Session 7B

Resistance to Crop Protection Agents

Monitoring, Mechanisms and Management 2

Chairman:	Dr Kevin Gorman *Rothamsted Research, Harpenden, UK*
Session Organiser:	Dr Ralf Nauen *Bayer CropScience, Monheim, Germany*
Platform Papers:	7B-1 to 7B-4

Acaricide resistance in two-spotted spider mites, *Tetranychus urticae*

T Van Leeuwen, L Tirry
Ghent University, Faculty of Bioscience Engineering, Coupure Links 653 B-9000 Ghent, Belgium
Email: thomas.vanleeuwen@ugent.be

R Nauen
Bayer CropScience, Research Insecticides, Building 6220, Alfred Nobel Str. 50, D-40789 Monheim, Germany

Tetranychus urticae is capable of rapidly developing resistance to many acaricides used for its control. A very high level of resistance to many compounds has developed after one to four years of use, which often also induces a high degree of cross-resistance. Resistance development is accelerated by the great fecundity, inbreeding, and very short life-cycle of the mites, resulting in many generations per year especially in warmer conditions. Spider mites also appear to have a high mutation rate, which favours the development of resistance alleles. The phenomenon of arrhenotoky, resulting in haploid males, leads to faster fixation of possible resistance alleles, and recessive traits are immediately visible in the population.

Although some progress has been made in understanding acaricide resistance in mites, especially tetranychids, knowledge of the mechanisms associated with acaricide resistance has not kept pace with that of insecticide resistance studies in insects. Limited in-depth studies have been published concerning acaricide resistance in *T. urticae*, with the exception of the genetics of acaricide resistance. Conventional methods of genetic analysis, adapted for the arrhenotokous reproduction of spider mites, revealed that often single major genes control specific types of resistance although important exceptions have also been published. Dominance of resistance refers to the phenotypic expression of a resistance trait in heterozygotes, relative to the expression in homozygote parents. Knowing the degree of dominance is essential for predicting the spread of resistance in the field. If applied rates can effectively kill the heterozygotes, resistance acts as a recessive trait, and spreads slowly. In contrast, when dominance is high and heterozygotes survive the toxicant, resistance spreads rapidly through the alleles present in heterozygotes. The number of genes involved is equally important in the management of resistance in the field. Monogenic resistance is more likely to spread than polygenic resistance, particularly when pesticide exposure varies across time or space.

If a polygenically resistant individual emigrates to a susceptible population, the resistance alleles are likely to be diluted by hybridization. Genetically fixed mechanisms of pesticide resistance in spider mites were similar to those found in pest insects and include enhanced metabolic detoxification of acaricides through esterases, glutathione S-transferases or cytochrome P450-dependent monooxygenases, and/or a mutated target site conferring target site resistance.

Resistance management attempts to conserve susceptibility towards pesticides through strategies aimed at either overcoming resistance to currently used compounds or preventing the development of resistance to existing or new pesticides. As resistance development is a selection process, discrimination by applied pesticides between susceptible and resistant individual target pests is essential.

Discrimination between genotypes can be countered either by enhancing the survival of susceptible homozygotes or by overpowering or reducing the fitness of resistant individuals before they become too common.

The survival of susceptible homozygotes can be achieved by reducing overall exposure (strategic placement of a pesticide, short-lived deposits, less frequent applications, creation of refuges) or by reducing up-take at the insect-insecticide interface through lowered application rates (though this may favour the survival of heterozygotes, too).

Overpowering or reducing the fitness of resistant individuals entails applying chemicals at sufficiently high concentrations to kill all resistant genotypes present. Since this scenario seems highly unlikely in practice, the high dose tactic is generally refined to one that aims at eradicating heterozygotes, thereby rendering resistance genes effectively recessive under field conditions.

The most important challenge now facing researchers, pesticide manufacturers, and others concerned with pest control is to develop the most effective tactics against current or perceived resistance problems. Agrochemical producers have confirmed their commitment to tackling resistance by establishing inter-company technical bodies such as IRAC (Insecticide Resistance Action Committee) to administer industrial support and create an environment favourable for the implementation of resistance management strategies.

The value of molecular-based technologies for detection of target-site resistance in weeds to ACCase and ALS inhibiting herbicides

J Wagner
University of Hohenheim, Weed Science Department, 70593 Stuttgart, Germany
Email: jeanwgnr@uni-hohenheim.de

H Menne, B Laber
Bayer CropScience, Industriepark Hoechst H872, 65926 Frankfurt, Germany

Herbicide resistance is conferred on individuals within a population by any of a number of mechanisms, each of which is under genetic control. Knowledge of the genetic basis of herbicide resistance contributes to a better understanding of herbicide resistance evolution in agroecosystems. Once the genes and their alleles responsible for resistance are known, the use of molecular-based technologies facilitates the diagnosis of resistance traits in the field. This supports integrated weed management strategies to retard the evolution of herbicide resistance as well as weed science issues of resistance mechanisms and population genetics. The mechanisms of herbicide resistance in plants include the modification of the herbicide target site (target-site resistance) and mechanisms such as increased metabolic activities, reduced uptake and translocation, which keep the active ingredient away from the target (referred to as nontarget-site resistance).

Target-site resistance is monogenically inherited and is the better known resistance mechanism. Genes or gene fragments of targets such as acetyl-CoA-carboxylase (ACCase) and acetolactate-synthase (ALS) are known for different weed species. Amino acid changes at well-known positions within the protein ACCase (Ile_{1781}, Trp_{1999}, Trp_{2027}, Ile_{2041}, Asp_{2078}, Gly_{2096}) and ALS (Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Trp_{574}, Ser_{653}) are known to confer resistance to ACCase and ALS inhibiting herbicides, respectively, and have been detected in different weed species worldwide. All amino acid changes are coded by single nucleotide polymorphisms (SNPs) on the target genes and can be detected either by direct sequencing of PCR-fragments or alternatively by the use of specific SNP detection methods. Two different procedures for detecting SNPs for target-site resistance in weeds are described in the literature; the allele-specific PCR (PASA) and the dCAPs method. A novel technology is Pyrosequencing®. This method for DNA sequencing is suitable for high-throughput SNP analysis and detects SNPs in the context of the surrounding sequence. Another advantage over classical SNP detection methods is the possibility to screen tri- or tetra allelic SNPs with one assay. The technology was adapted to genotype SNPs of ACCase and ALS genes in leaf samples of individual or pooled seed samples of herbicide-resistant black grass (*Alopecurus myosuroides* Huds.) and silky bentgrass (*Apera spica-venti* (L.) Beauv). The evolution of resistance to ACCase inhibiting herbicides is increasing in European populations of *A. myosuroides*. The proportion of target-site resistance in a population varies from one region to another. The role of agronomic and inherent factors for regional variation still remains to be elucidated. The most widespread target-site mutation in *A. myosuroides* is the exchange of isoleucine by leucine at amino acid position 1781. Recently the first reports of target-site resistance to ALS-inhibitors in both, *A. myosuroides* and *A. spica-venti*, added a new quality to resistance evolution. A proline to threonine or proline to serine change on position 197 and tryptophan to leucine change at position 574 of ALS was detected in biotypes of both species.

For each resistance allele of the diploid species *A. myosuroides* and *A. spica-venti,* three genotypes can appear in the field; susceptible, heterozygous and homozygous resistant plants. In field populations of *A. myosuroides* with resistance to ACCase inhibiting herbicides and *A. myosuroides* and *A. spica-venti* with resistance to ALS inhibiting herbicides, high proportions of heterozygotes were detected. Target-site resistance to ALS and ACCase inhibiting herbicides is co-dominantly inherited. Resistance factors for heterozygous and homozygous plants are expected to be different, indicating a difference in the selection of resistant genotypes by herbicides. The evolution begins with a single heterozygous genotype in the field. The proportion of hetero- and homozygous plants detected in fields can serve as an indicator for the inbreeding status. Genetic analysis combined with modelling will provide a valuable tool to predict the time of initiation and future of resistance evolution under given practical conditions.

The strength of molecular-based technologies is the potential for precise and fast analysis of herbicide resistance in actively cultivated fields, so the farmer can even react within the growing season. Molecular-based technologies operate at DNA level, which is independent from environmental influences. The potential for prophylactic resistance measurement to optimize support for anti-resistance weed management by avoiding "bad applications" is great. This can overcome or delay the evolution of herbicide resistance and the information collected about the status of the spatial and temporal development of allele frequencies in field populations are valuable. The current weakness of molecular-based technologies is their limitation to the detection of known mutation sites only. Novel mutations for target-site resistance and the alleles for non-target-site resistance need to be elucidated first. Therefore molecular-based technologies will not replace greenhouse assays to prove phenotypic resistance in the near future.

One opportunity associated with molecular-based resistance detection is the easy implementation of new scientific findings of resistance genetics into a running monitoring procedure. Increasing the knowledge about population genetics and about the spread of resistance mechanisms in weed populations are further opportunities of using molecular-based technologies in herbicide resistance research and management. The disadvantage of molecular-based technologies is their cost, which may prevent their wide-spread use. Furthermore, the acceptance and accurate implementation of molecular-based technologies needs communication between all parties involved. As long as herbicides remain the primary method of weed control, farmers are reluctant to adopt strategies based on integrated weed management. The dependence of farmers on sophisticated molecular-based decision support systems could promote the adoption of integrated weed management strategies.

Pyrethroid resistance and its management in European populations of pollen beetles, *Meligethes aeneus*, in winter oilseed rape

R Nauen
Bayer CropScience, Research Insecticides, Building 6220, Alfred Nobel Str. 50, D-40789 Monheim, Germany
Email: ralf.nauen@bayercropscience.com

Winter oilseed rape is one of the most important crops in several European countries. In Germany, for example, the acreage has more than doubled in the last 15 years, reaching more than 1.5 million hectares in 2007 with the federal states of Mecklenburg-Western Pomerania and Saxony-Anhalt being the main areas of oilseed rape cultivation. One quarter of the global harvest is produced within the EU. One of the reasons for this is the strong demand for rape oil in the food industry and for the production of biofuel.

Winter oilseed rape is attacked by a number of invertebrate pests of agronomic importance, including *Psylliodes chrysopcephala* (cabbage stem flea beetle), *Ceutorhynchus assimilis* (cabbage seed weevil), *Ceutorhynchus pallidactylus* (cabbage stem weevil), *Ceutorhynchus napi* (rape stem weevil), *Dasineura brassicae* (brassica pod midge) and *Meligethes aeneus* (pollen beetle). The latter, *M. aeneus*, is of major importance and known to be quite destructive if no control measures are taken once the treatment threshold is reached. After emerging from over-wintering sites, adults start to infest oilseed rape plants in mid-March until May, and can damage the flowering parts by feeding and oviposition. In particular, feeding larvae cause bud abscission. The consequence of these infestations are pod-less stalks and dramatically reduced yields, so the farmers need to control pollen beetles to keep numbers low and to avoid economic damage.

Pyrethroid insecticides have a long tradition in pollen beetle control throughout Europe. However, after the ban of other classes of insecticides such as organophosphates (OPs), they became the sole chemical class of insecticides for pollen beetle control in countries such as Germany (99% in 2005). Irrespective of the fact that OPs were banned, pyrethroids have often been used preferentially by farmers due to their good knock-down efficacy and their better environmental image than OPs. As a consequence of the increase in acreage of winter oilseed rape, pollen beetles increased in numbers and so, too, did pyrethroid applications.

The first documented case of reduced pyrethroid susceptibility in pollen beetles was reported in 1999 in the Champagne region in North-Eastern France but anecdotal reports on pyrethroid resistance development date back to 1997. A similar phenomenon was observed in Scandinavian countries such Denmark and Sweden. First rumours of reduced pyrethroid efficacy in Germany came in 2001 in the western federal state of Rhineland Palatinate. These rumours were confirmed in 2002, and since then pyrethroid resistance has spread all over Germany; since 2006, it has affected more than 50% of the winter oilseed rape acreage. We conducted pyrethroid resistance monitoring bioassays using a so-called adult vial test, utilizing pyrethroid coated glass vials where beetles are exposed to pyrethroids by contact. Efficacy was assessed after different elapsed time intervals, e.g. 1h, 5h and 24h. This method is known as IRAC Method no. 11 (www.irac-online.org) and is based on monitoring methods recently developed by Syngenta and Bayer CropScience. The monitoring was carried out with pollen beetle samples from different European countries.

Our monitoring studies in 2005/2006 revealed that particularly in the UK and Austria, pyrethroid-susceptible populations are absolutely dominant, whereas in Germany, France and Poland the majority of the populations (>70%) showed decreased susceptibility to pyrethroids. In other countries such as Belgium, some pyrethroid-resistant populations were found but to a much lesser extent than in France.

One of the major questions addressed during our monitoring studies concerned pyrethroid cross-resistance in populations coming from different regions in Europe. For this purpose vials were coated with different pyrethroids such as lambda-cyhalothrin (reference), deltamethrin, alpha-cypermethrin, etofenprox and bifenthrin. All compounds at 100% of their field-rate provided 100% mortality in several pyrethroid-susceptible pollen beetle strains collected in the UK. In a second step, 42 populations collected in Germany, France, Belgium, Austria and Poland were checked for their responses to all pyrethroids mentioned above, and a correlation analysis was performed (Table 1).

Table 1. Correlation analysis for pyrethroid cross-resistance in pollen beetles. Efficacy of lambda-cyhalothrin in adult vial tests (IRAC method 11) was correlated with those pyrethroids shown in the table (42 populations were tested in total).

Parameter	Deltamethrin	Cypermethrin	Etofenprox	Bifenthrin
Number of XY pairs	42	42	42	42
Pearson r (95% confidence interval)	0.8415	0.8362	0.5192	0.6579
P value	P<0.0001	P<0.0001	0.0004	P<0.0001
P value summary	***	***	***	***
Is the correlation significant?	Yes	Yes	Yes	Yes

Our results clearly indicated, as expected, cross-resistance between the whole chemical class of pyrethroid insecticides in pollen beetles. However, the extent to which the different pyrethroids are affected seems to be different. Nevertheless it is questionable if switching from one pyrethroid to another would be a long-term solution to the problem, as they all act on the same molecular target-site, the voltage-gated sodium channel in the insect central nervous system; so continuous exposure may select for target-site resistance known as kdr (knock down resistance).

Biochemical studies were also performed with pyrethroid-susceptible and pyrethroid-resistant populations. No differences were found in the overall esterase activity and glutathione S-transferase activity but monooxygenase levels were significantly different. This suggests a metabolic mechanism of resistance contributing to the observed differences in pyrethroid susceptibility. Molecular diagnosis for a mutation in the voltage-gated sodium channel known to confer target-site resistance in several other insect pests did not reveal an amino acid change.

Thiacloprid, a new insecticide belonging to the neonicotinoid family was recently introduced to the German market for pollen beetle control and was shown to be completely unaffected by the mechanisms conferring pyrethroid resistance (this was also confirmed for organophosphates). This classifies thiacloprid as an excellent option for rotational use with pyrethroids in resistance management strategies for sustainable pollen beetle control in the future. Only the rotation between insecticides of different modes of action should be considered as a valuable long-term strategy in future resistance management tactics.

Piperonyl butoxide restores the efficacy of *Bacillus thuringiensis* toxin in transgenic cotton against resistant *Helicoverpa armigera*

R V Gunning
New South Wales Department of Primary Industries, 4 Marsden Park Road, Calala, NSW, Australia 2340
Email: robin.gunning@dpi.nsw.gov.au

V Borzatta, E Cottage
Endura SpA, 5-40121 Bologna, Italy

L M Field, G D Moores
Rothamsted Research, Harpenden, Herts. AL52JQ, UK

Introduction

A recent pest management strategy exploits the use of the insecticidal proteins of *Bacillus thuringiensis* (*Bt*) in genetically engineered crops. These are now grown in many countries to control insect pests such as the cosmopolitan cotton (old world) bollworm, *Helicoverpa armigera*. Transgenic cotton cultivars expressing the *Bt* toxin Cry1Ac (INGARD®) have been grown in Australia to control *H. armigera* since 1996, and a cultivar expressing genes encoding for two toxins, Cry1Ac and Cry2Ab (BOLLGARD II®), has been grown commercially since late 2003. Resistance to Cry1Ac toxin in a field derived strain of Australian *H. armigera* has, however, been reported (Gunning *et al.*, 2005; Gunning *et al.*, 2006).

Resistance was shown to be associated with elevated esterase levels. Such non-specific esterases, a class of serine hydrolases found in the insect gut, have been implicated in many cases as an insecticide-resistance mechanism due to their ability to both hydrolyse insecticidal esters and sequester xenobiotics. *In vivo* and *in vitro* studies on Cry1Ac-resistant *H. armigera* demonstrated that esterases could bind to both the Cry1Ac pro-toxin and the activated toxin, suggesting that sequestration was the basis of the observed resistance

Insecticide synergists are compounds with little or no toxicity that, when applied with an insecticide, will suppress metabolism-based resistance and increase efficacy of the insecticide. One of the best-known insecticide synergists is piperonyl butoxide (PBO). One mode of action of PBO is the inhibition of esterase enzymes, which are responsible for resistance in some insects, including Australian *H. armigera*. Since Cry1Ac resistance reported in Australian *H. armigera* was mediated by esterases, the aims of the experiments reported here were to determine whether PBO could "cross-over" from synergism of conventional insecticides to synergism of *Bt* toxins expressed in transgenic cotton. PBO is also known as an inhibitor of another detoxification enzyme system (microsomal oxidases) in insects. To determine if observed synergistic effects of PBO could be explained solely by esterase inhibition, an experimental PBO analogue, 5-[2-(-butoxy-ethoxy)-ethoxymethyl]-2,3-dihydro-benzofuran (EN16/6) lacking the intact methylenedioxyphenyl ring essential for oxidase inhibition, was also used.

Results

Feeding bioassay techniques on third instar (30 – 40mg) *H. armigera* assessed any diet with

synergistic effects of PBO or EN 16/6 on Cry1Ac. Cry1Ac resistant and susceptible larvae were fed an artificial diet containing formulated Cry1Ac (MVP®). For synergism experiments, larvae were dosed topically with 10 µg of PBO or EN 16/6 prior to exposure to the Cry 1Ac diet. Both PBO and EN 16/6 act as effective synergists of Cry1Ac in the resistant strain. When PBO was applied, the observed resistance factor was lowered from 150-fold and 275-fold to 7.2-fold and 12.5-fold at the LC_{50} and $LC_{99.9}$ respectively. The PBO analogue, EN 16/6, was marginally more effective as a Cry 1Ac synergist, with resistance factors of 1.8-fold and 2.5-fold for the LC_{50} and $LC_{99.9}$ respectively. Neither PBO nor EN 16/6 synergised Cry1Ac against the susceptible strain.

Conventional and transgenic cotton leaves were treated with PBO by dipping into an emulsifiable concentrate diluted with water (0.1% PBO). After drying the leaves, first instar *H. armigera* were placed on the leaves and allowed to feed. Approximately 70% of resistant larvae survived on untreated Ingard cotton, whereas there was no survival on Ingard® cotton treated with PBO. A smaller percentage (10%) of the resistant larvae also survived on Bollgard II® cotton, but there was no survival following PBO treatment. There was no survival of susceptible larvae on transgenic cotton, with or without PBO.

Model substrate studies (α-naphthyl acetate) showed that esterase activity from Cry1Ac resistant *H. armigera* was inhibited strongly by both PBO and EN 16/6, although no significant esterase inhibition occurred in the susceptible strain. Biacore® biosensor studies confirmed that binding occurred, between esterase from Cry1Ac resistant *H. armigera* and PBO.

Discussion

The discovery that *Bt* toxin resistance can be overcome by an established insecticide synergist, PBO, may have considerable practical implications for future control by *Bt*. The mechanism of synergy between PBO and Cry1Ac appears to be inhibition of the esterase iso-enzymes that are either sequestering or metabolising the Cry1Ac toxin. Synergy between EN 16/6 (a non-oxidase inhibiting PBO analogue) and Cry1Ac toxin, adds considerable weight to this hypothesis. The ability of PBO to "cross over" from synergising conventional insecticides to synergism of *Bt* cotton represents a considerable breakthrough for the effective management of transgenic crops against resistant pests in the future.

References

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