Posters

Using plant secondary metabolites to engineer enhanced crop protection - progress with two contrasting approaches.
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Plants produce a very wide variety of so-called "secondary metabolites", many of which function in protecting the plant from pests or diseases. Two very different classes of compound, with quite different modes of action, are being studied at the biochemical and molecular level, with a view to exploiting them for the protection of crop plants.

Brassicas, and some other plants, contain glucosinolates: S- and N-containing compounds derived from amino acids. Glucosinolates are degraded when plant tissues are disrupted, to release isothiocyanates and other noxious products. However, some of these breakdown products are used by specialist pests and pathogens to identify the host plant. The biochemistry of glucosinolate synthesis in oilseed rape is being studied to identify the key regulatory steps, and those which control the final spectrum of stored compounds. Knowledge of these enzymes and their regulation will allow strategies to be devised for engineering the glucosinolate content of oilseed rape, to enhance defence against non-specialist pests and disrupt host recognition by specialist organisms. There is also a need to engineer the glucosinolate content of seeds, to enhance the value of seed meal as an animal feed without compromising the resistance of the young seedlings to infection and herbivory.

The glucosinolate system is not passive, but changes during development (Doughty *et al.*, 1991) and responds to stress, insect attack, or fungal infection. Glucosinolate synthesis increases markedly under such conditions, and the speed and extent of this response varies between varieties (Porter *et al.*, 1991, Wallsgrove *et al.*, 1993). Certain abiotic elicitors mimic this response, and this may be a way of enhancing resistance to attack or infection. This defence response also has implications for S and N nutrition and amino acid supply.

The first steps in glucosinolate biosynthesis are catalysed by monooxygenases, converting amino acids to N-hydroxyamino acids and then oximes. Several enzymes have been identified, each apparently with a strict substrate specificity, and these have some characteristics comparable to the flavin-linked monooxygenases found in animal tissues (Bennett *et al.*, 1993). This similarity is being exploited to identify the genes coding for these enzymes, helped by the availability of synthetic rape lines lacking one or more of the enzymes. These substrate-specific monooxygenases are likely to control the entry of amino acids into the pathway, and are thus prime targets for altering the spectrum of glucosinolates in a plant or tissue. Other enzymes in the pathway with potential roles in regulation are also being investigated, particularly those linked to sulphur metabolism.

A quite different approach is being taken with the iridoid monoterpenes found in catmint, *Nepeta racemosa* (formerly *N.mussinii*). This plant makes a monoterpene (nepetalactone) with the same stereochemical configuration as the sex pheromone of the hop aphid (*Phorodom humuli*), albeit in a different oxidation state. We have investigated the biosynthesis of nepetalactone, and identified three enzymes involved in the conversion of geraniol to the end product. The genes coding for these enzymes are being cloned, for use in heterologous expression systems and in enhancing terpene synthesis in catmint tissue cultures. Either system can then be used for large-scale production of nepetalactone for reduction to the lactol and subsequent use in field traps for the aphid (an approach which has already been successfully field tested).

A partial clone of the gene for the first enzyme (a cytochrome P450 - Hallahan

et al., 1992) has been isolated, and a *Nepeta* leaf cDNA library is being screened for the full-length gene. Pure protein of the second, an alcohol dehydrogenase, has been prepared, and antibodies are being raised and partial amino acid sequences determined. Using information from this the cDNA library is being screened for the appropriate gene. A more direct approach is also being used, screening for monoterpene alcohol dehydrogenase activity in bacteria and yeast transformed with the *Nepeta* cDNA library. The third enzyme, a monoterpene cyclase, has been identified in tissue extracts, and purification and characterisation of this enzyme is in progress. Yeast and bacterial expression systems are being evaluated for over-production of all these enzymes, both for detailed biochemical studies of the proteins and for potential use in nepetalactone production.

The aim of all this work is to understand the biochemical pathways involved in the synthesis of these potentially useful secondary metabolites. Such an understanding will be used to direct manipulation of the expression of relevant genes, either in the crop plant (oilseed rape and other Brassica crops) or in culture and fermentation systems that can be used for industrial scale production (nepetalactone). In the first case, a more resistant crop will be produced requiring lower inputs. In the second, an environmentally friendly chemical will be produced, with very species-specific action, to control a pest which is becoming increasingly insecticide-resistant. The same general strategy may also be used against other aphids, by chemical manipulation of the core iridoid monoterpene.

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Molecular Genetic Analysis of Fungicide Targets in the Maize Smut Pathogen, *Ustilago maydis*

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The fungal plant pathogen, *Ustilago maydis* (DC) Corda offers many advantages as a model organism for studying the molecular basis of inhibitor action. It is amenable to both biochemical and classical genetic studies, and, more recently, to techniques for analyzing and manipulating genes. It is now possible routinely to move DNA into and out of *U. maydis* cells and to gain expression of the genes transferred into the cells. Furthermore, allele targeting and gene inactivation techniques serve to enhance the value of this pathogen for molecular genetic investigations. These developments in fungal gene technology are of particular relevance to fungicide research because not only can genes encoding known inhibitor targets be identified and manipulated but new antifungal targets can be evaluated by studying the effect of null mutations on growth and pathogenicity.

Two examples which demonstrate the utility of *U. maydis* for studying the molecular basis of inhibitor action are presented.

The first concerns the identification of a gene, *oxr*-1B , (Georgopoulos *et al.*, 1972, 1975) conferring resistance to the carboxamide fungicide, carboxin. This gene was isolated by transferring a plasmid genomic library, containing the resistance gene, into a carboxin-sensitive *U. maydis* strain and then selecting transformants that exhibited enhanced resistance to carboxin (Keon *et al.*, 1991). The carboxin resistance gene was identified as an open reading frame of 885 bp. Comparison of the amino acid sequence derived from this sequence with data base accessions revealed significant identity between the *U. maydis* carboxin resistance gene product and the iron-sulphur subunit of succinate dehydrogenase from a number of other organisms. Succinate dehydrogenase is the established target for carboxin action (White & Georgopoulos, 1991). Comparison of the deduced sequence of an iron-sulphur protein subunit from a carboxin-sensitive *U. maydis* strain with that of the carboxin-resistant form, revealed a single amino acid difference. This mutation occurred within a cysteine-rich region of the protein, and involved the replacement of a histidine residue in the carboxin-sensitive form by a leucine in the carboxin-resistant form (Broomfield & Hargreaves, 1992). This region of the protein is associated with a high energy iron-sulphur centre and carboxin is known to inhibit succinate dehydrogenase activity by preventing the reoxidation of this centre (Ackrell *et al.*, 1977). Resistance to carboxin may, therefore, be explained by a conformational change within this iron-redox centre, which allows electron transfer to occur in the presence of carboxin.

The second example centres on the *ERG2* gene. This gene encodes $\Delta^8{\to}\Delta^7$ sterol isomerase, an enzyme of the ergosterol biosynthesis pathway. This enzyme is thought to be an important target for the morpholine group of sterol biosynthesis inhibitors (Baloch *et al.*, 1984). However, disruption of the yeast *ERG2* gene is not detrimental to either the growth or the viability of this organism (Ashman *et al.*, 1991). This finding, therefore, casts doubt on $\Delta^8{\to}\Delta^7$ sterol isomerase being a primary site for the action of morpholine fungicides. We are attempting to clarify the position of this enzyme as a fungicide target in plant pathogenic fungi. To do this we have isolated the *ERG2* gene

from *U. maydis* and from the rice blast fungus, *Magnaporthe grisea* (anamorph: *Pyricularia oryzae*). The identity of the *U. maydis* gene was confirmed by complementation of a *U. maydis* mutant (*Erg2*) defective in sterol $\Delta^8 \rightarrow \Delta^7$ sterol isomerase activity (Bailey *et al.*, 1992). The amino acid sequence derived from both these genes share a high degree of similarity to the yeast *ERG2* gene product (*U. maydis* - 64.8%, *M. grisea* - 65%). Gene disruption and allele replacement are now being used to determine whether a null mutation within the *ERG2* gene affects either growth or pathogenicity of these pathogens.

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Development of *Lacanobia oleracea* resistant transgenic tomato plants.

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The aim of this work is to create plants which are resistant to attack by invertebrates through *Agrobacterium*-mediated genetic modification. Specifically, we intend to modify genetically *Lycopersicon esculentum* (tomato) so that it has increased resistance to the tomato moth (*Lacanobia oleracea* (L); Lepidoptera: Noctuidae). The tomato moth is a polyphagous glasshouse pest which is capable of causing severe damage to tomato crops (Lloyd, 1920). The effective use of contact insecticides reduced the economic importance of this pest. However, the recent trend towards the use of biological agents for pest control is not compatible with the concurrent use of chemical insecticides. Therefore, the use of contact insecticides has declined as effective biological control agents have become available. It is envisaged that the tomato moth may once again become a serious pest of glasshouse crops. Under these circumstances, control of *L.oleracea* may have to rely on the use of parasitoids, genetically modified organisms, such as Baculoviruses and *L.oleracea*-resistant tomato plants, or a combination of these control methods.

Insect resistance in transgenic plants, was first achieved by expressing DNA encoding highly selective insecticidal proteins (Dulmage, 1981) from *Bacillus thuringiensis* (*B.t.*). Tomato, tobacco, cotton and maize plants containing the *B.t.* toxin gene exhibit tolerance to caterpillar pests in laboratory tests (Fischoff *et al.*, 1987; Vaeck *et al.*, 1987). Tomato plants transformed with a *B.t* endotoxin gene were found to have increased tolerance of *Manduca sexta*, *Heliothis virescens* and *Helicoverpa zea* lepidopteran larvae. Analysis of the field performance of these tomato plants (Delanney *et al.*, 1989) has found that they maintained this resistance to *M.sexta* and *H.zea*, and were significantly resistant to attack by *Keiferia lycopersicella* (tomato pinworm). However, reports of broad-spectrum resistance to *B.t* toxins are beginning to emerge (Gould *et al.*, 1992), which indicates the need to consider alternative gene products for crop protection. There are several naturally occurring mechanisms for plant protection, which confer resistance to a wide range of pests. One such mechanism involves the production of proteinase inhibitors. Levels of the trypsin inhibitor of cowpeas (*Vigna unguiculata*) have been found to correlate with field resistance to their major pest, *Callosobruchus maculatus* (Hilder *et al.*, 1987). Cowpea trypsin inhibitors (CpTIs) are small polypeptides (ca. 80 amino acids) belonging to the Bowman-Birk group of double-headed protease inhibitors. Gatehouse and Boulter (1983) found that these CpTIs were effective anti-metabolic agents in *Heliothis*, *Spodoptera*, *Diabrotica* and

Tribolium. Expression of a CpTI encoding gene in transgenic tobacco plants confers resistance to *H.virescens* (Hilder *et al.*, 1987).

We have obtained permission from the Agricultural Genetics Company , Cambridge, UK., to use the CpTI encoding gene to modify tomato plants. It has not been demonstrated that the CpTI gene can confer increased tolerance to attack by *L.oleracea*. Preliminary results will be presented in this poster. If these CpTI transgenic tomato plants are found to have increased tolerance to attack by *L.oleracea*, then they will be used as a positive reference standard with which to compare other potential *L.oleracea*-resistance genes which we are in the process of isolating.

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CLONING OF A HOUSEFLY SODIUM CHANNEL GENE LINKED TO PYRETHROID RESISTANCE.

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The voltage-gated sodium channel is an integral membrane protein which generates the rising phase of action potentials in excitable cells by creating a sodium ion selective pore in response to changes in the transmembrane electric field (Hille, 1984). It is also the major target site for pyrethroid insecticides and DDT which exert their toxic effects by modifying the normal function of the channel through an alteration in the gating kinetics and a dramatic slowing of inactivation (Soderlund & Bloomquist, 1989). This is manifest as hyperexcitability, bursts of action potentials and nerve blockage, the symptoms varying according to the pyrethroid, nerve preparation and species. The mechanism by which this is achieved and the binding site for pyrethroids on the channel are not known.

The intensive use of pyrethroids over the last 20 years has led to the development of resistance in many insect species, which is often associated with a reduced sensitivity of the nervous system to these toxins (Georghiou, 1990). This form of resistance was originally described in the housefly and termed knockdown resistance (*kdr*). Several alleles of *kdr* have since been identified including the *super-kdr* factor, which confers up to 250-fold resistance to pyrethroids such as deltamethrin (Farnham *et al.*, 1987). Pharmacological studies involving the sodium channel ligands veratridine and batrachotoxin have indicated a reduced affinity for deltamethrin in these resistant strains (Pauron *et al.*, 1989), suggesting that resistance is conferred by alterations in the sequence/structure of the sodium channel protein that reduce its affinity for pyrethroids.

Recent advances in the cloning of sodium channel gene sequences from vertebrates and *Drosophila* has provided the opportunity to study the corresponding sequences of susceptible and *kdr*-type insects in order to identify the changes that confer insensitivity. The situation is complicated however, by the discovery of two separate sodium channel-like genes in *Drosophila*; *para* located on the X-chromosome and DSC1 (*sch*) on chromosome 2 (Loughney *et al.*, 1989, Salkoff *et al.*, 1987). Both show sequence homology to vertebrate sodium channels, but only the *para* gene has known physiological function based on the analysis of mutants associated with this locus. We have therefore focused our efforts on the cloning of *para*-like gene sequences from the housefly genome. This was achieved by low stringency heterologous probing of a housefly adult head library using a *para* cDNA probe (Williamson *et al.*, 1993). The first clone recovered from this library, pSCP2, contained sequences encoding the domain IV and C-terminal regions of a sodium channel and showed a striking similarity to the corresponding region of the *para* sodium channel protein (99% identity). This compared with only 52% identity to the same region of the predicted *sch* protein. pSCP2 therefore contains part of a sodium channel gene, equivalent to the *Drosophila para* gene, which we have designated *Msc*.

Restriction length polymorphisms (RFLPs) were detected at the *Msc* locus in susceptible, *kdr* and *super-kdr* housefly strains and exploited to show that this gene is involved in resistance (Williamson *et al.*, 1993). The RFLPs were identified using Southern blots of *EcoR*1-digested DNA and

are readily detected in the DNA from single flies, allowing their use as molecular markers for the *Msc* sodium channel gene. Linkage between the *Msc* RFLPs and resistance, as determined by topical bioassay with a discriminating dose of DDT, was assessed in the offspring of controlled crosses between the susceptible and resistant strains. The results indeed showed tight linkage between the *Msc* gene and resistance for the *kdr* and *super-kdr* genotypes, providing the first clear genetic evidence that resistance is associated with the sodium channel (Williamson *et al.*, 1993).

Our work is now directed towards the complete cloning and sequence analysis of the coding regions of this gene from both susceptible and *kdr* flies in order to identify the alterations that confer resistance. The sodium channel is a 260kD protein comprising a single polypeptide chain of around 2000 amino acids and therefore has a coding requirement of 6kb. Since the *Drosophila para* gene is known to have a complex organisation with more than 25 introns spread over 60kb (Loughney *et al.*, 1989), we are using cDNA cloning techniques to generate a series of overlapping clones that covers the full 6kb coding sequence of the *Msc* gene. Good progress is being made in this objective and cDNA clones covering over 4kb of channel coding sequence have now been mapped and sequenced. Corresponding sequences are being amplified directly from the resistant strains using polymerase chain reaction (PCR) techniques and have revealed differences between susceptible, *kdr* and *super-kdr* genes. The functional significance of these mutations can be assessed using *in vitro* expression systems once the full coding sequence of the wild-type gene is available. The identification of the alterations that cause resistance will not only provide fundamental information on the molecular basis of resistance, but will also highlight the region(s) of the channel that interact with pyrethroids and so offer new insights into their molecular mode of action.

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BIOCHEMICAL AND MOLECULAR ANALYSIS OF MALATHION RESISTANCE IN CULEX TARSALIS AND LUCILIA CUPRINA

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ABSTRACT

Malathion resistance and the role of malathion carboxylesterase (MCE) has been investigated in the mosquito *Culex tarsalis* and in the Australian sheep blowfly *Lucilia cuprina*. An esterase gene implicated in organophosphate resistance in other mosquito species is not amplified in our malathion resistant strain. Resistance is due to a qualitatively altered MCE in resistant mosquitoes, but in blowflies, it is due to a quantitative change in MCE.

The development of insecticide resistance poses a serious threat to our efforts to control pest insect populations. Resistance to organophosphates has been associated with high levels of esterase activity in the aphid *Myzus persicae* and the mosquitoes *Culex quinquefasciatus* and *C. pipiens*. The overproduction of esterases, which account for up to 3% of the total soluble protein in aphids and 6-12% of the protein in mosquitoes, is due to the amplification of a particular esterase gene in each species (see Devonshire and Field, 1991). In malathion resistant (R) strains of the mosquito *C. tarsalis* and the Australian sheep blowfly *Lucilia cuprina* resistance is due to an increased malathion carboxylesterase (MCE) activity. As suggested by the analysis of organophosphate-resistant aphids and other mosquito populations, increased MCE activity in our resistant pest species similarly may be due to gene amplification. To test this hypothesis we have initiated a biochemical and molecular analysis of malathion resistance in these species.

The *C. tarsalis* R strain is 150 times more resistant to malathion and has a 20-fold higher MCE activity than the susceptible (S) strain. Attempts were made to identify amplified DNA sequences in the R strain by the in gel renaturation technique (Roninson,1983). Although this method has been used successfully to identify amplified DNAs in *C. quinquefasciatus*, *C. pipiens* and *Musca domestica* (Mouches *et al.* 1985), greatly amplified fragments unique to the R strain were not observed in our mosquitoes. Because there are up to 250 copies of the esterase B1 gene in *C. quinquefasciatus* R insects, the B1 cDNA clone (pEst5; kindly provided by C. Mouches) was hybridized to Southern blots of R and S *C. tarsalis* genomic DNA. DNA which hybridized to the heterologous esterase B1 cDNA was not amplified in the *C. tarsalis* R mosquitoes. This cDNA was also used to isolate the corresponding cDNA from a *C. tarsalis* R strain library. The resulting clone Bif (likely esterase B3) showed 91% amino acid identity with the *C. quinquefasciatus* esterase B1. An estimation of Bif esterase gene copies on Southern blots in both *C. tarsalis* strains gives the same low number. Again, the gene does not appear to be amplified in the R strain. Northern analysis, however, indicates that expression levels in the two strains may be different.

In order to more directly assess the molecular mechanism of malathion resistance in *C. tarsalis*, MCE was purified to homogeneity from the two populations. It is a monomer of 59 kDa with a

different subcellular localization in R and S mosquitoes. In contrast to the abundance of particular esterases in organophosphate resistant aphids and other mosquitoes, MCE accounts for only 0.016% of the total extractable protein in our R and S strains (Whyard *et al.* 1993). Kinetic measurements of the enzyme from both populations showed that a unique MCE is present in the R strain mosquitoes which can hydrolyze malathion 18 times faster than the enzyme from S insects. Therefore, malathion resistance in these insects is due to a qualitatively altered esterase and not gene amplification.

Malathion resistance in *Lucilia cuprina* has been genetically mapped to a single locus on the fourth chromosome in an esterase gene cluster (Hughes and Raftos, 1985; R. Russell, unpublished). Purification of the 60.5 kDa MCE from R and S strains of *L. cuprina* showed that it had identical kinetic constants for several different substrates and inhibitors. The enzyme is unusual in that it hydrolyses malathion faster than any other insect esterase yet isolated. In contrast, the esterase implicated in organophosphate resistant aphids and *C. quinquefasciatus* mosquitoes shows little hydrolysis of insecticides and presumably confers resistance by sequestering the toxicants. A ten-fold increase in resistance in the *L. cuprina* R strain coincided with a nine-fold increase in total MCE content, to 0.05% of the total protein. Therefore, in this case, malathion resistance in blowflies is due to a quantitative change in the esterase responsible for the detoxification.

Malathion is a commonly used pesticide because it has relatively low mammalian toxicity and a short residual life. In order for it to continue to be used despite the development of resistance, appropriate synergists will have to be sought. We are hopeful that such a synergist can be identified since in neither species does MCE represent a large percentage of the protein (in contrast to the over-abundance of esterases in aphids and other mosquito species). This study has demonstrated that insects have a variety of ways to become resistant to insecticides and that the resistance mechanism may not be predicted by studies of related species. We thus have to continue efforts to understand the molecular basis of resistance in target pest populations to assist in their management.

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THE MARKET FOR BIOPESTICIDES

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ABSTRACT

Chemical pesticides make people nervous. Attitude surveys show that around two-thirds of all Americans rank chemical pesticides among their most serious concerns about food safety, despite the fact that they are in hundreds of thousands of times more danger from microbial contaminants in food than from pesticides. This anxiety should provide the perfect incubation medium for the new and growing market in non-chemical products for crop protection and crop production. For several decades, despite optimistic predictions, the volume and value of biopesticides remained small and is still less than 1% of the total world market for agrochemical crop protection of $20-$25b. In the past, products had narrow target spectra, a number of them did not work very well and yet most were more expensive than chemical equivalents. Recent improvements in the products have led to significant growth in the markets which may signal the start of the long predicted bright future for non-chemical farming.

THE OPPORTUNITY

Food has been in short supply through most of history; people ate what they could. Now, in the affluent Western world, most products, especially food, are in surplus and the consumer is king. Consequently, retailers compete for custom, manufacturers court retailers, suppliers pursue manufacturers and so on back to the farmer. Farmers were and are asked to produce large quantities of high-quality cheap food. Chemicals, fertilizers and pesticides, have made this possible. But many 'scares', about food and about the environment, have made consumers almost indiscriminately anxious about all chemicals, additives as well as pesticides. In response, many retailers started selling organically produced food. Experience to date shows that consumers are unwilling to pay the 'organic premium' nor will they purchase blemished produce. This creates a significant market opportunity for non-chemical crop protection agents, including biopesticides.

Similar considerations pertain in non-food pest control. Cotton production is one of the largest pesticide opportunities in the world and products based on *Bacillus thuringiensis* and viruses have been targeted here. Disease-carrying insects are most cost-effectively controlled by DDT, a chemical no longer allowed in most countries, so alternatives including *B thuringiensis* var. *israelensis* and *B sphaericus* must be used. Forest management requires insect control over vast areas; a number of governments have prohibited the use of chemicals creating high-volume opportunities for other varieties of *B thuringiensis*. Amenity pest control, household and garden pest and disease control all offer high value-added potential for non-chemical control.

THE MARKET

Despite these opportunities, the market for biopesticides still represents only $60-$130m per year, well under 1% of the $20b crop protection market. The majority of all sales of biopesticides are of products based on *B thuringiensis*, an aerobic gram-positive spore-forming bacterium, which also remains the focus of the majority of research on biopesticides. Sales of *Bt* products have increased steeply, up by at least 80% from 1989 to 1992, and current indications are that this increase is continuing. Sales of various formulations in 1992 were between $45-60m. The market is divided between Abbott (US) at 45%, Sandoz (Switzerland) and Novo (Denmark) with 25% each and all the other products and companies sharing the 5% balance. This market has been similarly divided for some years.

Some developments have occurred which may lead to further change: Solvay (Belgium), a long-time participant in *Bt* originally through Biochem and later through Duphar is now part of Novo; CRC, a relatively recent Italian entrant now owned by Caffaro, has closed. Ecogen, Mycogen and Bactec (all US) have entered the market, as has the specialist producer Fermone (US). Ciba-Geigy (Switzerland) is entering the market with a product developed by Horticulture Research International (HRI). Ciba have moved vigorously to obtain a key position in non-chemical crop protection by forming Ciba-Bunting with Buntings, the UK's leading insect producer. Ciba will also sell nematodes from Biosys (US), which in turn has bought pheromone producer Agrisense-BCS from Phillips/Dow Corning. DuPont has been selling *Bt* from Novo and has joined Crop Genetics International to launch viral insecticides. Bayer (Germany) launched and received German approval for its entomopathogenic fungal propagules and EcoScience (US) received EPA approval for its fungus-based cockroach control system. The number of new companies selling biopesticides and the number of new products is growing and the speed of growth is increasing. Table 1 gives an indication of product proliferation.

Table 1	Active ingredients in commercial products currently available for crop protection (Worldwide Directory of Agrobiologicals 3rd edition)	
Active ingredient	Number of products
Bacteria	104
Nematodes	44
Fungi	12
Viruses	8
Protozoa	6
Insects	107

Although only *Bt* products yet have significant sales, the prospects are better now than ever before. Both the technology and public and corporate attitudes have developed favourably for biopesticides and the market is now beginning to grow strongly.