOSSYM predicts the response of the field crop to variations in the environment and to cultural inputs. Specifically the model responds to weather inputs of daily total solar radiation, maximum and minimum air temperatures, daily total wind run, and rainfall and/or irrigation amount. The model also responds to cultural inputs such as preplant and within-season applications of nitrogen fertilizer, row spacing and within row plant density as they affect total plant population, and cultivation practices. Before the model can be used on a management unit (a management unit is a field or collection of fields that are all managed the same), certain initialization factors are required: a vertical description of the predominant soil hydraulic properties in the form of the water retention curve, the bulk density by horizon down to two meters in depth, carry-over nitrogen, organic matter content of the soil by horizon, and the initial soil moisture content. The water retention curve only has to be obtained once for

---

1/ The mention of companies or trade names is for information only and does not signify an endorsement by United States Department of Agriculture or a warranty of products mentioned.

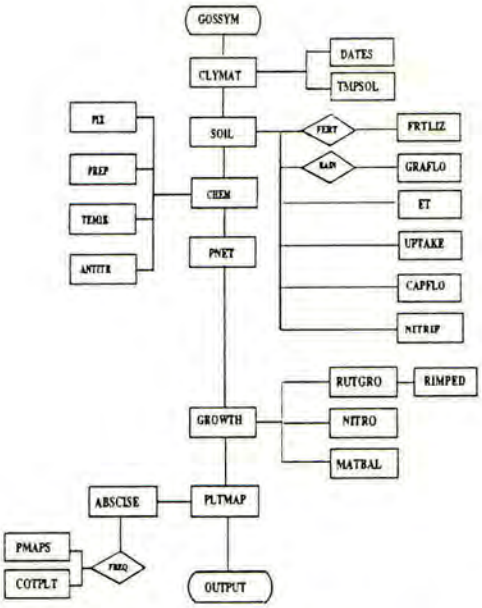


FIGURE 1. Flow chart of the GOSSYM cotton crop simulation model showing the organization of the model and program flow.

the soil type. Farmers typically measure carry-over nitrogen and organic matter before planting each growing season so this is not a major additional requirement. A daily time step is used in the model to calculate plant growth parameters. Up to ten iterations a simulated day are required for some processes such as redistribution of soil moisture by hydraulic conductivity. For a more detailed explanation of the GOSSYM model, see Baker et al (1983) and Whisler et al (1986). For validation results, see Fye et al (1984) and Reddy et al (1985). Since GOSSYM is a detailed, process level crop simulation model of cotton, it can be used for many purposes (McKinion, 1980). From a research viewpoint, the model's foremost use is as a tool for understanding the cotton plant and its interaction with the environment. The model is used as a guide to research since it very readily points to gaps in knowledge and to areas where the model performs poorly, leading to new experiments to obtain the missing knowledge or to correct model structure to improve performance. The model is used as a communication media among researchers. There is no ambiguity when one has to express physiological or physical processes as mathematical equations in a computer program. The model can also be used to evaluate potential characters in plant breeding programs, taking only the most promising characters to the field for trials (Landivar et al, 1983). The model can be used to evaluate the effect of chemicals which affect the physiology of the plant (e.g., systemics, plant growth regulators, defoliants, boll openers). The ability of accurate, validated simulation models to predict the performance of a system in response

to its environment leads to practical applications, i.e. management of crops using simulation models.

THE COMAX SYSTEM

The COMAX (CrOp MAnagement eXpert) system (Lemmon, 1986; McKinion and Lemmon, 1985; and McKinion et al, 1987) is an expert system environment that was developed explicitly for working with the crop simulation models developed by our modeling team. COMAX is a forward-chaining, rule-based system which contains an inference engine, a file maintenance system for the simulation model requirements, a database system for the knowledge base, and a "user friendly" menu driven system for user interaction. The GOSSYM/COMAX system is more properly called a model-based-reasoning system than an expert system. The inference engine applies the rules to set up weather and cultural practice input data files used by the GOSSYM program, to execute the GOSSYM program, and to interpret the model results to make recommendations on water usage, nitrogen usage, and timing of crop termination chemicals. Thus, the COMAX program has captured the expertise of the modeling team's ability to run the GOSSYM model and to interpret its results. A schematic of the GOSSYM/COMAX system with the companion automated on-farm weather station is shown in Figure 2. For more detailed information on COMAX see Lemmon (1986).

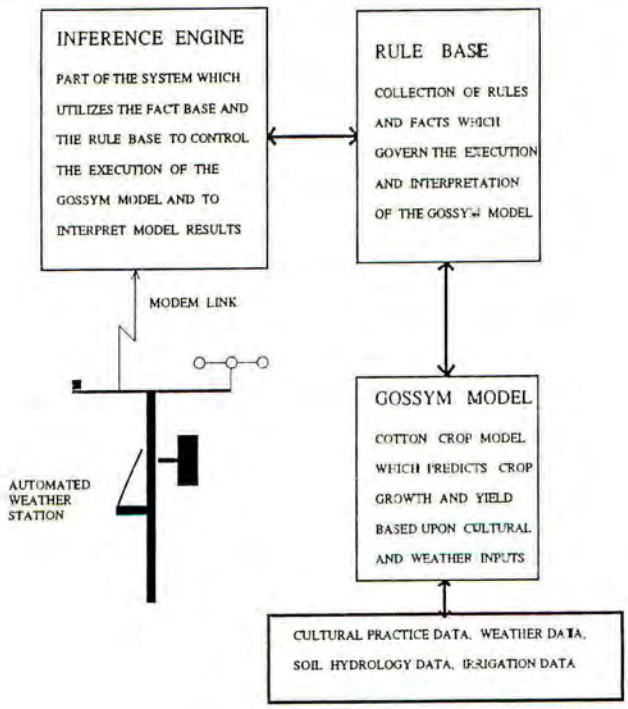


FIGURE 2. Schematic showing the organization of the GOSSYM/COMAX system including the automated weather station..

The interdisciplinary team of scientists who developed GOSSYM are able to use the model directly to manage timing and amount of nitrogen application and timing and amount of irrigation to maximize yield while minimizing inputs. Because of the accuracy of the model, critical water and nitrogen stress periods can be identified up to a month in advance using hypothetical weather data files. These hypothetical weather files are actually historical weather files collected close to the system will be used. These weather files consist of weather patterns having attributes of HOT-&-DRY, COOL-&-WET, TYPICAL, and OPTIMUM. This ability to forecast stress periods provides time for the crop manager to make management decisions and provide inputs to alleviate the stress, if possible. The model can also be used to forecast physiological maturity so that crop termination chemicals can be applied. COMAX was developed to enable farm managers and extension agents to use the GOSSYM model in the above areas of nitrogen management, irrigation management, and crop termination.

While the GOSSYM model was originally developed on large mainframe computers, it can be run on IBM PC/AT's and 100% compatibles equipped with 640 KB of memory and an 80287 math coprocessor. GOSSYM is written in ANSI 1977 FORTRAN and takes about 270 KB of memory and 15 minutes of computer time to simulate a full 150 day season. COMAX requires an additional 3 MB of extended memory. COMAX was developed on a Symbolics 3670 LISP Machine in COMMON LISP, and is delivered to users on the AT using Gold Hills Computer's GCLisp. Both programs are resident in memory concurrently with GOSSYM in the 640 K DOS partition and with COMAX in memory with the GCLisp environment above the one megabyte boundary. COMAX consists of approximately 12,000 lines of LISP code, including the inference engine and the rulebase of about 70 rules.

In addition to the AT computer, the GOSSYM/COMAX system requires an automated weather station to be located on the farm site. The weather station collects hourly and daily weather information that is used by the GOSSYM/COMAX system. Weather information from the current growing season is captured by the automated station and transferred to the AT microcomputer by modem. The GOSSYM/COMAX system uses weather data from the current growing season to simulate conditions that the crop has experienced. From this initial trajectory which lengthens as the season progresses, the expert system selects various weather scenarios from stored historical weather files to simulate conditions until the end of the season.

After the 1987 growing season, a joint meeting of the GOSSYM/COMAX developers and of the pilot test users was held. A questionnaire was distributed which requested each of the users to evaluate the net worth on a per hectare basis of the GOSSYM/COMAX

system. The response was a dichotomy of experienced and inexperienced users. The new users (ones with less than one season's experience using the system) rated the value of GOSSYM/COMAX at $100 per hectare. The experienced users had a much higher rating of $350 per hectare. Not one of the users was willing to give up use of the system. A summary of the results of the questionnaire is given in Table 1. Table 1 shows the net benefit averaged across all users to be $68.44 per acre (or $169.05 per hectare). With the United States having approximately 5-6,000,000 hectares of cotton under cultivation, the universal usage of the GOSSYM/COMAX system could have very large economic implications. Using the lower figure for land usage and the lower figure for

Table 1

Composite benefits estimated by users of 1987 Version of GOSSYM/COMAX·

FARM STATES	HECTARES	NITROGEN TOTAL VALUE	WATER TOTAL VALUE	HARVEST TOTAL VALUE	INSECTICIDE TOTAL VALUE	PIX TOTAL VALUE	BENEFIT PER HECTARE	TOTAL VALUE
MS	834	$91,700	$4,000	$35,000	$84,850		$227	$189,170
LA	28		1,400				50	1,400
TX	61					900	15	900
CA	120	29,180					243	29,180
FL	4	300					10	300
GA	206	-7,990#					-47	-7,990
TOTAL	1253							$211,960
BENEFIT PER USAGE		146	49	15	193	25		
AVERAGE TOTAL VALUE/HECTARE		$211,960/1253 = $169.16						

return, a total minimum that could be expected for the cotton industry is a $500,000,000 increase in profits. Using the $350/hectare and 6,000,000 hectares figure, total increase in profits could be $2,100,000,000. These benefits primarily arise from optimization of irrigation inputs and fertilizer inputs. Because the GOSSYM/COMAX system capabilities are being greatly expanded into other areas such as plant growth regulators, cotton quality issues, pest management, we strongly believe the higher figure is very conservative.

TECHNICAL SUPPORT FOR GOSSYM/COMAX

In the fall of 1988, the USDA Federal Extension Service established the GOSSYM/COMAX Information Unit (GCIU) with an initial core group of three scientists. The purpose of this team is to serve as the technical support group for GOSSYM/COMAX and to promote technology transfer to the State Extension Services, cotton consultants, and commercial cotton growers. The GCIU trained all users outside of the states of Mississippi and Texas, which had already set up their own training teams. Over the short term, two to three years, the GCIU will be expanded to add more scientific expertise to provide for technical support for expanding the usage of GOSSYM/COMAX. In the long term, the support and delivery of the GOSSYM/COMAX system will have to be accomplished through private enterprise. There are currently approximately 30,000 commercial cotton growers in the United States. A reasonable economic market penetration target is 30 to 40% of the total number of growers. At even 30%, this represents a potential of 9,000 growers. Through the GOSSYM/COMAX pilot project for initial delivery and testing of this system, there are only 5-6 full time personnel and an additional 12 part time personnel supporting the current 210 users. The numbers speak for themselves. Either there will be a fairly massive buildup of State and Federal support personnel, or the system will be given to private enterprise for delivery. Given the current status of the State and Federal Governments' budgets, the latter is the only feasible alternative. We foresee that the commercialization of the GOSSYM/COMAX system will most likely be accomplished through delivery and support provided by adequately trained cotton consultants. These consultants will be backed up by a first line of technical support provided by a private company with expertise in managing computer software, providing in-depth technical training, and providing technical support via telephone "hot-lines". A second level of "hot-line" telephone support will be provided by the GCIU. If problems still cannot be solved, a third and final level of "hot-line" telephone support will be provided by the research team.

SUMMARY

The GOSSYM/COMAX system has been pilot tested on commercial farms and experiment station plots since 1984. Starting with one commercial farm in Mississippi and one farm in South Carolina, the program has expanded to 170 locations in all fourteen cotton growing states across the Cotton Belt in the United States. After the 1987 growing season, commercial users of the system rated its value from $100 per hectare for new users to $350 per hectare for experienced users. In 1988, the program expanded to 150 sites and in 1989 the program expanded again to a total of 210 sites and over 300 trainees. With up to 6,000,000 hectares of cotton grown in the United States, the current potential of the GOSSYM/COMAX system if universally applied could approach a net benefit of $2.1 billion to the American cotton industry.

REFERENCES

Baker, D. N., Lambert, J. R., and McKinion, J. M. (1983) GOSSYM: A simulator of cotton crop growth and yield. S. C. Expt. Sta. Bull. Technical Bulletin #1089, S. C. Agricultural Experiment Station. December, 1983.

Finkelstein, L. and E. R. Carson. (1985) Mathematical modelling of dynamic biological systems. Research Studies Press. New York, New York. 355 p.

Fye, R. E., Reddy, V. R., and Baker, D. N. (1984) The validation of GOSSYM: Part 1 - Arizona conditions. Agricultural Systems, 14 (1984) 85-105.

Landivar, J. A., Baker, D. N., and Jenkins, J. N. (1983) Application of GOSSYM to genetic feasibility studies: I. Analysis of fruit abscission and yield in okra-leaf cottons. Crop Science 23:497-504.

Lemmon, H. E. (1986) COMAX: An expert system for cotton crop management. Science, 233 (1986) 29-33.

Maisel, H. and G. Gnugnoli. (1972) Simulation of discrete stochastic systems. Science Research Associates. Chicago, Illinois. 465 p.

McKinion, J. M. (1980) Dynamic Simulation: A positive feedback mechanism for experimental Research in the biological sciences. Agricultural Systems, 5 (1980) 239-250.

McKinion, J. M. (1982) Modeling, experimentation, verification and validation: Closing the feedback loop. Trans. of ASAE 25(3):647-653.

McKinion, J. M. (1987) MODVEX: A MOdel Development and Validation EXpert. Trans. of the ASAE, Vol. 30, No.4 pp.1126-1130, 1987.

McKinion, J. M., Baker, D. N., Lambert, J. R., and Whisler, F. D. (1987) Expert systems for model validation, development, and application in cotton crop management. In Proceeding of the 7th International Workshop on Expert Systems and their Applications, Avignon, France, May 13, 14, and 15, 1987. pp 197-206.

McKinion, J. M. and Lemmon, H. E. (1985) Expert systems for agriculture. Computer and Electronics for Agriculture, 1 (1985) 31-40.

Phillips, D. T., A. Ravindran, and J. J. Solberg. (1976) Operations research: principles and practice. John Wiley and Sons, New York.

Reddy, V. R., Baker, D. N., and Jenkins, J. N. (1985) Validation of GOSSYM: Part II - Mississippi conditions. Agricultural Systems, 17 (1985) 133-154.

Whisler, F. D., Acock, B., Baker, D. N., Fye, R. E., Hodges, H. F., Lambert, J. R., Lemmon, H. E., McKinion, J. M., and Reddy, V. R. (1986) Crop simulation models in agronomic systems. Advances in Agronomy, Vol 40. pp 142-208.

COTTON SIMULATION MODELS AS RESEARCH TOOLS FOR INSECT CONTROL

C. R. FLÜCKIGER

Research & Development Department, Agricultural Division
Ciba-Geigy Ltd., 4002 Basel, Switzerland

ABSTRACT

The possibility of using cotton simulation models as a research tool for insect control is analysed. The cotton plant model has a great potential to produce valid simulations, but major developments are still necessary to upgrade the Heliothis model. Available knowledge about the Heliothis biology is included and knowledge which is missing but necessary to upgrade the model is identified.

INTRODUCTION

During the last 15 years models have been developed for cotton which have resulted in efficient summaries of current knowledge about interrelated biological, chemical, physical and economic factors in this crop. These models provide an excellent theoretical framework for capturing knowledge. They enable the researcher to guide his research in areas such as optimizing pesticide use to maximize short and long term profit, or evaluating the potential of a control agent with a new mode of action. This system approach is a problem solving methodology that is particularly useful for guiding the generation of knowledge and for synthesizing the information into useful forms (Teng 1987). In this paper we present some of these models and their uses in Ciba-Geigy.

The objective of this project was to study both the validity and the usefulness of such models to support our research effort in insect control. The models that we used either originated from governmental institutions or were constructed in-house.

MATERIALS AND METHODS

Different cotton simulation models were examined for their potential use as a research tool (Flückiger et al., 1988). Figure 1 shows which models are used: (1) unmodified, (2) modified in Ciba-Geigy, (3) constructed in Ciba - Geigy. As can be seen we used parts both of the GOSSYM and CIM model to construct GOSINS. In addition we developed FEEDKILL and the user interface.

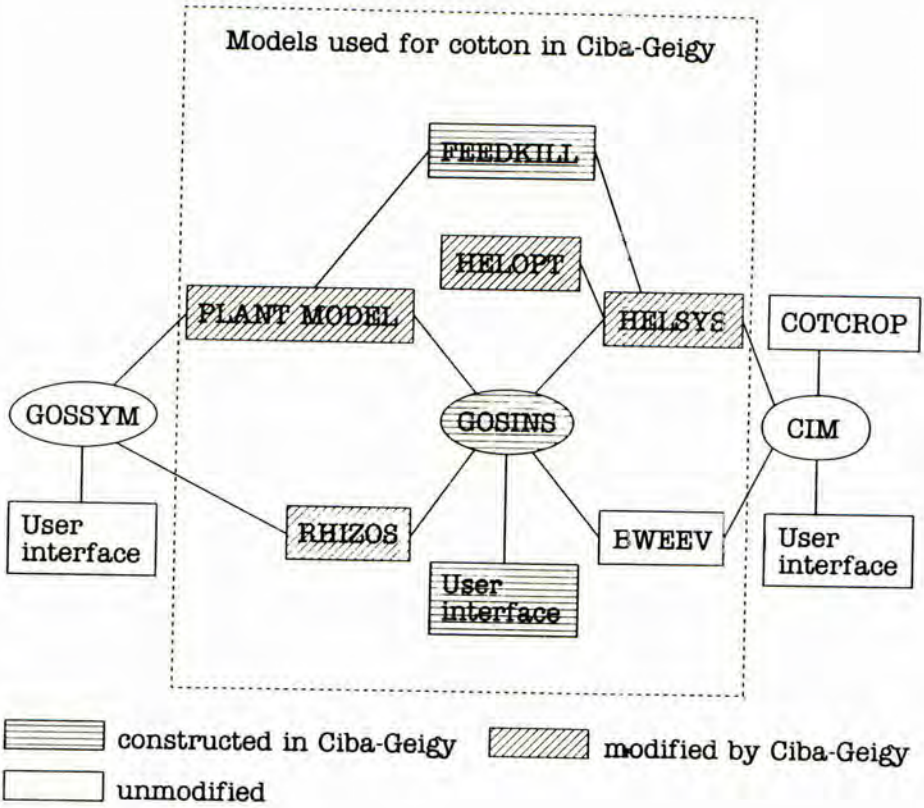


FIGURE 1: Models used as research tools for Insect Control in Ciba-Geigy.

GOSSYM

Different simulation models that have been developed for cotton are: SIMCOT (Baker et al., 1972), COTTON (Stapelton et al., 1973), COTSIM (Wang et al., 1977), FRUITING MODEL (Curry et al., 1980), FRUITING FORMS (Wallach 1980), SIRATAC (Hearn et al., 1981), BOLL PERIOD (Wanjura and Newton, 1981), KUTUN (Mustaers 1982), GOSSYM (Baker et al., 1983), COMAX (Lemmon 1986), COTTAM (Jackson et al., 1984) and COTCROP (Brown et al. 1985). GOSSYM is a dynamic model because photosynthesis, respiration and growth change rapidly with temperature, light intensity, nitrogen availability, and plant water status. Except for pollination and organ abscission, the plant processes are continuous. For calculation, discrete time steps are used, which vary in length, depending on the process being simulated. GOSSYM consists of a plant model and a soil process simulator, called RHIZOS.

The plant model contains pools of nitrogen and labile carbohydrates which arrive via the transpiration stream and the photosynthetic processes, respectively. These materials flow to the leaves, stems, fruits, and roots. Various losses occur as a result of the natural plant processes: senescence and abscission occur in response to physiological stress. The model describes the redistribution of nitrogen within the plant. The initiation of organs on the plant occurs at rates depending on temperature and on the physiological status of the plant. In general, the plant's response to environmental factors are as follows : photosynthesis depends on light intensity and canopy / light interaction and is reduced by water stress. Respiration depends on temperature and plant biomass. Growth is a function of temperature, tissue turgor and metabolic supply. The supply / demand ratios for carbohydrate and nitrogen are used as indices of stress-induced time delays for morphogenetic events. While morphogenetic development is driven by temperature, it is affected indirectly by those factors determining the supply of and demand for carbohydrate and nitrogen. Thus severe moisture stress and a heavy boll load may combine to stop new node formation, while a mild moisture stress which reduces growth (demand) more than supply (photosynthesis) may have no effect on, or may cause relative increase in the morphogenetic development (Baker et al., 1983). RHIZOS, the soil model, was designed to provide the plant model with the necessary parameters from the soil.

HELOPT

An optimization software, developed by L.G. Brown (Mississippi State University), allows the number of Heliothis virescens and H.zea adults (originating either from pupae or immigrated) at a given time of the season to be estimated, based on field observations of eggs and larvae. This software has been intensively tested, and the finished version can guarantee the optimal solution under even extreme circumstances (Koukolik 1988). It consists of two parts: (1) The Heliothis model HELSYS (2) A Linear Programming model. Our changes in algorithms were concentrated mainly on the LP optimization system.

Different simulation models that have been developed for Heliothis spp. are: HELSIM (Stinner et al., 1974), MOTHZV (Hartstack et al., 1976), HELSYS (Brown et al., 1979), SIRATAC (Ives et al., 1984) and TEXCIM (Legaspi et al., 1989). HELSYS (applied in our concept) was developed by Brown and Hogg (Pieters et al. 1981). The model keeps H.virescens and H.zea separated, because they differ in rates of development and ovipositing and their resistance to insecticides is different. The model registers the life stage of each species by day of appearance. Development of the Heliothis spp. is temperature dependent with the distribution, based on field data, of the number of degree days spent in each life stage. In the model, fecundity is a function of temperature and moth age, and mortality can be caused by insecticide applications, predators and other natural causes. The number of eggs and larvae destroyed by predators is proportional to the number of predators present. The actual number of predators is treated as an exogenous variable subject to the effect of insecticides. Development of the larvae is not affected by the availability of feed.

It is not feasible to measure the number of adults that fly into a field early in the season, and so initiating the HELSYS model is difficult. An attempt was therefore made to use the counts of eggs and/or larvae during some part of the season, and to estimate from these data the number of adults that flew into the field earlier in the season. To solve this problem a Linear Programming model was used with the objective of determining the entering adult population that results in the minimum absolute difference between the simulated and observed counts of larvae and/or eggs. The permissible days for adults to enter the field are selected by the user based on his experience. The number of eggs and larvae that result from adults initiating the simulation are calculated using the HELSYS model. Preparing the PC-version the input/output dialogue was complemented by the information panels and the transfer of results into a graphical presentation part.

FEEDKILL

FEEDKILL, a model of the mortality of the larval population of Heliothis spp. caused by the spray application of a feeding agent was developed (Randazzo 1987). The complete model consists of a combination of HELSYS, which includes a description of the larval life cycle, and the larval mortality model FEEDKILL. This model contains a description of the major events in the larval mortality process (figure 2). These are : 1. Spray application of the compound and its distribution over the plant (fruits and leaves); 2. Daily update of the residue of the compound on the plant; 3. Distribution of the available population of larvae over the plant; 4. Feeding activity of the larvae and concomitant accumulation of the compound in the larvae; 5. Larval mortality caused by compound accumulation. An important new model aspect here, one which has not been considered in previous models of this kind, deals with larval behaviour patterns in the field, both with respect to their feeding habits (how much is eaten and which plant parts are fed on), and with respect to the parts of the plant where they prefer to spend much of their life in this development stage. This aspect is of practical importance because insecticide sprays are distributed unequally over different plant parts, and the larvae have clear feeding site preferences. A larva may feed in an unsprayed square and survive, or on a leaf, where it will be killed due to the high insecticide concentration.

It would make this paper too voluminous if all data that have been used to construct this model were presented. Two aspects, however, should be mentioned. (1) After a standard insecticide application only a small amount of active ingredient can be found on fruits where Heliothis spp. larvae feed (especially on older cotton). The leaves are the plant organs with the highest insecticide concentrations (Fischer et al., 1985). (2) Heliothis eggs are layed mostly on leaves of cotton, whereas the larvae feed mostly on squares and bolls (Flückiger 1985, Scheurer 1983).

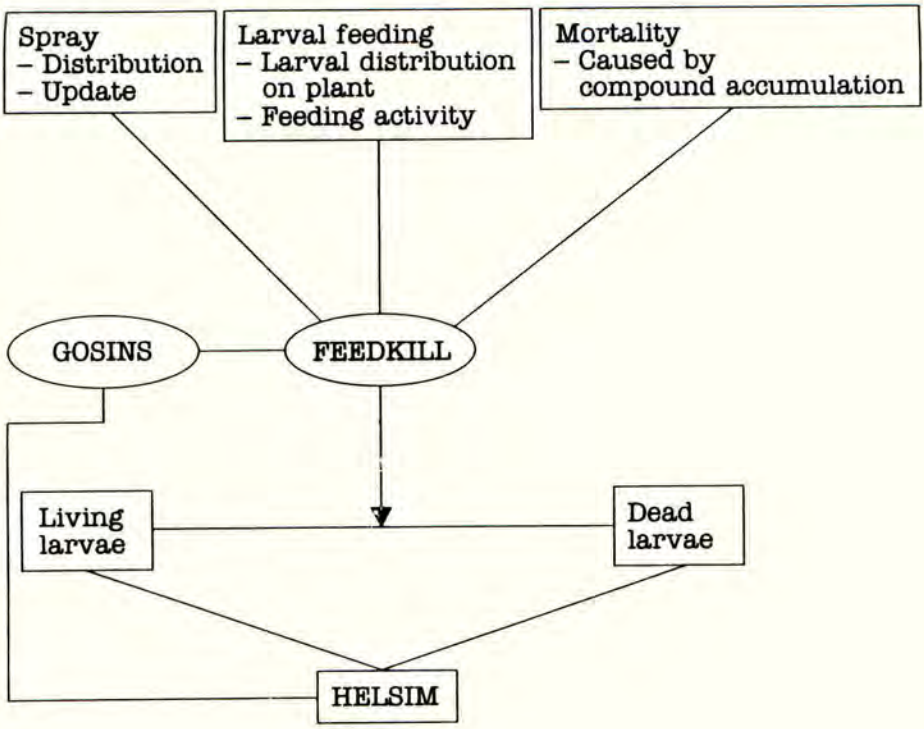


FIGURE 2: Simplified graphical representation of FEEDKILL.

GOSINS

GOSSYM is purely a plant model and lacks pest models. HELSYS for H.virescens and H.zea and BWEEV for Anthonomus grandis from the CIM package were selected for combination with GOSSYM (version 1987). We chose GOSSYM because it is a dynamic model that is mechanistic at the process level. HELSYS was chosen mainly because of its simple and clear structure. The development of insect populations depends partially on the development stages of fruits on the cotton plant as feed source, as does the pest damage. The latter is also influenced by nitrogen and carbohydrate stress, as well as pest damage suffered previously. The main difficulty in combining the two packages arose from the fact that their fruit development models differ in conception, in number of fruit age groups, and in the calculation of physiological age limits and physiological stresses. This necessitated a kind of double book keeping for the fruit inventory and keeping track of different units (per plant, m2 or acre). The difficulties were increased by poor documentation of the latest versions in both packages, requiring a detailed analysis of all relevant subroutines to determine the definitions of crucial variables.

In GOSINS, our combination, the parts of both packages that were used were left untouched as far as possible, and their combination was achieved by a single subroutine as interface (Brugger 1988). Modelling the development of plant structure, fruit development and fruit abscission had to be restructured. The data acquisition and on-line dialogue were adapted to our needs. The reporting of results allows different choices of detail and allows the user to follow on screen the daily development of the most pertinent plant and insect variables. Figure 3 illustrates the different units of GOSINS.

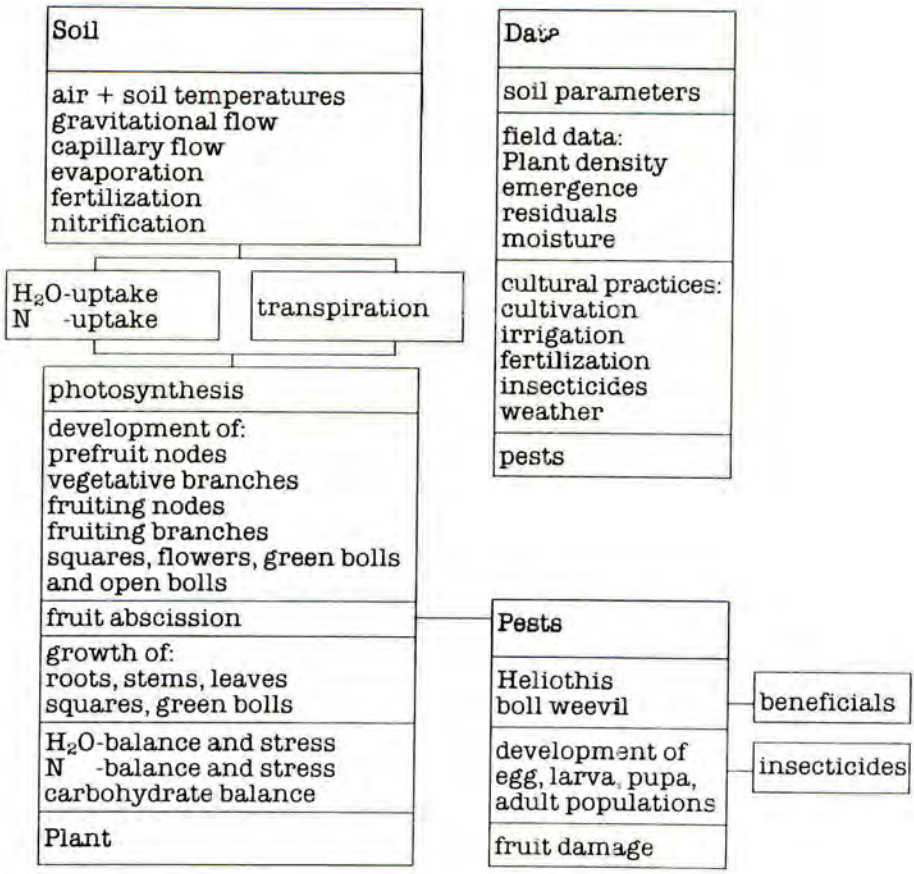


FIGURE 3 Schematic representation of GOSINS

GOSINS /GOSSYM

Six data sets of cotton were used to compare the results of the simulations with the observed plant data from the fields. The input data used to make the simulations are shown in Table 1. The observed values all originate from fields in which pests were controlled. Figures 4-5 show the comparison of simulated and observed square and boll densities.

TABLE 1

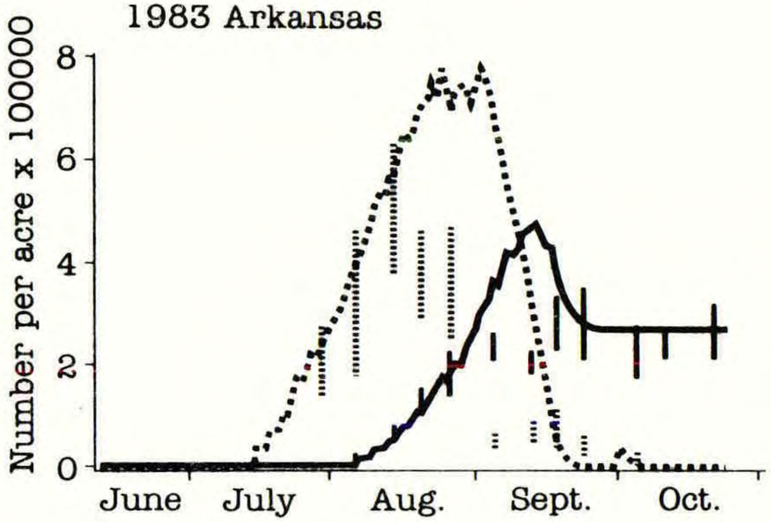
Input data used to make the simulations. The soil was characterised by a number of parameters measured at the different sites. Weather data that are not shown here were also used. Irrigation was only performed in ARK 83 (3 inches on 07/27 and on 08/15).

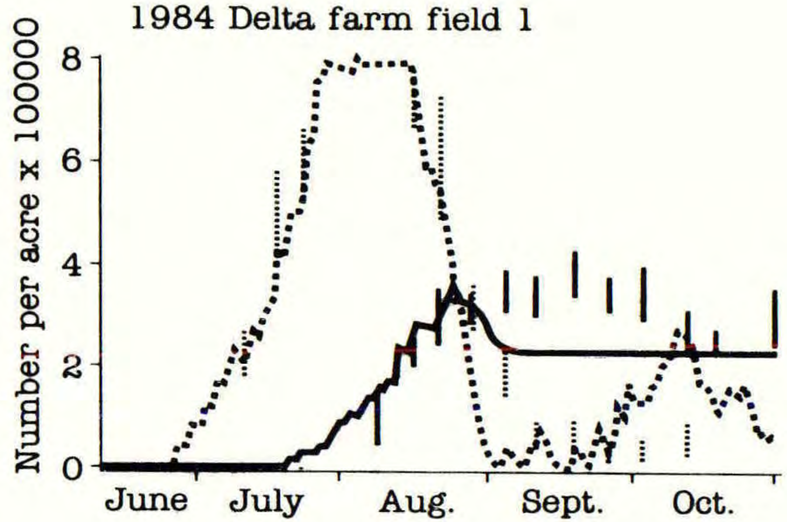
Field Year	Soil	Emergence harvest	Plant pop./acre	Fertilization Date	NO3	NH4
ARK 83	Arkansas Silt Loam	06/03 10/23	36315	05/02	27.0	27.0
DF1 84	Dundee Silt Loam	05/22 11/06	40580	05/17 06/26 07/27	30.0 22.5 20.0	30.0 22.5 20.0
DF5 84	Bosket Loam	05/21 11/06	29162	05/14 06/26 07/27	30.0 22.5 20.0	30.0 22.5 20.0
DF9 84	Bosket Loam	06/20 11/06	36177	03/27 06/26 07/12	35.0 22.5 20.0	35.0 22.5 20.0
DF9 85	Bosket Loam	05/01 09/19	36614	03/13 06/24 07/12	40.0 0. 0.0	40.0 24.0 16.0
DF9 86L	Bosket Loam	05/28 10/01	54880	03/06	35.0	35.0

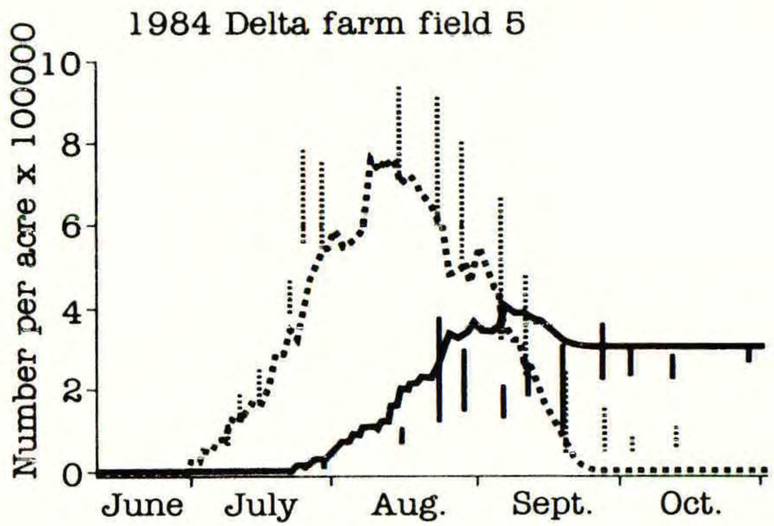
HELOPT

Eggs

The model can optimize initial values for moths on some of the days predetermined by the user, so that the measured density values of first generation eggs that are used as an input to the model can be obtained accurately in a simulation (figure 6). One can try to forecast the egg peaks of the second and third generation by calculating egg values throughout the season, using the same values, that were obtained with the first generation egg peaks as input. Figure 7 shows that, obviously, the simulation model is not precise enough to do this accurately. In figure 8 an attempt is described where eggs from the first two generations were used as an input to the optimization system. In this situation the results show a better fit of simulated and observed values for the second generation (figure 8) than if values of egg densities of the first generation only are the input (as a result of an optimization compromise).







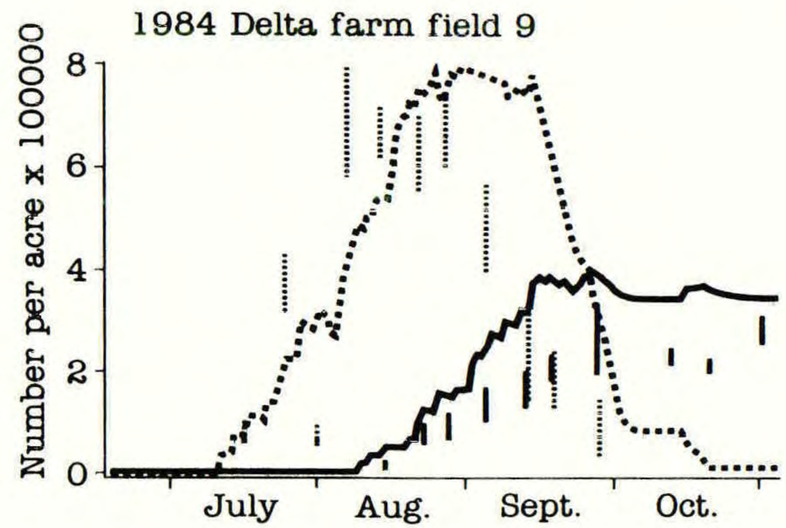


FIGURE 4: Simulated and observed number of squares (dotted lines) and bolls (continuous line). Vertical lines indicate the 95% confidence interval of the observed value.

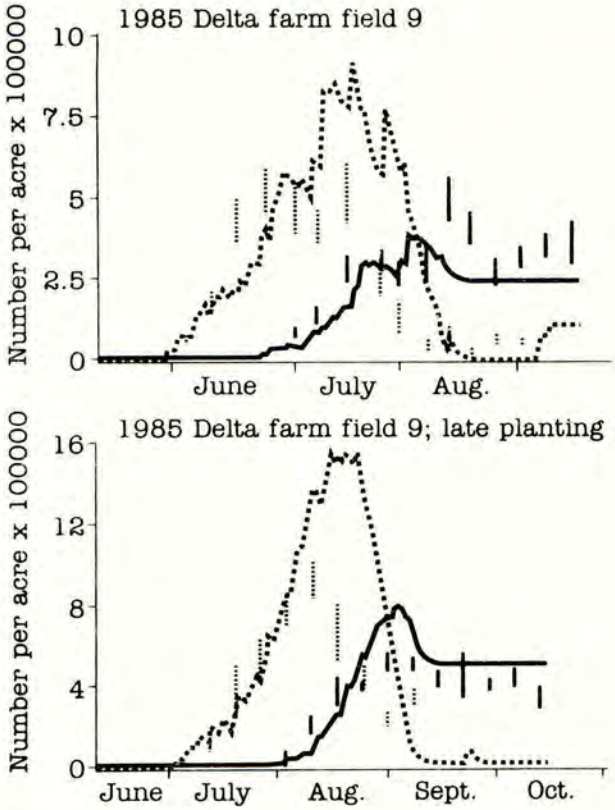


FIGURE 5: Simulated and observed number of squares (dotted lines) and bolls (continuous lines). Vertical lines indicate the 95% confidence interval of the observed value.

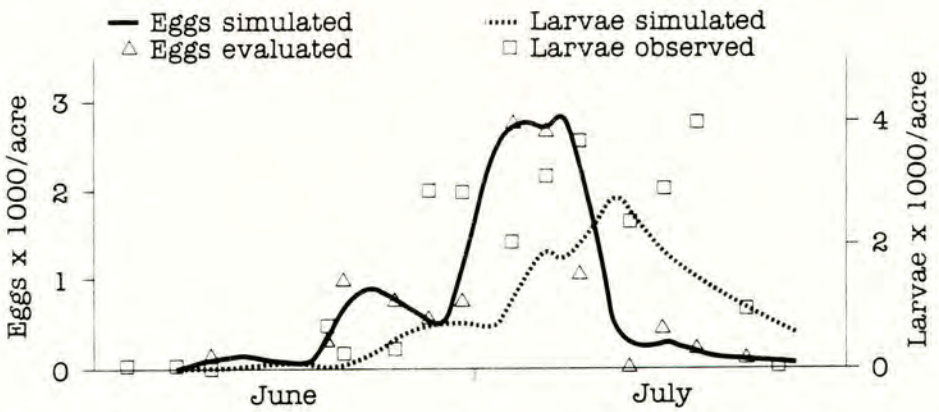


FIGURE 6: Simulated and observed number of eggs and larvae of Heliothis zea and H. virescens.

Larvae

Using the larval counts only as optimization input is a guarantee of a reliable solution where simulated and observed data are almost identical for one insect generation (due to the smoothing effect of the optimization program). An attempt was also made to forecast larval densities based on egg densities as an input to the model. Figures 6 and especially 8 show that a precise forecasting cannot be made, due to the shortcoming of the HELSYS model.

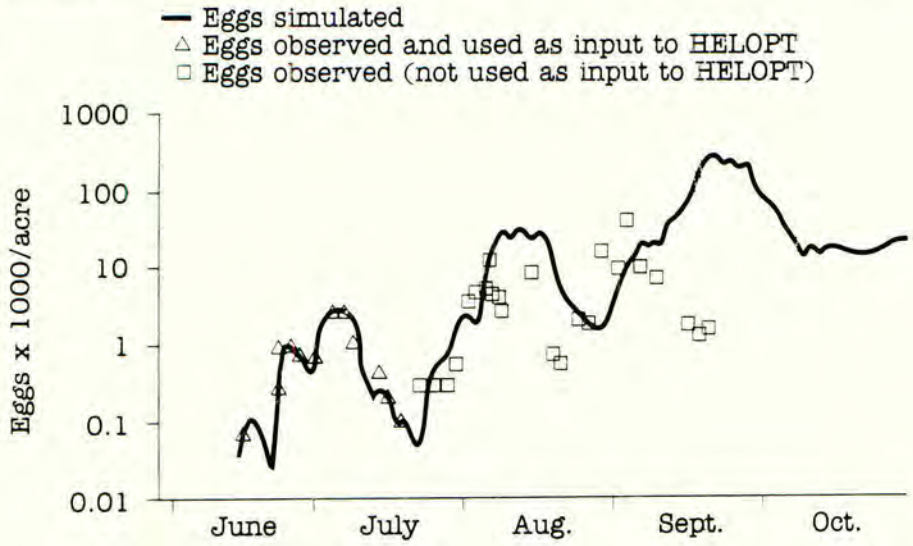


FIGURE 7: Forecasting the number of eggs laid by second and third generation of Heliothis zea and H. virescens, based on observed egg numbers from the first generation.

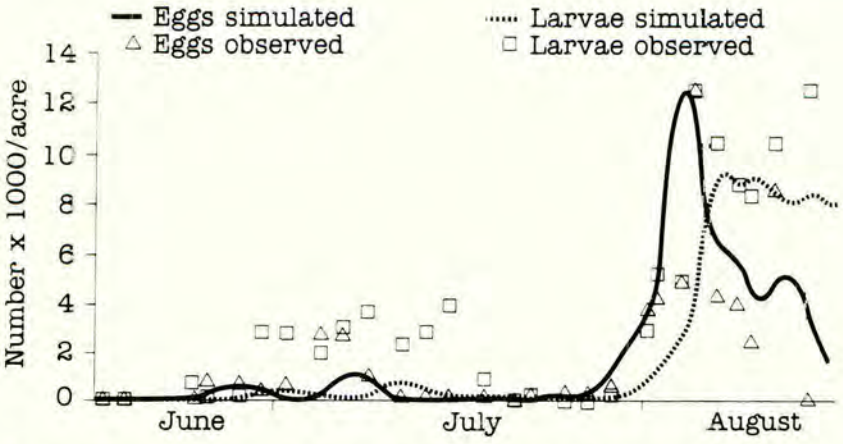


FIGURE 8: Initiation of HELOPT with data from the two first generations of Heliothis zea and H. virescens.

FEEDKILL

From other simulations that we made, it became obvious that the performance of a feeding agent against Heliothis spp. in the field can be influenced by the status of the cotton plant (Figure 9). If the status of the cotton is such that young attractive leaves are present (young cotton or cotton with much new foliar growth), larvae are more likely to feed on contaminated leaves than if the cotton has only old, unattractive leaves. Then larvae feed on fruits with little spray deposit. Field trials confirm the validity of our hypothesis that the status of the cotton plant can influence the performance of a feeding agent against Heliothis spp..

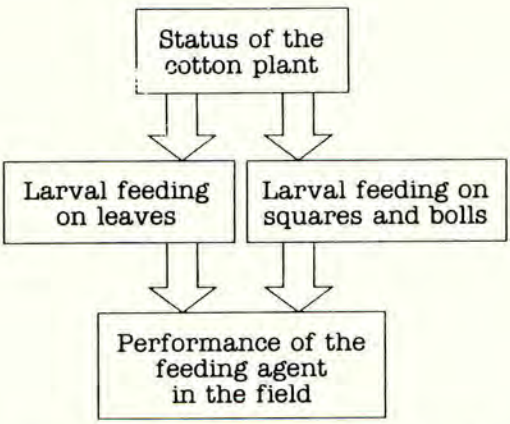


FIGURE 9: Relationship between status of the plant and the performance of the feeding agent.

DISCUSSION

GOSSYM /GOSINS

The comparison of simulated and observed square and boll counts (figures 4-5) demonstrates that the model is not always accurate, but that the main tendencies in the development of the cotton plant can be calculated. In the validation work carried out here, no site-dependent calibration parameters were used to make the model fit the observed data from the field. Considering this, we support the statements (Fye et al., 1984 ; Reddy et al., 1985), that a general and process level cotton simulation model is feasible and may be very useful.

The results show that the method of Linear Programming can be used to fit simulated egg or larval counts to the observed ones. The system failed, however, to forecast a further generation of eggs. This demonstrates the restricted accuracy of the simulation model. Larval densities can be forecasted, based on egg data input of the same or the previous generation. The agreement between observed and simulated number of larvae was not quantitatively exact, but corresponded qualitatively, ie when peaks were observed in the field, they were also simulated. The unreliability in predicting eggs and larvae demonstrates the restricted efficiency of the simulation model. The fact, however, that simulated values can be fitted to the observed egg counts demonstrates the efficiency of the Linear Programming method used, which can compensate in these situations for the deficiency of the simulation model HELSYS. In Table 3, aspects of the biology of Heliothis spp. which have been investigated extensively, and also aspects that have to be further investigated to upgrade the system are listed. Therefore the requested improvements concern not the optimization part, but only the simulation model HELSYS.

We carried out this exercise with much more data than indicated in this paper, and our conclusions therefore are not only based on the results presented here.

TABLE 2

Present knowledge and knowledge necessary to upgrade the Heliothis model.

Present knowledge	Knowledge necessary to upgrade the model
– Longevity of different stages with regard to temperature	– Winter mortality
– Reproduction: Female oviposition	– Diapause
– Mortality at all stages	– Spring emergence
A.) With regard to insecticide applications	– Mortality with regard to biological factors
B.) With regard to adverse bioclimate	– Migration
C.) With regard to predation (crude approach)	– Longevity of different stages with regard to nutrition
– Damage estimation with regard to number of insects and plant organs present (partially known)	

FEEDKILL

FEEDKILL is not a model with direct practical value in pest management. The process of its construction however, which required a rigorous examination of the system, has been useful in generating guidelines for the investigation and understanding of the action of a feeding agent on Heliothis. The results with this model demonstrate how models rely on assumptions. Results of model applications must be interpreted relative to the assumptions and to the inherent limitations in a model's ability to mimic real system behaviour precisely. We have indicated in table 2 what information is available in the HELSYS model and what is lacking. Based on this knowledge, a model can still be useful, even though some of its aspects do not work. In the case presented here, the model helped us to obtain some hypotheses, which then had to be verified by precise field experiments. Thus the model was used here as a planning instrument for research.

CONCLUSION

This paper demonstrates that the cotton plant model has a great potential to produce valid simulation data, but that major developments are still necessary to upgrade some parts of the Heliothis model. For successful research use of a model that does not simulate data accurately it is a prerequisite that the strong and weak aspects of the model be identified. The model then can be used as a research tool. Hypothesises gained from model simulations have to be verified in the field. In this sense, the modelling activity becomes a planning instrument for research. The more valid models become, the easier it will be to use them as research tools and obtain valid hypothesises from them.

ACKNOWLEDGEMENT

The author thanks Drs. K. Brugger (GOSINS), M. Koukolik (HELOPT) and D. Randazzo (FEEDKILL) for model development and simulation work, H. Ngo from the Delta Farm for the collection of plant and insect data, Prof. F. D. Whisler for the soil analyses and F. Buholzer for reviewing the manuscript.

REFERENCES

Baker, D.N.; Lambert, J.R.; McKinion, J.M. (1983) GOSSYM : A simulator of cotton crop growth and yield. South Carolina Agricultural Experiment Station Technical Bulletin 1089, 134 pp.

Baker, D.N.; Hesketh, J.D.; Duncan, W.G. (1972) Simulation of growth and yield in cotton. I. Gross photosynthesis, respiration, and growth. Crop Science 12, 431-435.

Brown, L.G.; Jones, J.W.; Hesketh, J.D.; Hartsog, J.D.; Whisler, F.D.; Harris, F.A. (1985) COTCROP: Computer simulation of cotton growth and yield. Mississippi Agricultural and Forestry Experiment Station Information Bulletin 69, 117 pp.

Brown, L.G.; McClendon, R.W.; Jones, J.W. (1979) Computer simulation of the interaction between cotton crop and insect pests. Transactions of the ASAE 22, 771-774.

Brugger, K. (1988) Cotton Crop Management Simulation, GOSINS. Model description/ Model calibration. Ciba - Geigy, Internal reports.

Curry, G.L.; Sharpe, P.J.H.; DeMichele, D.W. (1980) Towards a management model of the cotton-boll weevil ecosystem. Journal of Environmental Management 11, 197-223.

Fischer, J.; Arnold, A.; Flückiger, C. (1985) Quantitative deposit distribution of plant protection sprays on cotton plants at three growth stages of cotton. Ciba - Geigy, Internal report.

Flückiger, C.R. (1985) The distribution of Heliothis eggs, larvae and damage within a cotton plant. Ciba - Geigy, Internal report.

Flückiger, C.R.; Randazzo,D.; Koukolik, M (1988) The use of models for cotton crop protection. OILB, WPRS Bulletin 11, 12-18.

Fye, R.E.; Reddy, V.R.; Baker, D.N. (1984) The validation of GOSSYM : Part 1 - Arizona conditions. Agricultural Systems 14, 85-105.

Hartstack, W.A.; Witz, J.A.; Hollingsworth, J.P.; Ridway, R.L.; Lopez, J.D. (1976). MOTHZY-2: A computer simulation of Heliothis zea and Heliothis virescens population dynamics. USDA, ARS-S-127 (User's Manual).

Hearn, A.B.; Ives, P.M.; Room, P.M.; Thompson, N.J.; Wilson, L.T. (1981) Computer-based cotton pest management in Australia. Field Crops Research 4, 567-579.

Ives, P.M.; Wilson, L.T.; Cull, P.O.; Palmer, W.A.; Haywood, C.; Thompson, N.J.; Hearn, A.B.; Wilson, A.G.L. (1984) Field use of SIRATAC: An Australian computer-based pest management system for cotton Protection Ecology 6, 1-21.

Jackson, B.S.; Arkin,G.F.; Hearn, A.B. (1984) Cotton fruiting model: calibration and testing. American society for Agricultural Engineers Paper No. 84-4542.

Koukolik, M. (1988) Optimization of the Heliothis Population initiation from the field observation data. Ciba - Geigy, Internal report.

Legaspi, B.A.C.; Sterling, W.L.; Hartstack, A.W.; Dean, D.A. (1989) Testing the interactions of pest-predator-plant component of the TEXCIM model Environmental Entomology 18, 157-163.

Lemmon, J.H. (1986) COMAX: An expert system for cotton crop management. Science 233, 29-33.

Mutsaers, H.J.W. (1982) KUTUN: A morphogenetic model for cotton (Gossypium hirsutum L.) Thesis, Agricultural University, Wageninen, 99 pages.

Pieters, E.P.; Abaky, K.S.; Brown, L.G.; McClendon, R.W. (1981) Use of computer game COTGAME in teaching entomology. Environ. Entomol. 10, 256-261.

Randazzo, D. (1987) The Ciba-Geigy spray application model. An estimation of the Heliothis larvae mortality caused by the spray application of a compound with feeding activity. Ciba - Geigy, Internal report.

Reddy, V.R.; Baker, D.N; Jenkins, J.N. (1985) Validation of GOSSYM: Part 2. Mississippi conditions. Agricultural Systems 17, 133-154.

Scheurer, R. (1983) Observations on the behaviour of newly hatched larvae of Heliothis virescens. Ciba - Geigy, Internal report.

Stapelton, H.N.; Buxton, D.R.; Watson, F.L.; Nolting, D.J.; Baker, D.N. (1973) COTTON: A computer simulation of cotton growth. Arizona Agricultural Experimental Station Technical Bulletin 123.

Stinner, R.E.; Rabb, R.L.; Bradley, J.R. (1974) Population dynamics of Heliothis zea (Boddie) and H. virencens (F.) in North Carolina: a simulation model. Environmental Entomology 3 , 163-168.

Teng, P.S. (1987) The systems approach to pest management. In: Crop Loss Assessment and Pest Management. Teng (ed.),St Paul: APS Press, 160-167.

Wallach, D. (1980) An empirical mathematical model of a cotton crop subject to damage. Field Crops Research 3, 7-25.

Wang, Y.; Gutierrez, A.P.; Oster, G.; Daxl, R. (1977) A population model for plant growth and development : Coupling cotton-herbivore interaction. Canadian Entomologist 109, 1359-1374.

Wanjura, D.F. ; Newton, O.H. (1981) Predicting cotton crop boll development. Agronomy Journal 73, 476-481.

TOWARD AN EXPERT SYSTEM FOR PEST SIMULATION MODELS: LESSONS LEARNED FROM APPLICATION

J. A. LOGAN

Department of Entomology and Department of Forestry, Virginia Polytechnic Institute and State University, Blacksburg, VA. 24061, USA

ABSTRACT

A previously described computer system that assists in development of pest simulation models has been recoded for execution in a Personal Computer (PC) environment. The PC version has been applied to simulation of a wide variety of pest systems, ranging from spider mites in agronomic crops to grasshoppers and pine bark beetles in Natural Resource systems. The program has also been applied to teaching graduate level classes in computer modelling. These applications have indicated that the system is fulfilling a legitimate need in the applied entomological community. Experience gained from application has resulted in substantive changes in both the system's capabilities and in the basic philosophy of how such a system should be structured. In this paper, I first briefly describe operation of the system and then detail several important modifications that have resulted from experience gained through application.

INTRODUCTION

Applications of computer modelling techniques have made important contributions to the development of modern insect control practices. In the United States, the evolution of computer simulation modelling occurred concurrently with the evolving concept of Integrated Pest Management. Computer simulation modelling was a central organizational paradigm of the Huffaker project (Huffaker et al. 1976), a research initiative that to a large degree established the direction of IPM research in the US throughout the 1970's and early 1980's.

Although computer modelling has played an important conceptual role in the development of IPM programs, often the value of models in field application has fallen far short of original expectations (e.g. Fleming 1988, Stone 1989). The reasons for this disappointing result are complex indeed, and an adequate discussion is beyond the scope of this contribution. However, at least some of the reasons are related to the fact that models are typically developed by specialists who possess skills far different from those of the typical applied entomologist. There often seems to be little common ground for communication between the "modelers" on the one hand and the field entomologists on the other. The uncoupling of model development from field application has led to a widespread perception that computer models represent an esoteric technology with little "real world" importance. Because serious consequences could result from this lack of communication, considerable effort has been expended over the years in attempts to make sophisticated computer modelling technology more readily accessible to the computer non-sophisticate.

Development of both general and specific IPM modelling ''languages'' occurred early in the history of systems analysis applications in pest management (i.e. Logan 1979). These early applications, however, were severely constrained by computer hardware and software structures of the day. Operating systems were machine specific for particular vendors, and often even for models within vendors. Therefore, it was difficult to transfer computer programs from one machine to another and clearly impossible to transport compiled programs. In general, computers of the 1970's were difficult to interact with and intimidating to the non-specialist.

Recent advances have resulted in an environment that is conducive to change in the way in which computers are applied in both IPM research and IPM application. Many of the earlier problems were resolved by adaptation of industry-wide standards for computer languages and the emergence of flexible, machine independent operating systems such as UNIX or DOS. The unrelenting advances in computing power, accompanied by a plummet in computer costs, have also led to the widespread availability of inexpensive, but nonetheless powerful, computers. Last, and perhaps most importantly, the realization of concepts in artificial intelligence and expert systems has altered dramatically the way in which humans interact with computers. In this paper, I discuss the continuing evolution of a computing environment that attempts to capitalize on these new ideas in order to make sophisticated modelling capabilities available to applied entomologists.

SYSTEM DESCRIPTION

My earlier (Logan 1979) interest in the problem of automating IPM model development was rekindled by experiences as a visiting scientist in New Zealand. In 1983 I was awarded a Research Fellowship by the New Zealand government to work with Ministry of Agriculture and Fisheries (MAF) scientists at Christchurch, Canterbury, NZ. Specifically, MAF was interested in developing models to aid in grass grub and sitona weevil control, and in more general terms, evaluating the importance of modelling applications to New Zealand pest management. It soon became clear to me that the majority of my time was spent developing computer code that, while requiring a certain technical facility, was largely routine and repetitive of previous modelling work. At about the same time, artificial intelligence and expert systems were becoming newsworthy topics in the popular press. The utility of an expert system that would (1) make modelling expertise (that was a scarce commodity in New Zealand) more readily available and (2) free my time for more valuable (and interesting) endeavors was patently obvious. A preliminary system (PMDS/PHNMOD) resulted that combined a interactive user interface with elements of computer inference (Logan 1988). This system was developed on a VAX computer with UNIX playing an important part in system structure. It has since been rewritten as PC-DOS programs, and I will be concerned primarily with the PC version of the system.

PMDS-PHNMOD is composed of two computer programs. The first (PMDS for Pest Model Development System) analyzes data, makes choices about the most appropriate mathematical constructs for model representation, and estimates parameters for important functional forms. The second program is the simulation model itself (PHNMOD for PHeNology MODel). PHNMOD is a self- contained program that provides a generalized life-system

representation based on a temperature-driven phenological paradigm. Communication between these two programs is accomplished by a PMDS-produced file that contains key information used as the knowledge base for the PHNMOD simulation. Both programs are characterized by a menu-driven user interface that emphasizes graphical representation of; data, the results from data analysis, and the results from model simulation. Flexibility in application is maintained by providing both executable and source versions of PHNMOD. The system is made available on a "shareware" basis, in which it is anticipated that prospective users will either return the documentation and disks or remit $30.00 to cover expenses.

Phenology

Perhaps the most basic requisite of poikilothermic organism living in temperate environments is the maintenance of an appropriate seasonality. In addition to basic ecological considerations, most IPM programs require accurate prediction of pest phenology. Predicting the occurrence of a particular life stage is often essential for efficacious timing of a pesticide application. Knowledge about both pest and beneficial insect phenology may also be critical for avoiding potentially disruptive chemical applications. Recognizing that phenology is basic to IPM, and that developmental processes are the basis of insect phenology, a major portion of PMDS is devoted to the analysis of temperature dependent developmental data.

The physiological basis for insect phenology is the temperature dependent developmental rate curve. Typically, developmental rate models are parameterized from a series of constant temperature experiments performed under controlled environmental chamber conditions. From these fixed temperature experiments, if certain assumptions are made, the model can be used to predict development for any arbitrary temperature cycle through application of Eq. 1.

$$D_i = \int_{t_S}^{t_M} r[T(t)]dt \qquad (1)$$

where D_i is the developmental index or physiological age, r is developmental rate as a function of temperature (T), which is in turn a function of time (t).

PMDS either chooses an appropriate form for $r(T)$, or the program user can designate particular functional representations that the program will then attempt to fit to data. In either case, one of eight potential models can be used to describe the developmental rate curve. An inferential knowledge base is used by PMDS to determine which potential developmental rate curve is most appropriate for a particular life stage. This knowledge base contains information regarding (1) the physiological nature of developmental processes in poikilothermic organisms (2) information about the ecological/environmental circumstances under which the model will most likely be applied and (3) statistical information that relates the particular data set to the mathematical forms of the developmental rate functions (Logan 1988). Parameterization is accomplished by application of Powell's least squares algorithm (Powell 1965). Developmental rate model selection is conceptually designed to choose the best possible rate model given the existing data constraints

and the proposed modelling application. In practice, performance of PMDS appears to rival that of a human expert performing the same tasks. Lack of human intuition and insight are counterbalanced by the pedantic attempt to fit a wider variety of models than would typically be attempted by the human expert.

Depending on the availability of adequate data, PMDS can build a model that includes variation in developmental rates. The procedure used is that of the "same shape" principle of Sharpe et al. (1977). The program attempts to fit three flexible cumulative probability functions to both the normalized distributions of developmental rates and developmental times. The best of these six potential distributions models is determined by the adjusted coefficient determination (Kvalseth 1985). PMDS is capable of building a mixed model, with some life stages including the distribution of individual developmental rates, and others represented solely by the median individual.

Once the stage specific developmental rate models are determined, population demography is modeled by a cohort representation of the life cycle (Curry et al. 1978, Logan 1979, Shaffer and Gold 1985, Wagner et al. 1985, Logan and Amman 1986). A cohort is defined as all individuals in a particular life stage of approximately the same chronological age. In practice, this reduces to the individuals that endured a particular life stage during the same time step. Two pieces of information are maintained for each cohort, the current number of individuals in each cohort (n) and their median physiological age (a^*, defined as the summation of physiological time t^* since initiation of the cohort). The updating process for the cohort scheme is diagrammatically depicted in Fig. 1, computational details are provided in either Logan (1988) or Logan and Weber (1989). Implementation of the cohort updating scheme depicted in Fig. 1 results in a "sojourn time" model. Scaalje and van der Vaart (1989) discuss the relative strengths of this modelling approach.

Mortality

Mortality is clearly an important demographic process. A flexible way to model mortality is in particular critical to IPM models. Many key IPM questions involve catastrophic mortality resulting from control tactics such as pesticide applications. Mortality representation must provide the capabilities for (1) including catastrophic events and (2) multiple potential causes of mortality. PMDS includes these two capabilities by computing the conditional time-step probabilities of: (1) death, (2) remaining alive in the life stage, and (3) live emergence to the next life stage. A flexible basic modelling structure is provided by PMDS, and since source code is provided for PHNMOD, this structure can be modified by a user to more clearly represent a specific application. Once again, for computational details, the reader is referred to Logan and Weber (1989).

Recruitment

The final demographic function considered by PMDS/PHNMOD is recruitment. Since recruitment is highly dependent on the modeled species and environmental circumstances, PMDS/PHNMOD provides the bookkeeping structure for implementation of recruitment rules provided by the user. Initialization of a simulation run (in PHNMOD) is accomplished

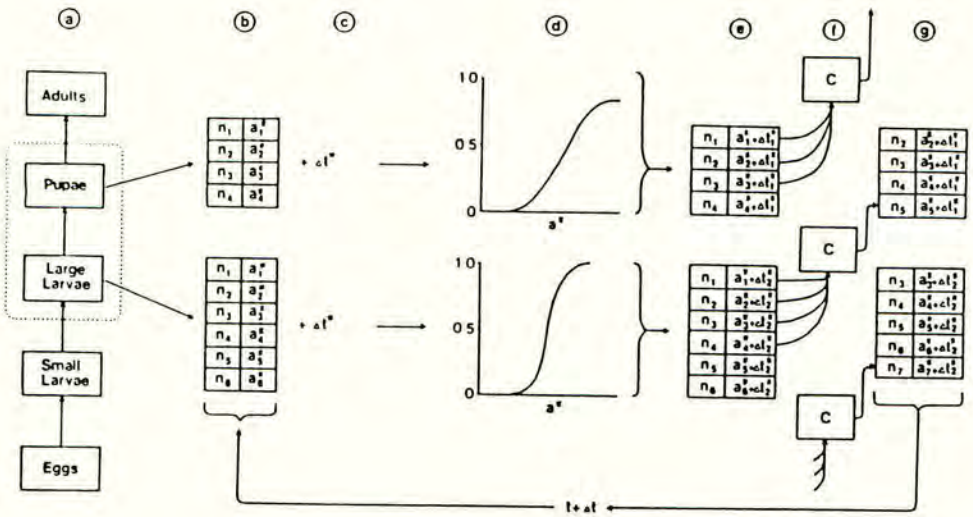


FIG. 1. Updating algorithm for a PMDS produced simulation of population phenology. The progression of population age structure is represented as (a) the flow of individuals through the various life stages. In order to accomplish this, each life stage is represented as (b) a vector of cohorts. Cohorts are updated over Δt by first computing Δt^* for each life stage, and (c) adding this to the a^* of each cohort. In (d), the stage-specific, cumulative emergence curve $[F^*(a^*)]$, is compared with (e) the updated physiological age. Depending on physiological age status, the proportion of each cohort that has completed the life stage is computed and in (f) a new cohort (c) is created from all individuals that have completed the previous life stage. Finally in (g), cohorts with $a^* \geq 1/A^*$ are dropped from the cohort vector and the summed emergence from the previous stage are included as the last entry in the cohort vector for the next life stage. The iterative nature of this process is indicated by the arrow from (g) to (b).

either through user supplied recruitment rules or by run-time initialization of life stage densities.

LESSONS LEARNED FROM APPLICATION

In general, the lessons learned from application of PMDS/PHNMOD fall into two categories, those that resulted from modification of the system to accommodate specific applications, and those of a more general philosophical nature that are independent of a particular application. I will first address those that resulted from application of the system to a specific problem.

Specific Applications

The first field application of PMDS/PHNMOD was in a Colorado potato beetle (CPB) IPM program headed by Dr. D. N. Ferro at the University of Massachusetts. Among the most important modelling goals of this program was evaluation of various pesticide application strategies. This application immediately focused on the mortality aspects of the model. In the original version of the system (as published in Logan 1988), the cohort structure was maintained by recording two attributes for each cohort, the initial number and the physiological age of the median individual. The number actually remaining in a cohort was computed from the crude probability of survival to the beginning of the time step. This method was efficient in computer time (it was unnecessary to update the actual number remaining in the cohort at each time step), but made it difficult to represent the catastrophic effect of a pesticide application. This situation was exacerbated by the need for an increasingly complex and realistic representation of an array of chemical and biological insecticides.

Effectively including complex pesticide effects required a basic change in model structure. In the revised PC version of the model, both survival and emergence are computed as probabilities conditioned on survival to the beginning of the time step. Computation of conditional rather than simple probabilities entailed a loss of computational efficiency (the actual number remaining in a cohort is updated at each time step) but resulted in an increased flexibility for representing ecological events. In effect, the revised mortality rule captures the complete history of a cohort by its current state. This rather simple change in program structure resulted in a vastly increased flexibility for representing mortality factors and other complex physiological processes such as diapause. The resulting model has become a central feature of Dr. Ferro's research and has led to publication of research dealing with basic CPB ecology (Voss et al. 1989), as well as control strategy evaluation (Ferro et al. 1989). The important lesson from this first application was the clear advantage of increasing ecological flexibility even at the cost of decreasing program efficiency. The validity of a general philosophy that maximizes program flexibility may seem patently obvious, but it actually represents a fairly subtle shift from the dated objectives of trying to optimize the efficiency of a specific program designed to perform a single task, to the more modern concept of designing object oriented programs useful for modelling the general characteristics of a broad class of phenomena.

A current application of PMDS/PHNMOD for modelling *Melanoplus sanguinipes* population dynamics has resulted in an illustration of the

power of the basic concepts of PMDS/PHNMOD as well as uncovered a specific ecological limitation in program design. The power of the concept was illustrated by the relatively smooth translation of an existing complex simulation model (Hilbert and Logan 1983) to a version that included the convenient user interface and powerful graphics capabilities of a PHNMOD model. However, an important limitation of PHNMOD became apparent when the original deterministic (life stages represented by the median individual with no variation in developmental processes) model was expanded to one that included variation in developmental processes. This deficiency involved the way in which diapause was modeled by Hilbert et al. A basic assumption of the bookkeeping algorithm in PHNMOD is the equivalency of individuals within a life stage. This condition is violated by the Hilbert et al. diapause model because an individual in the egg stage may simultaneously exist in two states, depending on the relative relationship between two developmental curves describing diapause development and embryogenesis. In order to overcome this limitation, PHNMOD was modified to include capabilities to stop and restart the simulation at any designated time step. It was then possible to link the PHNMOD model with a stochastic model that simulates diapause on an individual basis. Output from the diapause model is used to initiate spring emergence in the *M. sanguinipes* life systems model. The capabilities for convenient modification and/or linking PHNMOD models are facilitated because both the structure of PHNMOD and the source code (either FORTRAN or C) are provided with the system.

PMDS/PHNMOD is being used to analyze and model an increasingly wide array of insect life systems (e.g. mountain pine beetle, spider mites, etc.). Each of these applications raises specific modelling difficulties that must be overcome. However, the initial philosophy of PHNMOD was to provide computer code that was flexible and easy to interface with other simulation models. The entire system was designed to maximize flexibility and to capitalize on an object-oriented philosophy of system structure. Applications to new specific problems continue to provide insights into (1) new objects to included in the system (e.g. a distributed delay life-stage structure in addition to the rate summation algorithm of Fig.1) and (2) ways to increase both the flexibility and power of the system (e.g. continued development of flexible user interfaces, powerful graphics, and inference components to assist in the model development process).

General Principles

In addition to ideas gained through specific application of the system, more general principles are beginning to emerge. The first of these is that the basic idea of a model development "expert" system has apparently struck a responsive chord in the international entomological community. Although the availability of a PC version of the system has never been published in the open literature (the system described in Logan 1988 was specifically for a VAX computer operating under UNIX), I have received requests for over 50 copies of the PC version, including requests from over 10 foreign countries. Several overseas requests are from less-developed countries, a turn of events that is particularly gratifying. Application of expert-systems concepts on inexpensive, but nonetheless powerful computers, is particularly attractive in LDC environments where technical expertise and cash resources are both in short supply. The bottom line is that the concept at least seems to be

useful. The question that perhaps yet remains to be answered is: Is PMDS/PHNMOD a system that satisfies this need? The system has been made available for release on a cost-recovery basis. The expectation is that user feedback from the earliest stages of development will help guide evolution of the system in directions that more clearly meet the expectations of the user community.

One of the earliest lessons gained from application of the system was that sometimes the "expert's" concept of valuable inference does not coincide with that of the intended user. The original inferential capabilities of PMDS were designed to choose the most appropriate developmental model, given knowledge about arthropod developmental processes, ecological conditions, and data constraints. Through an early application in a teaching environment, it soon became apparent that entomology graduate students (at least) were rather more interested in making these "expert" decisions on their own. This particular user group did not wish to abdicate the responsibility of choosing a developmental model to an expert system but instead wanted to fit all possible models to data and then make the choice of appropriate models based on their own expertise. In other words, the appropriate division of labor was to place the drudgery (and in some sense technical expertise) of curve fitting on the system and to leave the scientific excitement of ecological reasoning to the user. I must admit that in my personal applications of the system I tend to follow the same strategy. PC's are now powerful enough for the intensive computing required to attempt fitting a large number of possible models to data. The evolving philosophy of PMDS/PHNMOD is to develop a system that provides powerful technical support capabilities that clearly enhance the ecological inference capabilities of pest managers.

Development of PMDS/PHNMOD has also led to interesting modelling research. Because the system assumes the burden of model development, it is possible to compare a relatively large number of competing model constructs. This has two distinct benefits. First, the best of a variety of representations can be chosen to model a particular insect species. Second, through a large number of applications, patterns begin to emerge that suggest one modelling approach may in general be superior to another.

Another example of modelling research that has resulted from PMDS/PHNMOD applications is evaluation of numerical techniques used to simulate basic processes. Two methods have become dominant for modelling variation in developmental processes. These are based on either distributed delays (e.g. Gutierrez, et al. 1984) or rate summation (e.g. Sharpe, et al. 1977, or Regniere 1984). The choice of a particular method is more likely related to chance events, such as where one took graduate training, than due to any compelling ecological or even computational reason. Although, both techniques clearly work, they are mathematically different and each approach has distinct numerical and analytical properties. There may well be reasons to choose one over the other for a given circumstance. This conjecture has led to extensive simulation research (Ravlin and Logan in progress) comparing these two approaches for a variety of ecological circumstances and computational goals. There are in fact different circumstances in which one approach is superior to the other, and PMDS is currently being expanded to include

the inferential basis to help determine which one is most appropriate for a particular application.

Along with the positive suggestions for improvement of the system that have been gained through interactions with users, inherent problems have also become apparent. Cost recovery is one such problem. PMDS/PHNMOD is supplied on a shareware, zero-profit-margin basis. The system is distributed on request with the provision that if the software is found to be useful, then the user will remit $30.00 US to cover costs of floppy disks, system documentation, and handling charges. There is, of course, no restriction on further duplication and/or distribution, except that it may not be done for commercial gain. If, on the other hand, the software is not found to be useful, return is requested. The response to this shareware approach has been less than gratifying. Less than 10% of people who have requested the system have responded with payment; none has returned the software package. Lack of response may be due to several reasons. The shareware notice is included in the Preface of the user's manual, and perhaps prospective users simply skip-over reading the Preface. It may be due also to the tradition of gratis technical support that has been supplied historically by land grant universities in the US. However, computer software in support of research seems to fall outside the realm of typical Extension activities. At any rate, I have yet to satisfactorily resolve the dilemma of making software widely available on the one hand and paying for this service on the other. Although commercial matters are typically distasteful for an academician, I have recently begun to include an invoice with copies of the system. Perhaps this dilemma will be resolved by being less subtle.

Another problem that is very much related to the academic setting is lack of recognition for software development when it comes to matters of promotion and tenure. The personal distribution of a user's manual and computer code does not even command a place in the grey literature, much less the refereed literature upon which academic recognition is based. As a result, even though the care and maintenance of a computer system such as PMDS/PHNMOD requires a considerable continuing investment of time and resources, the professional return on this investment is questionable. Writing a successful applications program can clearly become a mixed blessing. Realization of the promise that new technologies such as expert systems and artificial intelligence hold for IPM will require sensitivity on the part of research administrators to insure that contributions in nontraditional outlets receive the recognition that will insure continued application of these concepts.

FUTURE APPLICATIONS

PMDS/PHNMOD has resulted from the recognition that the same model structure was being applied to simulation of a diverse array of insect life systems. This realization led to an object-oriented approach that has demonstrated general value. However, through application to a wide variety of problems, it has become apparent that true utility requires maximizing the flexibility of the system This has resulted in a philosophical shift from the ideal of an expert system that could automatically design a model to that of a modelling environment that includes inferential capabilities at certain key points in the process of model development. The system is, therefore, becoming but one of an array of modelling tools that will be linked through an intelligent-user

interface. The interface will function as a navigator that helps to guide the user through a variety of modelling and data analysis tools. At the present time, we (in association with L. A. Weber) have developed tools to perform tasks that: (1) require technical expertise, such as curve fitting and assembly of computer code, (2) facilitate data interpretation through data base management and graphical analysis, (3) perform numerical analysis such as finding roots or integration of functions. All of these tools have a common feel in their user interface and graphics support. They also conform to the same input/output conventions. With the help of a flexible expert-systems shell, it is only a short step from PMDS/PHNMOD to the navigator concept.

REFERENCES

Curry, G.L., R. Feldman, and K.C. Smith. 1978. A stochastic model of a temperature-dependent population. Theoretical Population Biological 13: 197-213.

Hilbert, D.W., and J.A. Logan. 1983. A population systems model of the migratory grasshopper (*Melanoplus sanguinipes*), pp. 323-334. *In* W.K. Lauenroth, G.V. Skogerboe, and Flug (eds.) Analysis of ecological systems: State-of-the-art in ecological modelling. Elsevier, Amsterdam

Huffaker, C.B., R.F. Smith, A.P. Gutierrez. 1976. The need for systems analysis and its use in The US/IBP Integrated Pest Management Project. pp. 209-216 *In* R. L. Tummala, D. L. Haynes, and B. A. Croft, Modelling for pest management: concepts, techniques, and applications. Department of Entomology, Michigan State University, East Lansing.

Fleming, R.A. 1988. Difficulties implementing a modelling-based integrated pest management program for alfalfa. Memoirs Entomological Society Canada 143: 47-59.

Ferro, D.N., J.A. Logan, and R.H. Voss. 1989. Field applications of a simulation model for Colorado Potato Beetle (Coleoptera: Chrysomelidae). Environmental Entomology (submitted).

Gutierrez, A.P., J.W. Baumgaertner, and C.G. Summers. 1984. Multitrophic models of predator-prey intergetics. Canadian Entomologist 116: 923-963.

Kvalseth, T.O. 1985. Cautionary note about R^2. The American Statistician 39: 279-285.

Logan, J. A. 1979. SIMBUG: a pest management simulation language I. Maintenance and reference manual. Melanderia 31: 1-17.

Logan, J. A. 1988. Toward an expert system for development of pest simulation models. Environmental Entomology 17: 359-376.

Logan, J.A., And G.D. Amman. 1986. A distribution model for egg development in mountain pine beetle. Canadian Entomologist 118: 361-372.

Logan, J.A., and L.A. Weber. 1989. PMDS&PHNMOD: Insect simulation model development tool. Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg. 72pp.

Powell, J.D. 1965. A method of minimizing a sum of squares of nonlinear functions without calculating derivatives. Computer Journal 7: 303-307.

Regniere, J. 1984. A method for describing and using the variability in developmental rates for the simulation of insect phenology. Canadian Entomologist 116: 1367-1376.

Scaalje, G.B., and H.R. van der Vaart. 1989. Relationships among recent models for insect population dynamics with variable rates of development. pp. 309- 321 *In* L.L. McDonald, B.F.J. Manly, J.A. Lockwood, and J.A. Logan, Estimation and analysis of insect populations. Springer-Verlag, Heidelberg (in press).

Shaffer, P.L., and H.J. Gold. 1985. A simulation model of population dynamics of the codling moth *Cydia pomenella*. Ecological Modelling 30: 247-274.

Sharpe, P.J.H., G.L. Curry, D.W. DeMichele, and C.L. Cole. 1977. Distribution model of organisms development times. Journal Theoretical Biology 66: 21-28.

Stone, N.D. 1989. Knowledge-based systems as a unifying paradigm for IPM Proc. National IPM Symposium, Las Vegas, NV, USA (in press).

Voss, R.H., D.N. Ferro, and J.A. Logan. 1988. The effects of seasonal changes in reproductive and diapause activities on the population dynamics of the Colorado Potato Beetle (Coleoptera: Chrysomelidae) in Western Massachusetts. Environmental Entomology 17: 863-871.

Wagner, T.L., H. Wu, R. Feldman, P.H.J. Sharpe, and R.N. Coulson. 1985. Multiple-cohort approach to simulation development of insect populations under variable temperature. Annals of the Entomological Society America 78: 691-704.

5.
Posters

DNA RFLP (RESTRICTION FRAGMENT LENGTH POLYMORPHISM) ANALYSIS OF STRAINS OF THE ENTOMOPATHOGEN METARHIZIUM ANISOPLIAE.

N.D. PIPE, J.B. HEALE, B.W. BAINBRIDGE[1].

Plant Cell and Molecular Sciences Research Group, Kings College London, Campden Hill Road, London W8 7AH.

A.T. GILLESPIE.

Institute of Horticultural Research Littlehampton, Worthing Road, Littlehampton, BN17 6LP.

ABSTRACT

The black vine weevil, Otiorhynchus sulcatus, is an increasingly serious pest of ornamentals under glass and of strawberries, raspberries, blackcurrants and ornamentals grown outdoors in the U.K. and Europe. Effective biological control is possible with the entomopathogen Metarhizium anisopliae, eg. providing up to 93% mortality of O. sulcatus populations in glasshouse grown strawberries. Strain improvement (via parasexual crosses) involves attempts to increase pathogenicity and effective control. RFLP analysis provides a means of 'fingerprinting' or characterising wild-type Metarhizium isolates/strains, from differing geographical regions and insect hosts, as well as recombinants from such crosses. Starting with freeze dried, ground mycelium, total DNA was purified using a phenol/chloroform based extraction procedure, precipitating DNA with isopropanol. An hour long centrifugation at 13,000 rev/min removed high molecular weight polysaccharides, improving the restrictability of samples. Restriction endonucleases used separately for digests were EcoRI, BamHI and PstI, and resulting fragments were separated by agarose gel electrophoresis. Digests were transferred to nylon filters and fixed by baking at 80°C for 2 hours. The results from probing digests of M. anisopliae will be presented.

[1]Microbial Physiology Research Group.

SOME COMPLEMENTARY STUDIES OF BIOLOGICAL CONTROL WITH PSEUDAULACASPIS PENTAGONA (TARG. TOZZ.) IN GUILAN PROVINCE

A. HABIBIAN

Plant Pests and Diseases Research Laboratory of Guilan, P.O. Box 133, Anzaly, Iran.

H. BAYAT ASSADI

Plant Pest and Diseases Research Institute, Teheran, P.O. Box, 1454-19395, Iran.

ABSTRACT

In 1972, the white peach scale "Pseudaulacaspis pentagona appeared as a guaranteed pest on mulberry in Guilan province. Early investigations of biological control by the endoparasite Encarsia berlesei (Howard) (Aphelinidae) indicated that the rate of parasitism was around 30%.

The host, pest, parasite relationship is complex with the second nymphal instar of the white peach scale being more susceptible to attack by the parasite than the third instar. Our laboratory investigations show that parasitism of scale on mulberry branches is more than the level exhibited on other hosts such as potato or pumpkin. Low temperatures can cause high mortality of the parasite, 0 to 2°C for adults and -5°C for pupae and larvae. The number of eggs for each female parasite was recorded as being 20 to 30. White peach scale has three generations annually, the first begins in early May, the second around mid-July and the third in early September. Females overwinter as the third nymphal instar.

The ectoparasite "Aphytis sp." was also encountered in this study with 5% parasitism. Other natural enemies in this region are Aspidiotiphagus citrinus as parasite and Chilocorus bipustulatus L. as predator feeding on this harmful insect.

THE COMPATIBILITY OF THE ENTOMOGENOUS FUNGUS, METARHIZIUM ANISOPLIAE WITH HORTICULTURAL PESTICIDES

E.R. MOORHOUSE, A.T. GILLESPIE, E.K. SELLERS AND A.K. CHARNLEY[1]

AFRC Institute of Horticultural Research, Worthing Road, Littlehampton, West Sussex, BN17 6LP
1) School of Biological Sciences, University of Bath, Bath, BA2 7AY

ABSTRACT

The entomogenous fungus, Metarhizium anisopliae has shown potential for biological control of the black vine weevil (Otiorhyncus sulcatus, Curculionidae), a major pest of glasshouse and hardy ornamentals, and soft fruit. The commercial application of M. anisopliae will necessitate integration with other crop protection products, thus it is important to assess the compatibility of this fungus with horticultural pesticides. This has been examined in both the laboratory (in vitro) and the glasshouse.

In the laboratory, the fungicides chlorothalonil and zineb prevented spore germination when incorporated into Sabouraud dextrose agar (SDA) at the recommended rate. The other twelve fungicides and six insecticides examined had no effect on spore germination at the same rate. Mycelial growth of M. anisopliae on SDA plates containing; 0.1x, 1x and 10x the recommended rate of each pesticide was also studied. Fungal growth at the recommended rate was reduced by all the pesticides examined except propamocarb. Two of the fungicides; benomyl and carbendazim totally inhibited growth at all three rates. At 10x the recommended rate the fungicides; etridiazole, triforine and zineb and the insecticides; dichlorvos and hostathion also prevented growth.

In the glasshouse experiment, a 20 ml drench per pot of a spore suspension containing 10^7 M. anisopliae conidia per ml reduced the weevil population on Impatiens by 87%. This level of control was not significantly reduced by a subsequent pesticide application (at 16% egg hatch) of 20 ml per pot, at the recommended concentration. Larval control on pots treated with both M. anisopliae and the twelve fungicides and four insecticides examined, ranged from 82-98%. The insecticide diazinon (applied alone) reduced larval numbers by 100%. Two other insecticides; dichlorvos and cypermethrin and the fungicide pyrazaphos also reduced the weevil population by over 50%.

These experiments demonstrate the limitations of laboratory based in vitro screening programmes for the chemical compatibility of M. anisopliae. The implications of the results will be discussed.

DETECTION OF NERVE INSENSITIVITY TO CYPERMETHRIN IN RESISTANT AND SUSCEPTIBLE STRAINS OF *HELIOTHIS VIRESCENS*

R.T. GLADWELL

Department of Biochemistry & Physiology, School of Animal and Microbial Sciences, The University, Whiteknights, Reading RG6 2AJ.

ABSTRACT

The effect of cypermethrin on the spontaneous multiunit activity (MUA) of lateral nerves from a resistant (PEG87) and a susceptible (BRC) strain of *Heliothis viriscens* was examined *in vitro*. Third instar larvae (19-24 mg weight) of both strains were decapitated and the alimentary tract removed through a dorsal, midline, longitudinal incision. The preparation was bathed in a lepidopteran saline and the activity of the exposed lateral nerves was monitored by means of a suction electrode, amplified and recorded on magnetic tape for offline data analysis.

The effect of prolonged exposure (40 min) to trans-cypermethrin was examined in larvae from the susceptible strain. The response to the pyrethroid over a range of concentrations (10^{-8} to 10^{-6}M) was an initial increase in MUA followed by a decline to levels of activity below those observed in the pretreatment control period. The time course of these responses was dose-dependent, higher concentrations eliciting more rapid responses. The effect of high concentrations of trans-cypermethrin (10^{-6}M) on the MUA of larvae from a resistant strain was also measured. The response of these larvae was variable, some responded in a similar manner to the non-resistant larvae, some had a much smaller response while others were intermediate. These data clearly indicated the existence of a relative insensitivity of the nervous system to cypermethrin in some individuals of the PEG87 strain.

The variation in the neural MUA response to cypermethrin was further examined in the same preparation by exposing the nerves for successive 5 min intervals to increasing concentrations (10^{-9} to 10^{-7}M). The concentration at which a sustained increase in the MUA to at least five times the pretreatment control levels was recorded. Over 90% of the larvae of the BRC strain responded strongly to the lowest concentration of pyrethroid. The remaining individuals showed some resistance in that they responded only to the higher concentrations of cypermethrin. Preparations from larvae of the resistant strain showed a much more variable response. More than 25% of the larvae responded similarly to the non-resistant larvae, but the remainder demonstrated their resistance by only responding to higher concentrations. These data clearly demonstrate a difference in the sensitivity of the nervous system to cypermethrin between resistant and susceptible strains of *H. virescens*, as well as a considerable degree of variability in the degree of nerve insensitivity present in resistant individuals.

STUDIES OF THE ECOLOGY AND PREDATORY ABILITY OF THE HISTERID TERETRIOSOMA NIGRESCENS, A POTENTIAL BIO-CONTROL AGENT FOR PROSTEPHANUS TRUNCATUS.

D. P. REES

Storage Department, Overseas Development Natural Resources Institute, London Road, Slough, Berks, SL3 7HL, UK.

ABSTRACT

In the late 1970's, Prostephanus truncatus (Col.: Bostrichidae), a pest of erratic importance of farm-stored maize in Mexico and Central America, was accidentally introduced into Tanzania and a little later into Togo, West Africa. There it has become a pest of major importance of maize and dried cassava stored by subsistence producers and is spreading into neighbouring countries. Teretriosoma nigrescens (Col.: Histeridae) has been found closely associated with P. truncatus in farm-stores in Central America, but not in Africa, feeding on its juvenile stages. In farm-stored maize in Yucatan, Mexico, T. nigrescens was the most abundant and the only indigenous natural enemy present and was found only in stores infested with P. truncatus. Other natural enemies present were cosmopolitan species also known from Africa. Its presence may account for the lower pest status of P. truncatus in Yucatan than that observed in Africa. Significant populations of P. truncatus and T. nigrescens were also found in Yucatan living in forests, suburban gardens and sisal plantations far away from maize. The presence of the predator may offer the only practical way to control such pest populations which would be difficult or impossible to treat using conventional methods. Subsistence producers in both continents often accept as inevitable the presence of insects in their stored grain. In Africa, in spite of heavy losses, farmers are often reluctant, for financial, cultural and logistical reasons to shell maize and admix insecticides as currently recommended to control P. truncatus. If, by the introduction of T. nigrescens to Africa, losses due to this pest are reduced at least to Central American levels then a significant improvement to the agricultural economies of affected countries would be made, probably with minimal disturbance to traditional agricultural practices and customs.

EFFICACY OF SOME INSECTICIDES USED IN FILMCOATING OF CARROTS FOR CONTROL OF THE FIRST GENERATION OF CARROT FLY LARVAE (PSILA ROSAE F.)

A. ESTER AND J. NEUVEL

Research Station for Arable Farming and Field Production of Vegetables, 8200 AK Lelystad, The Netherlands.

ABSTRACT

Carrot crops can be damaged by the carrot root fly (Psila rosae F.), which can be a real problem for growers. Carrots growing with-out controlling the carrot root fly is impossible (Freuler et al. 1982). The carrot root fly in practice is controlled mainly by a soil application with insecticide and a crop spray later in the season.

Field experiments were carried out from 1986 to 1988 with the objective of achieving better control of the carrot root fly in the early season. The main aim was to examine the possibility of introducing insecticides into a filmcoating on the seeds (Halmer 1988). Using this method a saving can be obtained in the quantity of insecticides required. This is favourable from both the cost and the environmental viewpoint. Filmcoating of the seeds resulted in better protection of the crop from attack by the carrot root fly. Also, filmcoating leads to the possibility of pneumatic sowing and prevents the loss of fungicides and insecticides during transport and sowing.

Four different sites in the Netherlands were chosen having an extremely high population density of the carrot fly. The performances of carbo-furan, benfuracarb, chlorfenvinphos, furathiocarb, fonofos and bromophos in three doses in seed filmcoating were compared with conventional field application by spraying with chlorfenvinphos at 4 kg a.i. per ha. Chlorfenvinphos 25 gram a.i. per kg seed as a filmcoating in two different formulations and fonofos 25 gram a.i. per kg seed resulted in the same control level as the field application of 4 kg a.i. per ha. The insecticide fonofos, even at low concentrations, can give residue problems due to its persistence in the crop. Chlorfenvinphos 25 gram a.i. per kg seed does not exhibit these residue problems.

A STUDY OF THE BIOCHEMICAL NATURE OF PYRETHROID RESISTANCE IN THE TOBACCO BUDWORM (HELIOTHIS VIRESCENS) (F.)

E. J. LITTLE, A. R. McCAFFERY and C. H. WALKER,

Department of Pure and Applied Zoology,
School of Animal and Microbial Sciences,
University of Reading, Whiteknights, POBox 228,
Reading, RG2 2AJ.

ABSTRACT

A pyrethroid resistant (PEG87) and a susceptible (BRC) strain of the tobacco budworm (Heliothis virescens) were topically dosed at 3rd instar with trans and cis cypermethrin, each labelled with ^{14}C on the cyclopropyl or benzyl ring. BRC larvae were treated at a dose equivalent to an LD_5 for each isomer, while PEG87 larvae were treated at both an equal (LD_5 for BRC) and an equitoxic (LD_5 for PEG87) dose.

Penetration studies revealed that both cis (1-^{14}C cyclopropyl or first ring labelled ^{14}C benzyl) and trans (1-^{14}C cyclopropyl or first ring labelled ^{14}C benzyl) cypermethrin were absorbed at a faster rate into the susceptible strain of H. virescens as compared to the resistant strain over a 12h period. This effect was highly significant in PEG87 larvae treated with an equal dose. The times for 50% penetration in BRC larvae were 11h - trans cyclopropyl; 2h - trans benzyl; 5h - cis cyclopropyl and 3h - cis benzyl. The equivalent times for PEG87 larvae treated with an equal dose were 30h; 12h; 10h; 11h, respectively.

Studies on the metabolism of trans (1-^{14}C cyclopropyl) cypermethrin in both strains (BRC = 0.002µg/larva; PEG87 = 0.008µg/larva) indicated an increased rate of excretion in PEG87 larvae as compared to BRC larvae. The major metabolic product in the faeces of both strains was polar conjugated material, with PEG87 excreting more conjugate than larvae of the BRC strain. Analysis of the primary metabolites of trans (1-^{14}C cyclopropyl) cypermethrin present in the faeces, larval bodies and haemolymph of each strain indicates that the major route of detoxication is via a monooxygenase which leads to the formation of hydroxylated cypermethrin. Pretreatment with piperonyl butoxide (PBO) resulted in a reduction in the excretion of total radioactivity and conjugate from the PEG87 larvae. The absence of hydroxylated cypermethrin and an increases in the level of parent material in those larvae pretreated with PBO substantiates the importance of the monooxygenase system in the detoxication of cypermethrin in the resistant strain. Further studies on the metabolism of ^{14}C labelled trans benzyl, cis cyclopropyl and cis benzyl cypermethrin in BRC larvae and PEG87 larvae (treated with equal and equitoxic doses) will be reported.

THE PHARMACOKINETICS OF CYPERMETHRIN IN *HELIOTHIS VIRESCENS* (F.): TITRES OF PARENT COMPOUND IN NERVOUS TISSUE OF RESISTANT AND SUSCEPTIBLE LARVAE AT EQUAL AND EQUITOXIC DOSES.

I.J.WILKINSON and A.R.McCAFFERY

Department of Pure and Applied Zoology,
School of Animal and Microbial Sciences,
University of Reading, Whiteknights, PO Box 228,
Reading, RG2 2AJ.

ABSTRACT

Monitoring insect populations for resistant individuals is an essential tool for the efficacious and judicious use of pyrethroids, a group of compounds whose continued use is threatened by the development of this phenomenon. Such monitoring has previously relied on dose/mortality analyses, but the inadequacies of this method have initiated the search for rapid field bioassays for resistant insects. Logically, such bioassays should identify the mechanism of primary importance in determining the insect's tolerance of a particular compound. Thus a demonstration of the relative importance of resistance mechanisms is required. Such comparisons are, however, complicated by the incompatability of results of neurophysiological assays of nerve insensitivity and biochemical assays of detoxification enzyme activity, the two principle mechanisms for consideration in this study.

A quantitative pharmacokinetic approach, based on the following predictions, was used to resolve this dilemma:
(a) Total metabolic resistance will reduce the titre of pyrethroid reaching the nervous system (nerve titre) of resistant insects, so in comparison with susceptible insects the nerve titre should be lower at equal doses and similar at equitoxic doses. (b) Nerve insensitivity will allow a resistant individual to survive at the same nerve titre which kills a susceptible individual. Thus comparable nerve titres are expected at equal, but not at equitoxic, doses.

These predictions were tested by studying nerve titres of topically applied ^{14}C *cis*-cypermethrin in a resistant and a susceptible strain of third instar *Heliothis virescens* larvae at equal and equitoxic doses. The resistant strain is known to express both nerve insensitivity and metabolic resistance mechanisms, so judicious synergist applications were used to inactivate detoxification enzymes and hence quantify their effects on nerve titres. The final conclusions of this study will be presented.

TOXICITY AND SYNERGISM OF PYRETHROIDS IN VARIOUS LIFE-HISTORY STAGES OF THE TOBACCO BUDWORM (*HELIOTHIS VIRESCENS*)

A.R. McCAFFERY, E.J. LITTLE, H. EL-NAYIR, and J. HOLLOWAY

Department of Pure and Applied Zoology, School of Animal and Microbial Sciences, University of Reading, Whiteknights, Reading RG6 2AJ, UK

ABSTRACT

The toxicity of cypermethrin to resistant (PEG87) and susceptible (BRC) strains of *Heliothis virescens* was examined in various life-history stages. Foliar residue assays with formulated materials were used for first instar insects whilst topical applications of technical materials were made to the thorax of third instar insects and the eye of adults. At each stage the action of synergists which inhibit monooxygenase or esterase detoxication systems was examined.

Resistance factors of 1800 - 2100 to cypermethrin were found in neonate PEG87 larvae when compared to similar BRC larvae. Co-application of formulated piperonyl butoxide (PBO) and cypermethrin gave synergist ratios of around 13 to 17. This eliminated over 90% of the tolerance in these larvae suggesting a substantial involvement of monooxygenases in the resistance. Sub-lethal doses of OP compounds were poor synergists for cypermethrin.

Using third instar insects *cis*- and *trans*-cypermethrin were assayed separately and differences in their toxicity are presented. Extremely high resistance factors were obtained with the PEG87 strain. There was 200 to 600-fold synergism with PBO. DEF was an ineffective synergist for cypermethrin but co-adminstration of PB and DEF enhanced their action. In susceptible larvae both synergists were equally effective. Similar results were obtained with fenvalerate. It is likely that the monooxygenase system is a major resistance mechanism in third instar larvae of *Heliothis virescens*.

Resistance factors of around 250-fold were obtained with resistant moths. Females of both strains were more tolerant than males irrespective of the compounds being assayed. In the resistant strain synergist ratios of 9 and 14 were obtained with PBO for males and females respectively. This accounted for over 90% of the resistance suggesting that adults possess a resistance mechanism based on the monooxygenases.

The results of this work strongly infer that the use of compounds of the piperonyl butoxide type may have considerable value in the management of resistance in some strains of this insect.

ACTIONS OF CYROMAZINE ON INSECT CUTICLE

A.C.KOTZE AND S.E.REYNOLDS

School of Biological Sciences, University of Bath,
Claverton Down, Bath, BA2 7AY, UK.

ABSTRACT

Cyromazine (2-cyclopropylamino-4,6-diamino-*s*-triazine) is an insecticidal triazine that has insect growth regulator (IGR)-like effects.Its mode of action is unknown, but affected insects have been noted to display integumental lesions. Cyromazine is toxic to tobacco hornworm (*Manduca sexta*) caterpillars (LC_{50} ca. 5 mg kg^{-1} in artificial diet). Affected insects show reduced feeding and growth, impaired movement, a characteristic long and thin body shape, increased internal pressure within the body cavity, and ultimately lethal integumental lesions. Measurement of mechanical properties of loops of the body wall reveals that the integument is stiffer (ie less readily extensible) in cyromazine-treated insects than in controls. This increased stiffness is maintained in cuticle deprived of epidermal cells and soaked in buffer, indicating that the change is intrinsic to the cuticle itself. The cuticle's increased stiffness implies an increase in the extent of macromolecular interactions within it. In an attempt to define further these interactions we found that cyromazine treatment produced no change in the rate of incorporation of [^{14}C]-N-Acetylglucosamine into chitin nor of [^{3}H]-glycine into protein. SDS polyacrylamide gel electrophoresis revealed no differences in SDS-soluble proteins, either for total protein or for newly synthesised [^{3}H]-glycine labelled proteins. Cuticle content of total protein (per weight of cuticle) was unchanged although extraction of cuticle protein using a series of solvents (H_2O; 0.5M KCl; 2% SDS; 2M NaOH) showed the proportion of H_2O-soluble protein to be slightly increased while SDS-soluble protein was slightly decreased in treated insects. Amino acid analysis of whole cuticle revealed no marked differences between cyromazine-treated and control insects.These results show that the basic macromolecular composition of the cuticle is unaffected by cyromazine. Our working hypothesis is that cyromazine stiffens the cuticle by directly or indirectly inducing the formation of cross-links between existing cuticular macromolecules. The nature of these cross-links remains elusive.

STUDIES IN THE MANAGEMENT OF MAIZE INSECT PESTS USING SEVERAL INSECTICIDES DURING WINTER IN EAST UTTAR PRADESH, INDIA

PARAS NATH AND SUBASH CHANDRA

Department of Entomology and Agril. Zoology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi 221 005 (India)

ABSTRACT

Maize is grown predominantly during the rainy season in India but its cultivation throughout the year is increasing. The yields realised in various parts of the country during the winter season suggests that it might be possible to achieve the higher yields characteristic of the USA and European countries. The cultivation of maize on a more continuous basis has added new dimensions to insect pests attack. The magnitude of pest appearance and the severity of infestation in maize have resulted in the use of different methods of pest control. The maize growers of East Uttar Pradesh are using insecticides to check the insect damage during the winter season. To assist in the selection of products, 13 insecticides were screened in three different schedules, preventive, preventive + curative, and curative. The objective was to reduce the pest incidence and number of insecticide applications while still achieving the higher yields desired during the winter season. The study reveals that chlorpyriphos, monocrotophos, carbofuran, lindane and phorate were the highly effective insecticides in reducing the population of Mythimna separata Walker, Atractomorpha crenulata F., Marasmia trapezalis Guen. and Carpophilus sp. The insecticides applied under the preventative + curative schedule proved better than the curative schedule of application alone. Insecticide applied as a preventive measure proved to be the least effective.

TECHNOLOGY FOR MOLECULAR CLONING OF VIRULENCE GENES IN THE FUNGAL ENTOMOPATHOGEN METARHIZIUM ANISOPLIAE

L. BERNIER, I.C. PATERSON, R.M. COOPER, A.K. CHARNLEY AND J.M. CLARKSON

School of Biological Sciences, University of Bath, Claverton Down, Bath, BA2 7AY, U.K.

ABSTRACT

The Deuteromycete Metarhizium anisopliae parasitises over 200 insect species and is already used as a mycoinsecticide against several pests of agricultural crops. However, the potential for rational strain improvement is limited by ignorance of the molecular and biochemical basis of virulence and by the absence of a sexual cycle in this fungus. Our group has recently identified a serine protease (Pr1) as a virulence factor for M. anisopliae. Cloning and manipulation of the gene encoding Pr1 may lead to a better understanding of virulence in M. anisopliae, as well as to the production of more virulent pathotypes. The availability of a gene transfer system is a prerequisite for molecular studies of this type. We have successfully transformed protoplasts of M. anisopliae to benomyl resistance by using the cosmid vector pSV50 which contains a benomyl-resistance gene from the Ascomycete Neurospora crassa. Transformation of intact conidia with pSV50 was also achieved, at a higher frequency, by electroporation under high electric field strengths ($\geq$7 500 V/cm). All the transformants tested were stable and had arisen through integration of the cosmid in the host chromosomal DNA. The transformants also retained the ability to infect and kill test insects, fifth instar larvae of Manduca sexta. The benomyl-resistant phenotype persisted in reisolates from insect cadavers. Genomic and cDNA libraries from a wild-type strain of M. anisopliae have also been constructed in the lambda replacement vector EMBL3 and the expression vector λgt11, respectively. The libraries are currently being screened with molecular probes specific for Pr1. The development of an efficient gene transfer system and the identification of specific cloned DNA sequences will thus allow us to investigate the molecular basis of virulence in M. anisopliae.

A SIMPLIFIED APPROACH TO RESISTANCE TESTING FOR BEETLE PESTS OF STORED PRODUCTS IN THE FIELD

R. W. D. TAYLOR

Overseas Development Natural Resources Institute,
Storage Department, London Road, Slough, SL3 7HL

ABSTRACT

The increasing cost to chemical companies of developing and obtaining clearance for new insecticides, renders it essential that compounds in present use remain effective for as long as possible. Detection of resistance to contact insecticides at an early stage, can assist in the planning of pest mamagement strategies to overcome or delay the onset of resistance. An investigational programme at ODNRI, has shown that it is possible to adapt for field use, the standard FAO method of detecting resistance which employs filter papers treated with a discriminating dose of insecticide. Treated papers sealed in alumunium foil and a polythene envelope, retain their effectiveness for a period of at least six months when stored at 5^{o}C. To render the method more portable, perspex rings are used to confine insects on filter papers, rather than the more fragile glass variety usually employed in the laboratory. The original method for detecting resistance to contact insecticides described discriminating doses only for malathion and lindane. It has therefore been necessary to establish discriminating doses for additional insecticides now commonly used to protect stored products. It is hoped that the introduction of a field test method may result in much wider screening for resistance than hitherto, especially in developing countries.

WHAT IS DEATH ?

M.P. BRODERICK & L.D. LEAKE

School of Biological Sciences, Portsmouth Polytechnic,
King Henry I St., Portsmouth PO1 2DY.

ABSTRACT

Insects treated with pyrethroid insecticides exhibit two characteristic toxicological symptoms: knock-down and death. Numerous studies have attempted to correlate these symptoms, when whole animals are dosed, with the actions on isolated nervous systems; but with limited success. In this poster, we demonstrate a technique for long-term recording which uses an intact insect preparation on which simultaneous recordings can be made of both toxicological and neurophysiological effects. Continuous recordings of nerve activity were made from restrained 6th-instar larvae (c. 200mg wt) of *Spodoptera littoralis* during poisoning. Larvae were sampled sequentially from batches of 11 insects treated with a range of doses of cypermethrin (82ng - 540 ug/ insect); if nerve activity in one larva ceased, another one from the same batch was then used. Toxicological symptoms of the batch were noted at the same time, and also observed in replicate batches dosed with the same concentration.

Seven categories of toxicological symptoms were seen, viz: (1) hyperactivity, (2) re-gurgitation, (3) inco-ordination, (4) convulsions, (5) ataxia (largely immobile but mouthparts and legs moving), (6) semi-paralysis (apparently immobile but will move slightly if prodded) and (7) death. At lower doses (<740 ng/insect, i.e. ≏ LD95 and below), where recovery could occur, larvae could pass through poisoning to stage 6 and appear to be 'dead' before eventually recovering. Cypermethrin induced increases in nervous activity followed by repetitive firing; such activity continuing through stage 6 of the poisoning symptoms. Using doses of LD95 and above, this activity could be detected up to 5 days after initial dosing. At this time the animals had lost large amounts of body fluid (50-60% body wt) and appeared to be moribund, but were not actually dead as they would move slightly when prodded. At the highest dose (540ug/insect), death took longer to occur than at lower doses: more convulsions and less regurgitation (and, therefore, less water loss) were seen, suggesting that onset of death may be related to water loss. Actual death took much longer than many bioassay experiments suggest. Nevertheless, when the animal finally dies all activity in the nervous system has ceased; this, therefore, may be the best definition of death.