POSTER SESSION 7A

RESISTANCE: SCIENCE INTO PRACTICE

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Poster Papers: 7A-1 to 7A-10

Gene flow from Bt transgenic corn to nonBt corn: can refuges speed the evolution of pest resistance?

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ABSTRACT

Bacillus thuringiensis transgenic corn kills several pest species, but its usefulness is dependent on the prevention of pest resistance. To slow resistance to toxins in Bt crops, they are supposed to have high expression levels and nonBt refuges planted near the Bt crops. However, pollen from Bt crops such as corn can fertilize nonBt plants, causing toxin expression in the seeds or kernels of the nonBt plants. This paper examines how gene flow from Bt to nonBt corn affected toxin expression in nonBt refuges. Cry1Ab genes from Bt hybrids were spread for up to 32 m across nonBt refuge plots with moderate to low toxin levels throughout the nonBt refuge. These findings were used in a simulation model to determine the effects of Bt toxin expression in refuges on the development of resistance to Bt toxins by pests. Results indicate that refuges must be located at a distance, dependent on wind speed and pollen movement, from Bt plantings, in order to act as a refuge or they may actually increase the rate of resistance evolution in pests.

INTRODUCTION

Toxins produced by *Bacillus thuringiensis* transgenic corn hybrids kill several pest species, reducing injury to whorls, ears, and stalks. Their continued usefulness, however, is dependent on the prevention of pest resistance (Shelton *et al.*, 2002). Populations of all major pest species have evolved resistance to insecticides and several have evolved resistance to Bt sprays (McGaughey, 1985; Tabashnik *et al.*, 1990). To slow resistance to Cry toxins in Bt crops, nonBt refuges are planted with the Bt crops (Tabashnik, 1994). The strategy is to use highly toxic Bt plants to kill homozygous susceptible and heterozygous insects (Denholm & Rowland, 1992). Any surviving resistant homozygotes will mate with susceptible insects from refuges producing susceptible individuals that will be killed by Bt toxins (Roush & McKenzie, 1987). The strategy assumes that toxin expression is high enough to kill heterozygous insects, resistant allele frequencies are low, and random mating occurs between adults of different genotypes from Bt and nonBt plots (Gould, 1998).

Because corn is wind-pollinated, nonBt refuge plants near Bt corn can be pollinated with Bt pollen and will express Bt toxins. In theory, moderate to low doses could actually speed the evolution of resistance by allowing the survival of partially resistant heterozygotes. In planning refuge structure and placement in resistance management for insects in Bt crops, inter-mating of insects from Bt plantings and nonBt refuges has been stressed while the effects of gene flow to nonBt refuges has been ignored.

Toxin expression that only occurs in corn kernels will mainly affect ear-feeding pests such as corn earworm, *Helicoverpa zea* and fall armyworm, *Spodoptera frugiperda*. However, larvae of most species of borers, such as European corn borer, *Ostrinia nubilalis*, and southwestern corn borer, *Diatraea grandiosella*, the main targets of Bt transgenic maize, feed on ear tissues during their second (or later) seasonal generation. Because all target pests will come into contact with the Bt toxins in refuge plants that are within the halo of pollen from Bt plantings, nonBt plantings of small width will not act as refuges. Actually, for any refuge close to the Bt planting there will be a wide range of concentrations found throughout nonBt plantings rather than the high concentration of toxins required for the high dose strategy.

In this study, I measured the expression of Cry1Ab toxins in nonBt corn refuges, then used these measurements in a computer model to simulate the development of resistance to Bt transgenic corn by *H. zea*. Although *H. zea* is used as an example, any lepidopterous pest that Bt transgenic plants target will be affected in a similar manner. The effects of Bt gene flow on supposed nonBt refuges and on the development of pest resistance are then discussed.

MATERIALS AND METHODS

Six isogenic Bt/nonBt hybrid corn pairs were used in each of two replicate plots. Hybrids were from four different companies and included four Bt11 insertion event and two Mon810 insertion event hybrids, all genetically engineered with Bt genes to express the Cry1Ab protein toxin. Each of the two replicates was divided into six test plots. The first eight rows of each test plot were planted with one of the six Bt hybrids, then 36 adjacent rows were planted with its nonBt counterpart.

Ears were harvested when all hybrids were below 15% moisture and then dried to approximately 10% moisture. Samples were harvested from two rows of each Bt subplot, and rows 1-4, 8, 16, 24, and 32 of each nonBt subplot, (with row 1 being 0.965 m from the adjacent row of the Bt subplot and row 32 being 30.88 m from the Bt subplot). For all samples, kernels were removed from each ear and then ground to a fine powder.

Cry1Ab in each ground sample was quantified using EnviroLogix Inc. (Portland, Maine, USA) plate kits to perform Enzyme Linked Immunosorbent Assay (ELISA). Ground samples were placed in an extraction/dilution buffer for 24 h and then the extracts were added to the test wells of the ELISA plate. Test wells had been coated with antibodies raised against Cry1Ab toxin to which residues would bind and were then detected by the addition of horseradish peroxidase–labeled Cry1Ab antibody.

A single quadratic regression equation was fitted to a plot of Cry1Ab concentrations by distance from the Bt subplot for all nonBt hybrids. Distances for 1 to 76 rows were then inserted into the equation to obtain predicted Cry1Ab concentrations for each of these 76 rows.

A stochastic, generation-specific, simulation model as described by Chilcutt & Tabashnik (1999) was developed that included a population model and a Cry1Ab resistance evolution model for *Helicoverpa zea*. Three genotypes were included in the model, a homozygous susceptible genotype (SS), a homozygous resistant genotype (RR), and a heterozygote (RS). Mortality was varied in the model with values of 99, 90, and 60% for the SS genotype, 90, 60, and 30 % for the RS genotype, and a constant 1% mortality for the RR genotype.

To determine the effects of Bt toxin expression in refuge plants on resistance evolution, five *H. zea* larval subpopulations were included in the model, with one subpopulation feeding within the Bt planting and four subpopulations feeding within a 20% nonBt refuge. Each of the nonBt subpopulations was located within 1 to 19 rows of corn. For a 4-row refuge each subpopulation would be one row wide, whereas for a 76-row refuge each would be 19 rows wide.

The concentration used in the Bt subpopulation was a single value, an average for all Bt hybrid samples, whereas the Cry1Ab concentration in each of the four nonBt subpopulations was an average for all rows in that subpopulation and, therefore, changed depending on the number of rows in each subpopulation. At the end of each generation, all genotypes from all subpopulations mated randomly.

The model was then run with 10 different Bt plot and nonBt refuge plot sizes (number of rows) all with the refuge occupying 20% of the planting. The model was also run once with no refuge. For each of the 10 planting size values and the zero refuge, the model was run once for each of the set of SS/RS/RR genotype % mortality values, including 99/90/1, 99/60/1, 90/60/1, and 60/30/1. For each simulation, the model was run until the resistance allele frequency (R) at the end of a generation was above 0.5. This generation was considered to be the generation in which the population became resistant to Cry1Ab.

RESULTS

Cry1Ab toxin levels in nonBt plots of all hybrids decreased exponentially with increasing distance from Bt plots (Figure 1). This pattern was similar for all hybrid pairs, with some small row to row variations. Cry1Ab concentrations in Bt plots varied from 80 to 300 ng/g of dry kernel depending on the hybrid. In nonBt plots, Cry1Ab concentrations ranged from 88 to 307 ng/g at one row distance from the Bt plot (0.97 m), 1 to 7 ng/g at 16 rows distance (15.4 m) and 1 to 9 ng/g at 32 rows distance (30.9 m). This indicates that in the nonBt row adjacent to the Bt plot, Bt toxin levels are actually higher than in some Bt plots.

The model demonstrated that Bt gene flow to nonBt refuge plants will, in most cases, speed the evolution of resistance to Cry1Ab by *H. zea* (Figure 2). All values in Figure 2 are the number of generations to resistance in the presence of gene flow divided by the number of generations to resistance in the absence of gene flow (uniformly nontoxic refuges). Therefore, values less than one indicate that resistance occurs faster when Bt gene flow to refuges occurs than when refuges are uniformly nontoxic.

The results demonstrate that, except when susceptible homozygote and heterozygote mortality is low and the refuge physical size is large, resistance always occurs faster when gene flow occurs than when no gene flow occurs. Also, if the refuge is small in physical size (although always 20% of the corn planting) and if heterozygote mortality is nearly as high as for susceptible homozygotes, then resistance evolution will actually be faster than if there were no refuge.

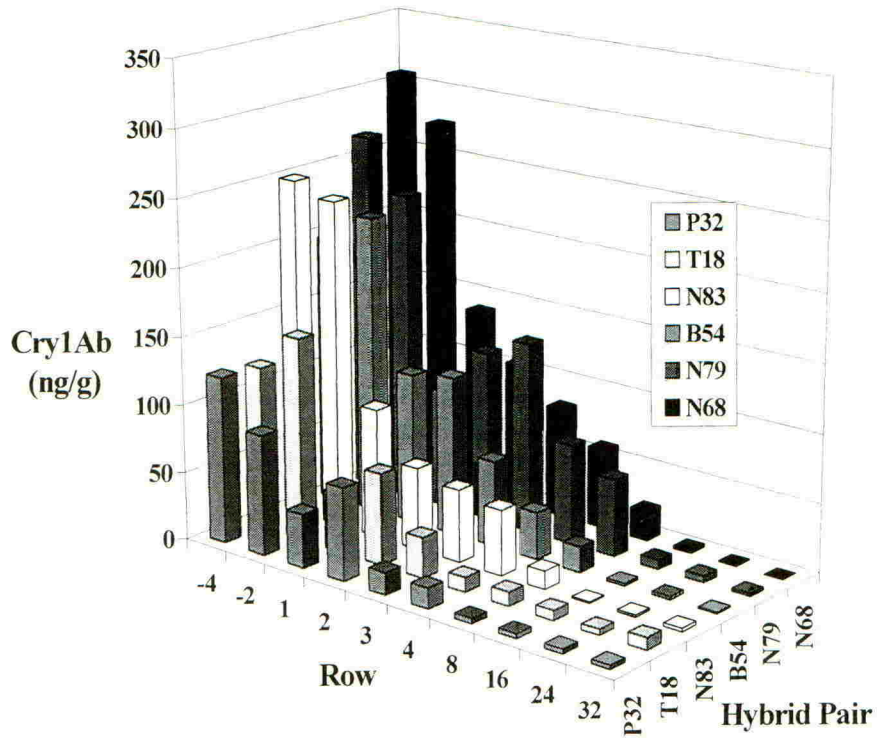

Figure 1. Average Bt toxin levels in corn kernels from two rows of Bt plots (-2,-4) and eight rows of an adjacent nonBt refuge at increasing distance from the Bt plot. Row spacing is 0.97 m.

DISCUSSION

The results indicate that pollen from Bt transgenic corn will produce moderate to low levels of Cry1Ab toxins in refuge plants for at least 30 m. The extent of these effects throughout a refuge depends on a number of factors including refuge size, shape, distance from the Bt crop, and wind speed and direction as well as similarity in planting times, and maturation times between Bt and nonBt corn hybrids.

The movement of Bt genes into nonBt refuges is extremely important to resistance management. The production of a range of levels of Bt toxins in nonBt corn ears within refuges is in direct contrast to the goals of a high dose-refuge strategy for controlling resistance. Not only is the refuge compromised by gene flow, but there is also no possibility of the uniform, high toxin concentrations required for the high dose strategy. Of course, even in a uniform planting of Bt corn, there is a wide range of toxin levels produced in leaves, silks, shanks, and kernels, as well as plant-to-plant variation throughout a field. These variations already call into question the probability that major corn pests always receive a high toxin dose when feeding on Bt corn. The addition of gene flow into refuges just increases the range of toxin concentrations throughout a planting, including very low concentrations that might not be present in uniform Bt corn plantings.

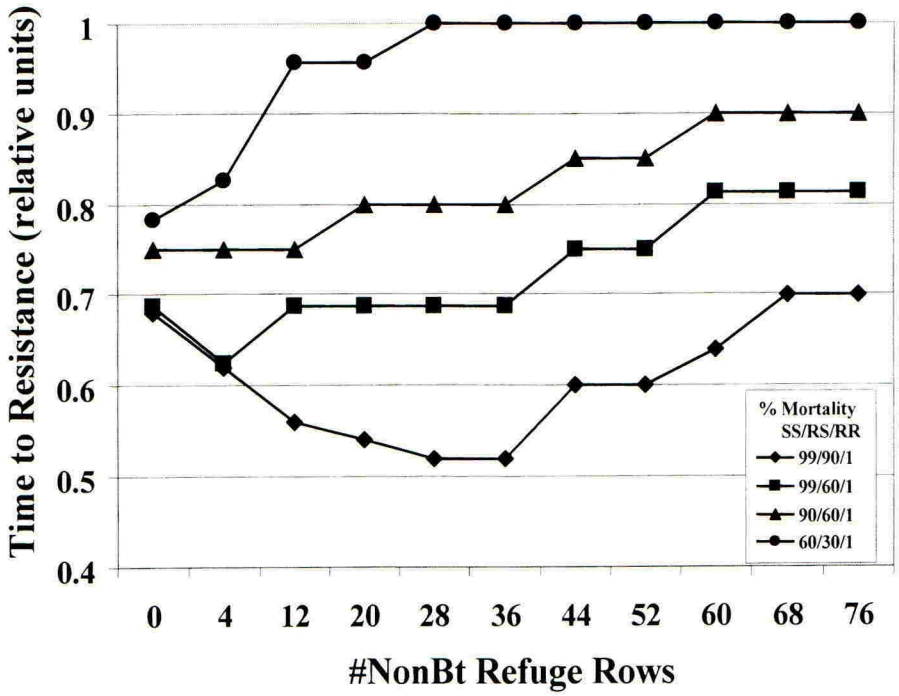

Figure 2. Amount of time it takes *Helicoverpa zea* to develop resistance to Cry1Ab toxins in the presence of toxic plants in a nonBt refuge. Time values are generations relative to a 20% nontoxic refuge (no gene flow). Lines represent different mortality values for susceptible homozygotes and heterozygotes, with resistant homozygotes always having 1% mortality.

I have shown here that the expression of Bt toxins in refuges will increase the rate of resistance development in ear-feeding pests over nontoxic refuges and could even increase resistance development faster than if a high dose were used with no refuge. The latter would only be possible if there were a population of a pest species that actually consumed high toxin doses throughout Bt plantings within the range of the population, a condition that is questionable at this point in time, but may be a factor in the future as hybrids with higher toxin expression are produced.

Several possible solutions to the problem of Bt gene flow into refuges include planting refuges in a manner that limits cross-pollination by Bt plants (Morris *et al.*, 1994), or planting refuges at different times than Bt plots, which Alstad & Andow (1995) have shown may slow resistance for other reasons. More information is needed to assess these tactics and their effects on mating between insects from refuges and Bt plots. Also, as with all current resistance management strategies, field tests of the effects of gene flow on resistance in pest populations is needed.

ACKNOWLEDGEMENTS

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Study of resistance to ALS inhibitors in the weed species *Echinochloa crus-galli*

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ABSTRACT

The aim of the study was the determination of whether resistance to ALS inhibitors occurred in the weed species *Echinochloa crus-galli*. Herbicide resistance represents an adaptive phenomenon resulting from repeated use of herbicides with the same mode of action. Resistance determination studies were performed in 2002, and material for the study was collected from different localities in the region of Vojvodina (Serbia), specifically Kamendin, Backi Maglic and Becej. Repeated use of the ALS-inhibiting herbicides, which are used very successfully against dicotyledonous and monocotyledonous weeds, has resulted in the occurrence of resistant biotypes of *E. crus-galli*. Results obtained from biological studies, whole plant tests and Petri dish bioassays, confirmed the presence of resistant *E. crus-galli*.

INTRODUCTION

The potential for adaptation by weed species to herbicides by single applications is weak. It becomes expressed after repeated use over a long period. Resistance development in some biotypes then becomes a major constraint on the use of herbicides that were previously efficient. The resistance phenomenon can not be recognized by visual assessment until it occurs in 1-10% of individuals in a field population, but in less than 0.1% of resistant individuals in laboratory conditions. Where only one weed species is present, it can usually be considered to have become resistant (Konstantinovic, unpublished).

The most frequently used ALS-inhibiting herbicides in our country are sulphonylureas and imidazolinones, of which imazethapyr was used in our studies. *Amaranthus retroflexus* was the first weed in Israel to develop resistance to ALS inhibitors (Sibony & Rubin, 2003). Our studies on resistance of the weed species *Echinochloa crus-galli* to ALS inhibitors are the first of this kind in our country. Up to now, there have been 80 reports of resistant of weed species to this mode of action and only one case in the genus *Echinochloa (E. colona)*, which was identified in Costa Rica in 1988 (HRAC, 2003).

MATERIALS AND METHODS

Seeds were collected from the localities Kamendin, Backi Maglic and Becej, which had a long history of imidazolinone and sulphonilurea herbicide use (over the last 10 years). A susceptible population collected from an area where no herbicides had been used was used as a reference population. Imazethapyr was used since it was one of the most frequently applied ALS inhibitors in the localities studied.

The most important individual factor for the initial determination of resistance is the level of non-susceptibility in the field. Consequently, we have used a method of visual assessment of imazethapyr efficiency to detect possible resistance.

There are several factors that can indicate possibility of resistance occurrence in field, such as:
i) level of control of other susceptible species,
ii) presence of live plants alongside dead ones,
iii) past experiences, i.e. previously successful control by the same treatment,
iv) herbicide history, i.e. repetition of the same herbicide treatment, or herbicide with the same mode of action,
v) resistance occurrence in the region,
vi) harvest,
vii) cultivation history, i.e. monoculture and minimum tillage (Moss,1995).

Studies were made on whole plants (Thurwachter, 1998) and Petri dishes bioassays (Clay & Underwood, 1990). Assays were performed in four replications and plants were treated with various doses of imazethapyr, representing. 40, 80, 100, 150 and 200 g a.i. ha^{-1}.

In whole plant studies, plants were grown in controlled conditions in pots from seed which was suspected to be imazethapyr resistant. There were 10 seeds per plot and the trial was set on chernozem, with 3.5% humus, in four replications, and assessments were done 3 – 4 weeks after treatment (pre emrgence herbicide application). In whole plant studies, efficacy was evaluated by measuring foliage fresh weight, as well as by counting emerged plants and assessing their vigour.

In the Petri dish assays, 10 seeds per dish were spread evenly over filter paper and 5 ml of imazethapyr solution added to saturate, but not flood, the filter paper (pre emergence herbicide application). There were four replications of each treatment. Dishes were kept at room temperature, out of direct sunlight. Germination and seedling condition were recorded at intervals up to 25 days from the start, with visual assessment of number of healthy and damaged seedlings in each dish. In Petri dishes bioassays, the lengths of epicotyls and hypocotyls of shoots were measured.

RESULTS AND DISCUSSION

Pot tests

At doses equivalent to 100 g ha^{-1} and above, none of plants from the Backi Maglic locality or the susceptible standard survived (Table 1), suggesting that the Backi Maglic population remains susceptible to imazethapyr. In contrast, some plants from both the Becej and Kamendin populations survived this herbicide dose, which resulted in 73% and 24% decreases in fresh weight for the two populations, respectively. Only a 44% reduction in fresh weight of plants from the Kamendin population was achieved by imazethapyr at the highest dose of 200 g ha^{-1}. This suggests that there may be some resistance in this population.

Table 1. Effects of doses of imazethapyr on foliage fresh weight and number of emerged plants of *Echinochloa crus-galli* in pot tests

	Imazethapyr dose (g a.i. ha^{-1})											
	0		40		80		100		150		200	
Locality	a	b	a	b	a	b	a	b	a	b	a	b
Becej	54.6	23	43.5	17	17.4	6	14.5	5	0	0	0	0
SED	3.0	-	2.2	-	1.5	-	1.3	-	-	-	-	-
Kamendin	52.1	29	46.8	27	40.2	19	39.8	12	31.2	8	29.4	6
SED	5.2	-	3.4	-	1.2	-	2.3	-	0.8	-	0.2	-
Backi Maglic	45.3	28	38.4	17	15.9	2	0	0	0	0	0	0
SED	4.1	-	2.7	-	0.2	-	0	-	-	-	-	-
Susceptible standard	49.6	27	31.5	19	12.5	1	0	0	0	0	0	0
SED	3.5	-	2.4	-	0.3	-	0	-	-	-	-	-

a, foliage fresh weight (mg per plant); b, total number of emerged plants.

Petri dish bioassays

After 25 d in Petri dishes, damage to emerged plants occurred at some imazethapyr concentrations. At 0.15 and 0.2 mg l^{-1}, there were no undamaged plants in the Becej population (Table 2). There was 30-45% damage to plants from Backi Maglic, depending on the concentration used. Only plants from Kamendin were less susceptible than the standard susceptible plants. This decrease in susceptibility of the Kamendin population was confirmed by hypocotyl lengths (Figure 1) and epicotyl lengths (Figure 2), which were greater in plants from this population, after treatment at 2.0 mg l^{-1}, than in those from other populations, including the standard susceptible.

The Petri dish bioassays provide some confirmation of the whole-plant data, suggesting that biotypes from the Backi Maglic locality are still susceptible to imazethapyr at field doses of 80 and 100 g ha^{-1} but that there is some resistance in the population at the Kamendin locality. The situation at Becej is less clear, since the Petri dish bioassay indicated good susceptibility although there was some plant survival at 100 g ha^{-1} in the whole-plant tests.

The decreased susceptibility in the *E. crus-galli* population from Kamendin is a result of more intensive use of ALS-inhibiting herbicides in that locality. Data from other studies support the suggestion that repeated use of herbicides with this mode of action increases the risk of resistance development.

Table 2. Effects of imazethapyr concentration on percentage of damaged plants 25 days after emergence in Petri-dish assays

Locality	Imazethapyr dose (mg litre^{-1})											
	0		0.04		0.08		0.1		0.15		0.2	
	%	SED	%	SED	%	SED	%	SED	%	SED	%	SED
Becej	0	0	14.7	1.5	13.5	1.8	28.8	2.4	100	0	100	0
Kamendin	0	0	13.3	3.8	9.3	3.2	10.0	1.4	13.3	23	20.0	2.4
Backi Maglic	0	0	36.6	4.7	30.0	3.7	40.0	2.1	43.3	1.7	40.0	1.4
Susceptible standard	0	0	20.0	1.3	23.3	2.6	23.3	1.8	30.0	1.2	33.3	1.5

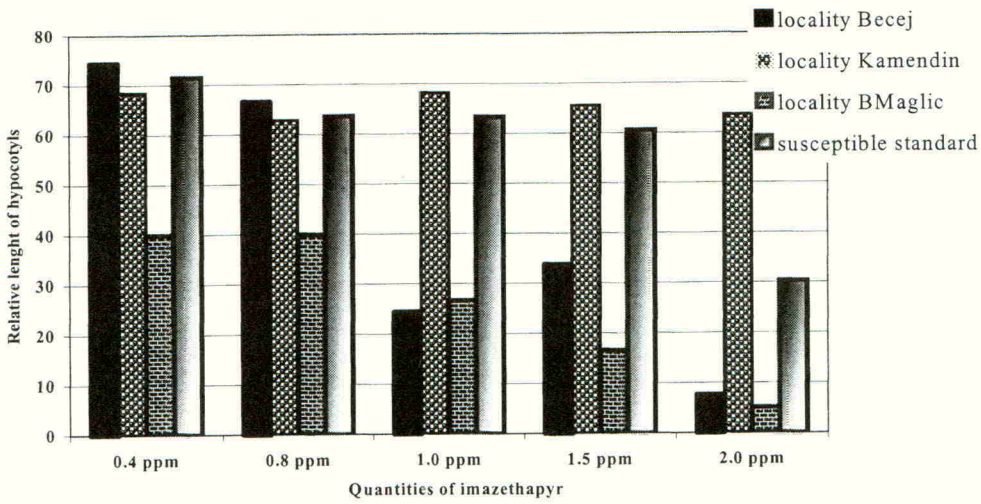

Figure 1. Effects of imazethapyr concentration on lengths of hypocotyls of seedlings of *Echinochloa crus-galli* from different localities in Petri-dish bioassays.

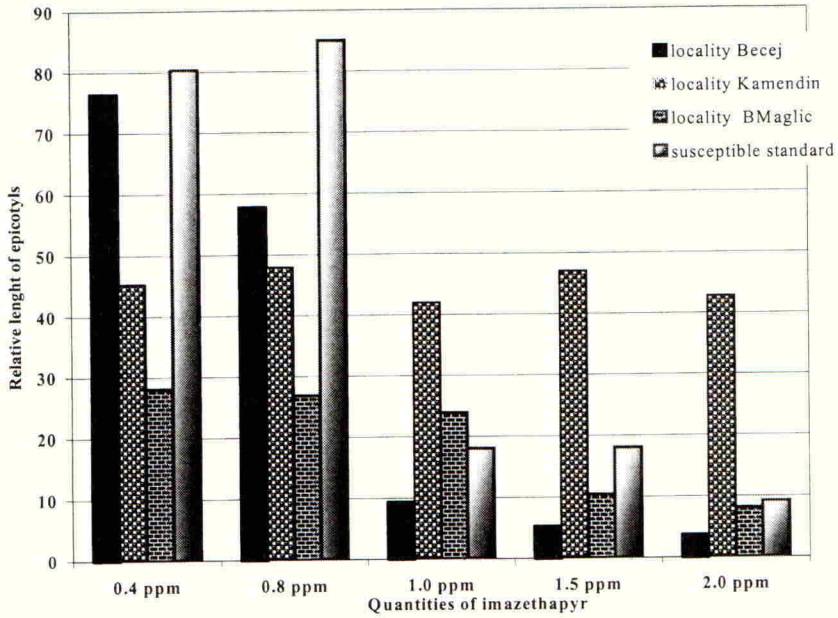

Figure 2. Effects of imazethapyr concentration on lengths of epicotyls of seedlings of *Echinochloa crus-galli* from different localities in Petri-dish bioassays.

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Differential sensitivity of Jordanian *Amaranthus retroflexus* populations to post-emergence herbicides

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ABSTRACT

The differential sensitivity of three Jordanian *A. retroflexus* populations to 2,4-D, glyphosate, and paraquat was examined in three greenhouse experiments. Herbicides were applied at rates that enclosed estimated ED_{50} doses for a control population in preliminary experiments. Results indicated that one population had a higher tolerance of 2,4-D. This population was very sensitive to glyphosate applications. The three populations were very sensitive to paraquat, but their responses to this herbicide were different. These experiments revealed the presence of diversified responses to commonly used herbicides in *A. retroflexus* populations, a fact that should be considered if site-specific herbicides are utilized more often in the future.

INTRODUCTION

Amaranthus retroflexus (redroot pigweed) is one of the most widely distributed weeds in arable crops worldwide. The species is present in more than 60 crops in 70 countries (Holm *et al.*, 1997). *A. retoflexus* is present in almost all parts and environments of Jordan (Abu-Irmaileh, 2000). Ghorbani *et al.* (1999) reported that *A. retroflexus* seeds germinate over a wide range of temperatures, water potentials, and burial depths. Interest in the biology of *A. retroflexus* increased dramatically after triazine resistance was confirmed in many biotypes of the species (Holm *et al.*,1997). *A. retroflexus* populations have shown resistance to photosystem II inhibitors, acetolactate synthase (ALS) inhibitors, ureas and amides (Heap, 2003).

Despite the relatively small area of Jordan, the country is characterized by diverse topography and environmental conditions. This has created a diversity in agricultural systems. Jordanian agricultural systems are characterized by limited reliance on chemical weed control. Nevertheless, 2,4-D, glyphosate and paraquat were used frequently over the past 20 years. No resistance of *A. retroflexus* to any of these herbicides has been documented (Heap, 2003).

The repeated use of herbicides with similar modes of action on the same weed population imposes selection for increased resistance within species that had been susceptible. Therefore, recognition, prevention, and management of herbicide resistance in all agricultural situations is imperative (Holt & LeBaron, 1990). In that regard, the differential response of *A. retroflexus* populations from different Jordanian locations to herbicides is not well defined. Our objective

in this research was to determine the sensitivity of three Jordanian *A. retroflexus* populations to 2,4-D, glyphosate, and paraquat herbicides through dose-response relationships and to diagnose any progression of herbicide resistance.

MATERIALS AND METHODS

Individual *A. retroflexus* inflorescences were collected in summer 2002 from three locations in Jordan, which are briefly described in Table 1. Seeds were stored for 7 months in paper bags at room temperature before use in experiments. Seeds from 10 random plants per location were combined to represent a population for the designated location.

Table 1. Geographical, environmental, and agricultural systems prevailing in Jordanian collection sites

Location	Latitude	Longitude	Altitude (m)	Average annual rainfall (mm)	Average upper & lower temperatures (°C)	Cropping system
Jordan University of Science & Technology (JUST)	32° 34′	36° 01′	560	235	23.7, 10.7	Barley-Fallow
Mushaqer Agricultural Reserach Center (MUSR)	31° 43′	35° 48′	85	358	22.9, 10.2	Wheat-Legumes
Faisal nursery (FAIS)	32° 12′	35° 53′	260	350	24.8, 11.1	Orchard and Nursery

Source: Jordanian Meteorological Department.

Experiments were conducted in spring 2003 at the Institute of Phytomedicine, University of Hohenheim, Germany. Seeds from the three populations were sown 0.5 cm deep in boxes (20 cm x 10 cm x 3 cm deep) containing compost. Boxes were placed in a dark cold chamber (4°C, 48 h) and then transferred to a greenhouse (24/18°C day/night). Individual seedlings were transplanted at the cotyledon stage to 10-cm pots filled with sterilized compost. Mercury halogen lamps were used to provide 300 $\mu E\ m^{-2}\ s^{-1}$ for a 16-h photoperiod.

A. retroflexus seedlings from the three populations were subjected to herbicide applications at the 4 or 5-leaf stages in three separate experiments. The dose causing 50% reduction in dry weight, referred to as the ED_{50}, was approximated for the three herbicides by conducting preliminary experiments on a control *A. retroflexus* population from Germany. Approximate ED_{50} rates were 155 and 4 g a.i. ha^{-1} for 2,4-D and paraquat, respectively, and was 86 g a.e. ha^{-1} for glyphosate. Therefore, we decided to apply 2,4-D (U 46® D-Flud) at 0, 8.75, 17.5, 35, 70,

140, 280, 560, 1120, 2240, 4480 and 35840 g a.i ha^{-1}. Glyphosate (Roundup Ultra®) was applied at 0, 13.125, 26.25, 52.5, 70, 105, 157.5, 210, 420, 840 and 1680 g a.e. ha^{-1}. Paraquat (Gramaxon Extra®) was applied at 0, 1.0937, 2.1875, 2.9166, 4.375, 8.75, 17.5, 35, 70, 280, 2240 and 4480 g a.i. ha^{-1}.

A spraying chamber equipped with a flat-fan nozzle (8004) calibrated to deliver 400 l ha^{-1} at 250 kPa was utilized. Treated plants were arranged in a completely randomized design with six replicates and monitored closely in the greenhouse. A very rapid response was observed in all three herbicide applications. The experiments were terminated when necrosis appeared with the low-dose treatments, approximately 24, 72, or 96 h after applications in experiments on paraquat, glyphosate, and 2,4-D, respectively. Plant foliage was harvested and their dry weights (48 h at 80° C) were recorded.

Statistical analysis followed procedures described by Seefeldt *et al.* (1995). The log-logistic curve was adopted to describe the response y to herbicide dose x by the mathematical expression of

$$y=f(x)=C+((D-C)/(1+\exp[b(\log(x)-\log(ED_{50}))]) \quad \text{Eq.1}$$

where C = lower limit, D = upper limit, b = slope, and ED_{50} = dose giving 50% response. Dry weight was analysed as a percentage of the average untreated control for the particular population. Thus, 100 was considered the upper limit for all equations.

The first non-linear analysis developed equations to predict the fit of three different dose-response curves, one for each population. The curves were allowed to differ in their lower limits, slopes and ED_{50} values. Then, three non-parallel curves that have a common lower limit were developed to fit dose-response data. To test whether the dose-response curves were parallel (i.e., had a common slope), another non-linear routine was performed that forced dose-response curves to have a common slope and variable ED_{50} values. Lack-of-fit tests were performed to make comparisons between any two models as described by Seefeldt *et al.* (1995).

RESULTS

2,4-D

Lack-of-fit tests indicated that it is not reasonable to assume equal lower limits for the response of the populations to 2,4-D applications. FAIS and MUSR populations were better described by the log-logistic model than was the JUST population (Figure 1). The estimated ED_{50} for the FAIS population was 510 g a.i. ha^{-1}, which is much higher than the estimated value in the preliminary experiment or the estimated values for the other two populations in this experiment. The log-logistic curve does not describe the data of the JUST population adequately due to high variability.

Glyphosate

Populations collected from JUST and MUSR were better described by the log-logistic equation than from the FAIS population. The FAIS population experienced approximately 40%

reduction in shoot dry weight in response to low doses of glyphosate. Thus, the log-logistic equation was considered unsuitable to describe the data for this population. Lack-of-fit tests indicated common lower limits and slopes, but not equal ED_{50} values for the JUST and MUSR populations (Figure 2). The estimated ED_{50} value for the MUSR population was greater than that estimated for the JUST population.

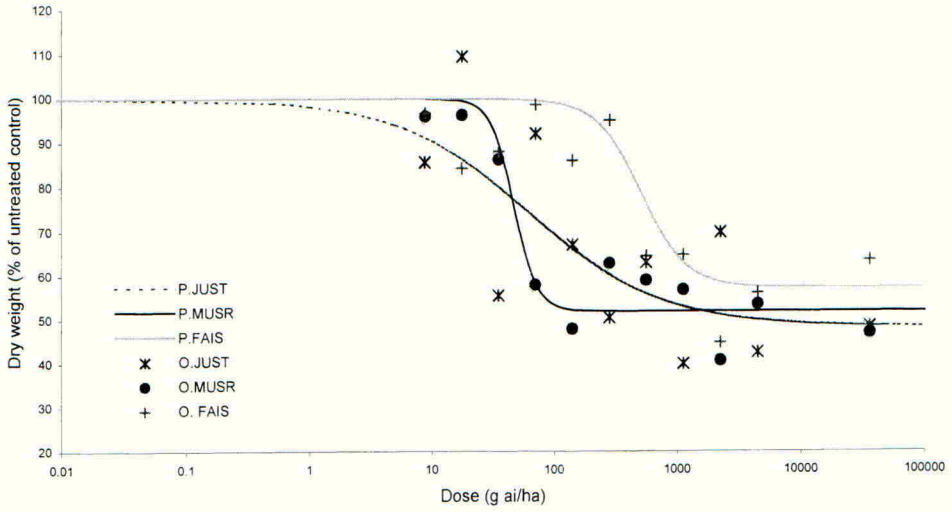

Figure 1. The log-logistic dose-response curves corresponding to differential sensitivity of three Jordanian *A. retroflexus* populations to 2,4-D applications. Parameters of Eq.1: for JUST population, D = 100, C = 48.3, ED_{50} = 63.4, and b = 0.80; for MUSR population, D = 100, C = 51.99, ED_{50} = 46.7, and b = 4.56; for FAIS population, D = 100, C = 57.4, ED_{50} = 510, and b = 2.36. (Abbreviations: O. = Observed; P. = Predicted).

Paraquat

Lack-of-fit tests between individual non-linear equation and a non-linear model that considered the lower limits common for the response of the populations to paraquat indicated that it is possible to assume equal lower limits. A further non-linear regression routine and lack-of-fit tests indicated that assuming equal slopes for the three populations is not appropriate (Figure 3). Estimated ED_{50} values ranged from 6.22 to 2.32 g a.i. ha^{-1}, which is very much less than the minimum recommended rate of 280 g a.i. ha^{-1} (Vencill, 2002).

DISCUSSION

Results of these experiments indicate variations in the responses of the three populations to herbicides commonly used in Jordan. However, the differences in response varied among herbicides and populations. The FAIS population had greater tolerance of 2,4-D, which can be

attributed to the genetic make-up rather than herbicidal response. Use of 2,4-D is very limited in nurseries and orchards and so shifts in tolerance would not have been expected.

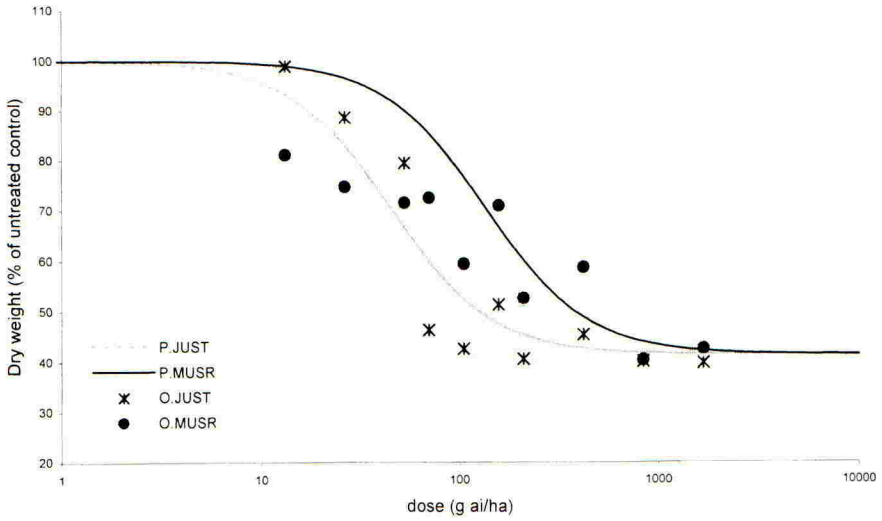

Figure 2. The log-logistic dose-response curves corresponding to differential sensitivity of two Jordanian *A. retroflexus* populations to glyphosate applications. Parameters of Eq.1: for JUST population, D = 100, C = 41.38, ED_{50} = 44.2, and b = 1.68; for MUSR, D = 100, C = 41.38, ED_{50} = 133.9, and b = 1.68. (Abbreviations: O. = Observed; P. = Predicted).

Results of the glyphosate experiment suggest that the three populations had somewhat different responses to this herbicide. This variation is also believed to be related to the genetics of the populations. The FAIS population, which was the most sensitive to glyphosate applications, as indicated by major dry weight reduction at low rates, was collected from a site where glyphosate applications are common. On the other hand, the relatively high ED_{50} value estimated for glyphosate in the MUSR population is thought not to be related to glyphosate application, which is uncommon in cereal-legume cropping systems.

For paraquat, the very high sensitivity observed in all three populations is more related to the experimental conditions and is not expected to be observed in field conditions. The growing conditions in our experiments apparently created plants that were very sensitive to this herbicide. Although variations in the estimated ED_{50} values for the three populations were minor, these values corresponded to different herbicide application rates in the experiment. This indicates that differential responses exist among the three populations to paraquat. Because associations between cropping systems and ED_{50} values could not be established, judging whether this variation is related to the frequency of paraquat use or not is not possible.

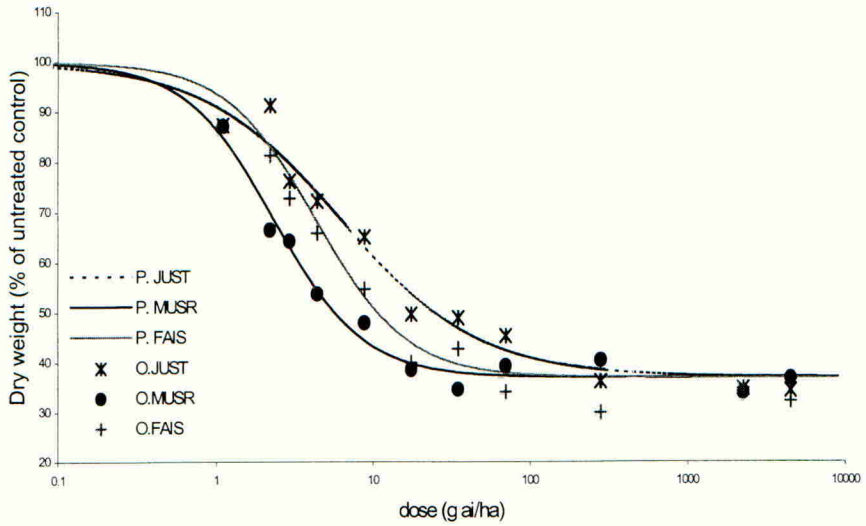

Figure 3. The log-logistic dose-response curves corresponding to differential sensitivity of three Jordanian *A. retroflexus* populations to paraquat applications. Parameters of Eq.1: for JUST population, D =100, C = 37.05, ED_{50} = 6.22, and b = 0.98; for MUSR population, D = 100, C = 37.05, ED_{50} = 2.32, and b = 1.53; and for FAIS population, D = 100, C = 37.05, ED_{50} = 4.29, and b = 1.49. (Abbreviations: O. = Observed; P. = Predicted).

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Characterisation of neonicotinoid resistance in *Bemisia tabaci* from Spain

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ABSTRACT

Three strains of the 'Q' biotype of the whitefly, *Bemisia tabaci*, from tomatoes in the Almeria region of southern Spain were tested for resistance to three neonicotinoid insecticides (imidacloprid, thiamethoxam and acetamiprid), two insect growth regulators (buprofezin and pyriproxyfen), as well as abamectin and diafenthiuron. Compared to a strain collected in 1994, two strains obtained in 2000 showed strong resistance to all three neonicotinoids. The more recent strains also resisted buprofezin and pyriproxyfen, through mechanisms considered genetically distinct from that conferring neonicotinoid resistance. All strains retained full susceptibility to abamectin and diafenthiuron. The response to imidacloprid of F_1 female progeny of reciprocal crosses between neonicotinoid-susceptible and -resistant strains was intermediate to those of females of the parental strains.

INTRODUCTION

The commercial introduction of neonicotinoid insecticides has provided agriculture with valuable new tools for controlling some of the world's most damaging crop pests. Many of the major pest species targeted by neonicotinoids, including aphids, whiteflies, planthoppers and Colorado potato beetle, have a long history of developing resistance to older insecticides. In most cases, neonicotinoids have proved to be unaffected by compounds used previously (Cahill & Denholm, 1999; Denholm *et al.*, 2002), and are therefore ideally suited as components of integrated pest management (IPM) and insecticide resistance management (IRM) strategies. However, the speed and scale with which neonicotinoids (especially imidacloprid) were taken up by growers have also led to concerns over the prospects and implications of selecting for resistance to neonicotinoids themselves (e.g. Cahill & Denholm, 1999).

In general, neonicotinoids have proved relatively resilient to resistance, with still very few cases of pests acquiring levels of resistance capable of compromising field efficacy. One of the most notable exceptions relates to the cotton or tobacco whitefly, *Bemisia tabaci* Gennadius, a highly polyphagous pest causing direct damage through feeding and by transmitting virus diseases to a wide range of arable and horticultural crops. Resistance of *B. tabaci* to imidacloprid was first reported in the intensive horticultural production system occupying over 40,000 ha near Almeria in southern Spain. A number of strains collected from Almeria in 1994 and 1995, and tested using a systemic leaf-dip bioassay, showed significantly reduced mortality at a diagnostic concentration of imidacloprid (Cahill *et al.*, 1996a). At the time there was no evidence of this impairing the performance of imidacloprid in the field. More recently, resistance has increased in potency with strains exhibiting cross-resistance to other neonicotinoids (Nauen *et al.*, 2002).

Developments in the Almeria region constitute a significant problem in their own right, but also highlight the importance of developing control strategies to reduce reliance on neonicotinoids and to exploit a greater diversity of insecticide classes. In this paper we report on the responses of two imidacloprid-resistant strains *B. tabaci* from Spain to a range of neonicotinoid and non-neonicotinoid molecules, and provide preliminary results of experiments investigating the genetic characteristics of resistance to imidacloprid.

MATERIALS AND METHODS

Insect strains and rearing method

All whitefly strains were reared at Rothamsted on cotton (*Gossypium hirsutum*; cv. 'Deltapine 16') under a 16 h photoperiod at 27°C and without exposure to insecticides. Adults used in bioassays were between 2 and 8 days old.

The three strains of *B. tabaci* for which results are reported were as follows: ALM-1 – collected in the vicinity of Almeria from a tomato crop in 1994; SPAN-R1 and SPAN-R2 – two strains collected near Almeria from tomatoes in 2000. Based on native polyacrylamide gel electrophoresis of non-specific esterases, all three strains conformed to the 'Q' biotype of *B. tabaci*, which predominates on the Iberian Peninsula and occurs through the Mediterranean Basin including the Middle East (Horowitz *et al.*, 2003a).

Insecticides

All insecticides were applied as formulated products diluted to the required concentrations in an aqueous solution of 0.01% of the non-ionic wetter 'Agral' (Zeneca, UK). Compounds tested were imidacloprid (SL formulation, 20% a.i., 'Confidor'), thiamethoxam (WG formulation 25% a.i., 'Actara'), acetamiprid (SG formulation, 20% a.i., 'Mospilan'), pyriproxyfen (EC formulation, 0.86% a.i., 'Knack'), buprofezin (SC formulation, 25% a.i., 'Applaud'), abamectin (EC formulation, 0.18% a.i., 'Vertimec') and diafenthiuron (EC formulation, 25% a.i., 'Polo').

Bioassays

Leaf-dip assays for imidacloprid, thiamethoxam, acetamiprid, abamectin and diafenthiuron against adult whiteflies were based on the method published by Cahill *et al.* (1995), with leaf discs being dipped in insecticide solutions and adults being confined to these treated surfaces in ventilated Petri-dishes. Mortality was assessed after 48 h exposure for imidacloprid, thiamethoxam and abamectin. Mortality for acetamiprid and diafenthiuron was scored after 72 h. Bioassays for assessing the response of nymphs to buprofezin followed the method of Cahill *et al.* (1996b). Adults were confined in clip cages to cotton leaves trimmed into rectangles of approximately 40 mm x 50 mm, thereby providing a synchronised cohort of eggs. Leaves were dipped 11 days later (when whiteflies were at the 2nd nymphal instar) into either the required concentration of insecticide or into a control solution, and mortality was assessed when surviving insects had reached late fourth instar, 22-25 days after oviposition. The ovicidal activity of pyriproxyfen was determined following the method of Ishaaya & Horowitz (1995). Adult females were again confined on cotton leaves for 24 h in clip-cages and removed 24 h

later. Leaves with eggs were dipped for 20 s into the required concentrations of formulated pyriproxyfen or in deionized water as a control. Eggs were counted one day after treatment and egg-hatch was determined 10 days later. All bioassays had 2-3 replicates per concentration, and each bioassay was repeated at least 3 times. Data from repeat bioassays were pooled for probit analysis to estimate dose-response lines and LC_{50} values.

Crossing experiments

Since different biotypes of *B. tabaci* often show partial or complete reproductive incompatibility, genetic crosses to investigate the inheritance of traits such as resistance should be performed on strains of the same biotype (Horowitz *et al.*, 2003b). For this work, we established reciprocal crosses between the ALM-1 (imidacloprid-susceptible) and SPAN-R1 (imidacloprid-resistant) strains, both of the Q biotype. Virgin adults were obtained by placing individual pupae with leaf material into individual wells of 96-well microplates until emergence (Horowitz *et al.*, 2003b). Males and females were then placed on cotton leaves in perspex leaf-boxes until the emergence of F_1 adult progeny. Due to the haplodiploid genetics of *B. tabaci* (Denholm *et al.*, 1998), females produced from reciprocal crosses were heterozygous for alleles from each parent, whereas male progeny were hemizygous for one of the maternal alleles. Responses of F_1 female progeny relative to those of their parents therefore provided a preliminary indication of the dominance of the gene or genes conferring resistance in the SPAN-R1 strain.

RESULTS AND DISCUSSION

Bioassays with neonicotinoids

LC_{50} values for ALM-1 (Table 1), collected from Almeria in 1994, were close to those obtained separately at Rothamsted for a standard susceptible strain (SUD-S) of *B. tabaci* that has been maintained in laboratory culture since 1978. Thus, although ALM-1 showed low levels of imidacloprid resistance at the time of collection (Cahill *et al.*, 1996a), it appeared to have reverted towards susceptibility in the intervening years. Both SPAN-R1 and SPAN-R2 showed strong (200-fold or greater) resistance to imidacloprid, which was also evident for the other two neonicotinoids tested, thiamethoxam and acetamiprid. This finding of cross-resistance encompassing several neonicotinoids is consistent with other studies on contemporary Spanish populations (Nauen *et al.*, 2002). The primary mechanism of resistance in Spain appears to be one of enhanced detoxification based on cytochrome P-450 dependent monooxygenases (Nauen *et al.*, 2002), which evidently show sufficiently broad substrate specificity to affect a range of molecules within the neonicotinoid class of chemistry.

Bioassays with other insecticides

LC_{50} values for the other four insecticides tested against ALM-1 (Table 1) were again an accurate reflection of baseline responses for these compounds. The two insect growth regulators (IGRs), buprofezin and pyriproxyfen, were both resisted by SPAN-R1 and SPAN-R2, with resistance levels for pyriproxyfen being 100-fold or more. These two compounds are, however, structurally and functionally very distinct from each other, buprofezin inhibiting chitin formation and pyriproxyfen being a juvenile hormone mimic affecting hormonal balance

and disrupting embryogenesis (Ishaaya, 2001). The level of pyriproxyfen resistance, in SPAN-R2 especially, was similar to those reported in *B. tabaci* on cotton in some areas of Israel, where it occurs independently of responses to buprofezin and neonicotinoids (Horowitz *et al.*, 1999; A R Horowitz, pers. comm. 2002). Thus, although IGR resistance occurred alongside neonicotinoid resistance in SPAN-R1 and SPAN-R2, it seems certain that the mechanisms involved are genetically independent and have evolved separately under selection with the respective control agents. LC_{50} values for abamectin and diafenthiuron were consistent across all three strains, disclosing no evidence of resistance and demonstrating that these compounds remain fully effective against neonicotinoid-resistant populations.

Table 1. Response of three Spanish strains of *B. tabaci* to neonicotinoid and non-neonicotinoid insecticides (Figures shown are LC_{50} values computed by probit analysis and expressed as ppm a.i.)

Insecticide	ALM-1	SPAN-RI	SPAN-R2
Imidacloprid	10	2200	>5000
Thiamethoxam	15	800	1400
Acetamiprid	3.5	60	210
Buprofezin	0.3	1.2	4.0
Pyriproxyfen	0.001	0.1	0.7
Abamectin	0.02	0.01	0.007
Diafenthiuron	52	53	68

Crossing experiments

Both reciprocal crosses between ALM-1 and SPAN-R1 produced substantial numbers of female progeny, proving that successful mating had taken place. Dose-response relationships for imidacloprid against females of both parental strains are shown in Figure 1. Mortality data for SPAN-R1 above 1000 ppm were erratic and implied important pharmo-kinetic constraints on bioassays that exceed this concentration. Mortality data for F_1 progeny of the reciprocal crosses were similar, and therefore pooled for presentation in Figure 1. These F_1 responses, although intermediate to those of the parental strains, appeared closer to those of ALM-1.

Further work is required to validate this result for a larger number of strains, and for neonicotinoids other than imidacloprid. The implications of heterozygote expression for the speed of resistance selection must also be interpreted with care, given that a primary consequence of haplodiploidy is that resistance genes arising by mutation are exposed to selection from the outset in hemizygous males, irrespective of intrinsic dominance or recessiveness (Denholm *et al.*, 1998).

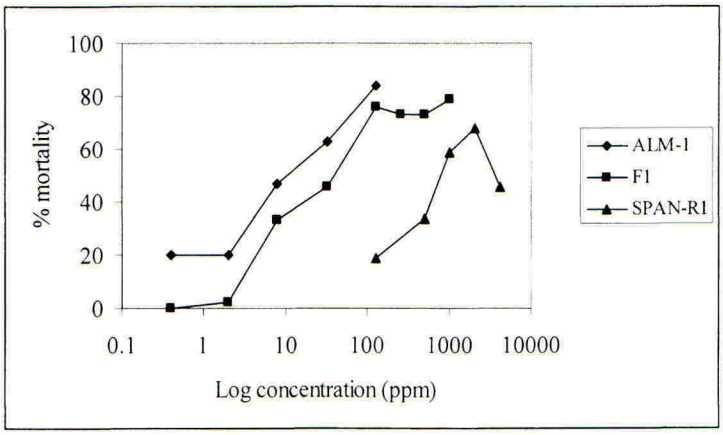

Figure 1. Response of parental females and F_1 female progeny to imidacloprid.

CONCLUSIONS

Although cases of neonicotinoid resistance in insects are still rare and relatively localised (Denholm *et al.*, 2002), developments with *B. tabaci* in Spain have highlighted the potential of pests to adapt and withstand exposure to this important group of insecticides. The status of whitefly resistance to neonicotinoids in other countries is less well documented, abut there are recent confirmed reports from Israel (A R Horowitz, pers. comm. 2003), Australia (R Gunning, pers. comm. 2002), Cyprus (M Hadjistylii, unpublished data), and isolated cases from greenhouses elsewhere in Europe (Nauen *et al.*, 2002). The homology of the underlying mechanism(s) to that present in Spain is largely unknown, as are the implications for cross-resistance between neonicotinoids and to unrelated molecules. Studies to compare the genetic and toxicological characteristics of resistance in strains from different parts of the world are clearly a priority, as is work to exploit lessons from regions such as the south-western USA where neonicotinoid resistance has so far been combated successfully (Denholm *et al.*, 1998, 2002; Li *et al.*, 2000). Management strategies based on the restriction and co-ordination of neonicotinoid use, coupled with alternation of chemical groups, currently offer greatest scope for sustaining the efficacy of neonicotinoids against *B. tabaci* and other target pest species (Denholm *et al.*, 2002).

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Negative cross-resistance between indoxacarb and pyrethroids in the cotton bollworm, *Helicoverpa armigera*, in Australia: a tool for resistance management

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ABSTRACT

Helicoverpa armigera is Australia's most important agricultural pest, especially on cotton. Insecticide resistance in *H. armigera* is, however, an enduring threat to the economic production of cotton in Australia and pyrethroid resistance has been of particular concern. Insecticide use against *H. armigera* on cotton is subject to an insecticide resistance management strategy and new insecticides such as indoxacarb are particularly important. Indoxacarb requires bio-activation to a toxic metabolite. Our studies show that, in Australian *H. armigera*, this is performed by esterase isoenzymes with conversion to the active metabolite of indoxacarb being correlated with esterase titre. Pyrethroid-resistant *H. armigera* has overproduced esterases and our results show greater indoxacarb conversion compared to susceptible strains. Indoxacarb had significantly better efficacy against more highly pyrethroid-resistant strains of *H. armigera*. Negative cross-resistance between indoxacarb and pyrethroid resistance should prove a valuable tool for the management of pyrethroid resistance in Australian *H. armigera*.

INTRODUCTION

The cotton bolloworm, *Helicoverpa armigera*, is arguably Australia's most important agricultural pest, especially on cotton and other summer crops. *H. armigera* has a long history of insecticide resistance in Australia (involving DDT, pyrethroids, carbamates, organophosphates and endosulfan), and resistance is an enduring threat to the economic production of cotton. Pyrethroid resistance in *H. armigera* has been of particular concern and has become fixed in some *H. armigera* populations. Pyrethroid resistance is mediated by overproduced esterase isoenzymes that metabolise and sequester pyrethroids (Gunning *et al.*, 1996)

Since the early 1980s, the use of insecticides against *H. armigera* on cotton has been regulated by a resistance management strategy to manage old chemistry such as the pyrethroids and the registration and deployment of new insecticides with novel modes of action such as indoxacarb. Indoxacarb is a potent sodium channel blocker with broad-spectrum efficacy against Lepidopteran larvae (Wing *et al.*, 1998). It requires bio-activation to an active, toxic metabolite, a decarbomethoxylated indoxacarb (DCJW). Studies indicate that indoxacarb conversion can be prevented by esterase inhibitors (Wing *et al.*, 1998). This paper describes how, in Australian *H. armigera*, indoxacarb is activated by esterase

isoenzymes and how, as a consequence, there is negative cross-resistance between pyrethroids and indoxacarb in the species.

MATERIALS AND METHODS

Insects and bioassay

A laboratory susceptible strain of *H. armigera* was used in these studies, as well as pyrethroid-resistant strains (obtained by selecting with fenvalerate). Third-instar contact-toxicity bioassays were used. Technical grade fenvalerate [(*RS*) -α-cyano-3-phenoxybenzyl (*RS*)-2-(4-chlorophenyl)-3-methylbutyreate] and technical indoxacarb [(*S*)-7-chloro-2,5-dihydro-2-[(methoxycarbonyl)indeno[1,2,-e]=[1,3,4]oxadiazin-2-ylcarbonyl]-´4-(trifluromethoxy) carbanilate] were dissolved in acetone and serially diluted concentrations prepared. Larvae were treated with 1 µl of solution. After dosage, the test larvae were held at 25° C with adequate food. Mortality was assessed 2 (fenvalerate) and 4 (indoxacarb) days after treatment. The data were analysed by probit analysis. Resistance factors were calculated as the ratio of the resistant LD_{50} / susceptible LD_{50}.

Esterase activity

Total esterase activity of pyrethroid-susceptible and resistant larval homogenates were detected using 1-naphthyl acetate as a substrate and kinetic assays (Gunning *et al.*, 1996). Larvae (3 – 4 mg) were homogenised in 2M phosphate buffer (pH 7.0) with 0.01% Triton X-100 (50 µl per 4 mg/insect tissue). Aliquots (10 µl) were transferred to a microplate and 240 µl of phosphate buffer (pH 6) containing 0.6% fast blue salt RR and 1.86% 1-naphthyl acetate added. Assays were run immediately on a BioRad 3550 microplate reader in kinetic mode at 450 nm.

Metabolism experiments

Larvae were homogenised in Tris buffer (pH 8, 0.05% Triton X-100). Indoxacarb was added to a final concentration of 10^{-4}M, total volume 200 µl. The homogenates were incubated for 140 min at 25°C. The reaction was stopped by the addition of 200 µl of very cold (-20°C) acetone. 200 µl of samples were loaded onto reverse phase, fluorescent, silica gel, thin layer chromatography plates. The running solvent was methanol:water (9:1) made up to 1% formic acid. Samples were co-chromatographed with indoxacarb and DCJW standards. Indoxacarb and DCJW were visualised under UV light and quantified by density scanning.

Similar experiments were performed with purified *H. armigera* esterase (from pyrethroid-resistant strains). Purified esterase was diluted in Tris buffer to concentrations of 1740, 870, 435, 218 and 109 mOD/min per 5 µl aliquot. A buffer only control was included.

RESULTS

Bioassay data

Bioassays showed a strong correlation between the toxicity of indoxacarb and pyrethroid resistance factor in the *H. armigera* strains tested (Figure 1). Negative cross-resistance between indoxacarb susceptibility and pyrethroids was therefore indicated. Indoxacarb had significantly greater toxicity against the more highly pyrethroid-resistant strains. There was an approximately 10-fold difference in the toxicity of indoxacarb between *H. armigera* strains and 150-fold resistance to fenvalerate.

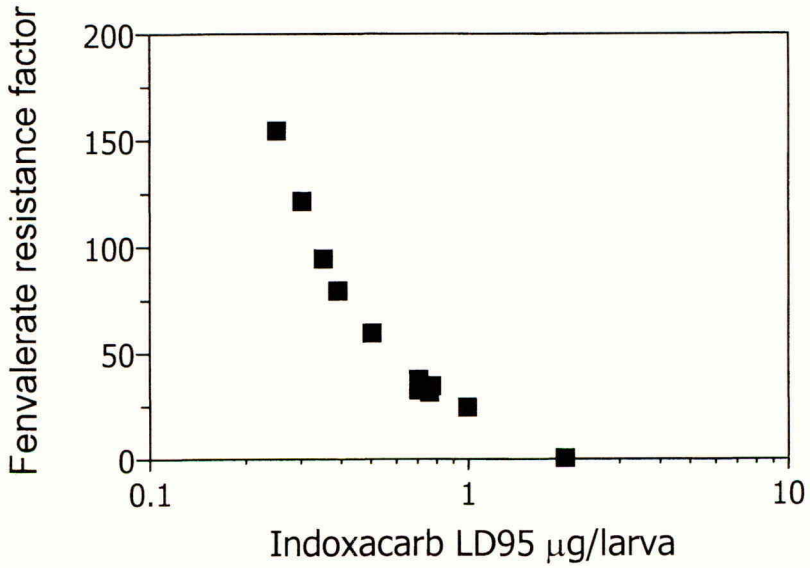

Figure 1. Toxicity of indoxacarb to strains of pyrethroid-resistant Australian *H. armigera.*

Esterase analysis

The mean esterase activity in each pyrethroid-resistant strain was determined and plotted against indoxacarb toxicity. Increasing esterase activity in the pyrethroid-resistant strains of *H. armigera,* gave rise to greater susceptibility to indoxacarb (Figure 2). Increasing esterase activity is correlated with increasing pyrethroid resistance factor (Gunning *et al.*, 1996).

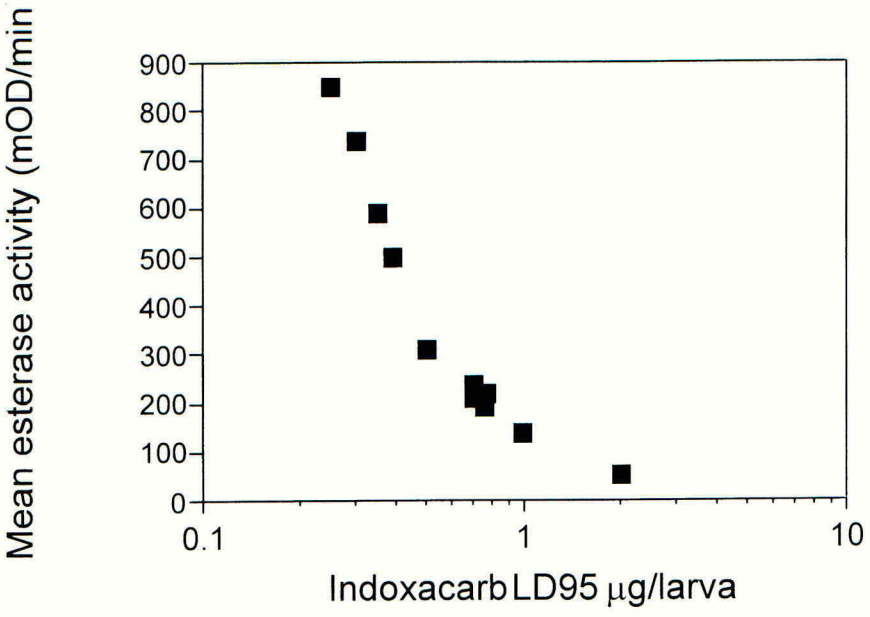

Figure 2. Relationship between total esterase and indoxacarb toxicity in pyrethroid-resistant strains of Australian *H. armigera.*

Bioactivation of indoxacarb by *H. armigera*

A good separation of indoxacarb and the metabolite DCJW was achieved by the thin layer chromatography solvent system and RF values for were 0.64 and 0.55 respectively.

When indoxacarb was incubated with *H. armigera* homogenate there was clear evidence of the formation of DCJW and that conversion was related to esterase concentration (Figure 3). Increasing esterase activity resulted in increased activation of indoxacarb to DCJW.

Indoxacarb was also incubated for 24 h with varying concentrations of purified esterase associated with resistance in *H. armigera*. Conversion to DCJW was directly proportional to esterase concentration and complete conversion could be achieved at higher esterase concentrations (Figure 4)

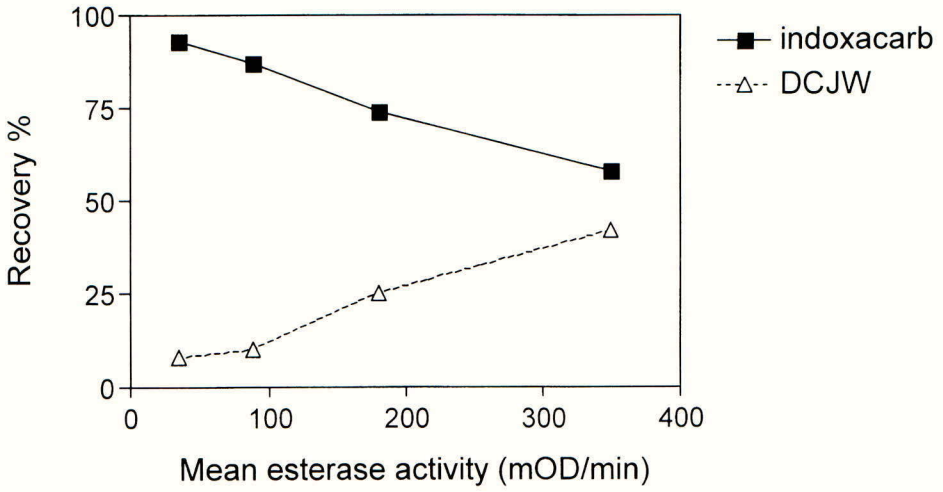

Figure 3. *In-vitro* activation of indoxacarb by *H. armigera* homogenates with varying concentrations of pyrethroid resistance-related esterase.

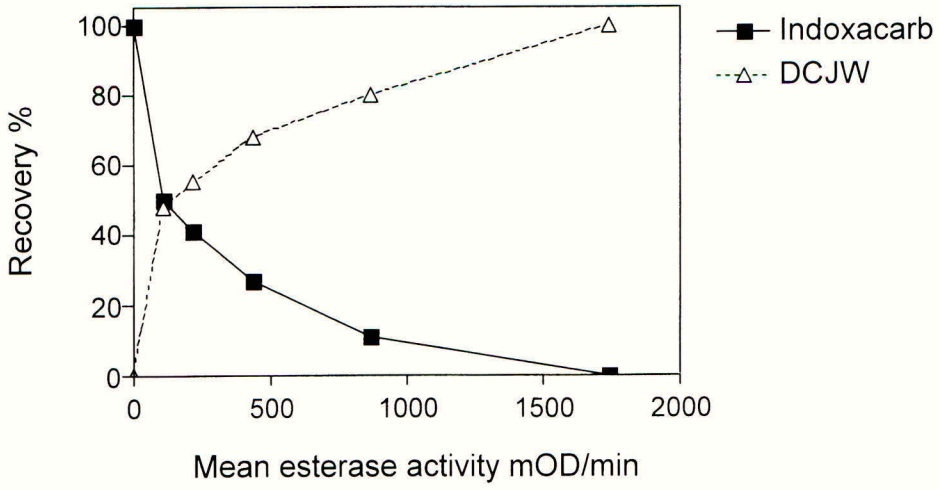

Figure 4. *In-vitro* activation of indoxacarb to DCJW by varying concentrations of purified *H. armigera* pyrethroid resistance-related esterase.

CONCLUSIONS

Our results show that, in Australian *H. armigera*, indoxacarb is bio-activated to the toxic metabolite after topical application of indoxacarb. *In-vitro* studies showed that in *H. armigera* indoxacarb was activated to DCJW by pyrethroid resistance associated esterase enzymes. Activation rates were proportional to the esterase titre.

Bioassays of strains of pyrethroid-resistant *H. armigera* showed that indoxacarb toxicity increased with an increasing pyrethroid resistance factor. The increased indoxacarb toxicity was correlated with increased titres of esterase associated with pyrethroid resistance in the *H. armigera* strains. Clearly, the negative cross-resistance exhibited between pyrethroid and indoxacarb in Australian *H. armigera* results from increased conversion of indoxacarb to the active metabolite by esterases also involved in the hydrolysis or sequestration of pyrethroids in resistant strains.

Despite resistance problems, pyrethroids have remained a valuable, low cost insecticide group for controlling *H. armigera* on cotton in Australia. However, pyrethroid performance has declined markedly in recent years. While indoxacarb is an excellent insecticide in its own right, negative cross-resistance to pyrethroids gives us an exciting tool to restore some measure of pyrethroid susceptibility. Indoxacarb has significantly greater toxicity to highly pyrethroid-resistant *H. armigera* and could be applied to reduce pyrethroid resistance levels. As indoxacarb is reliant on esterase for bio-activation in *H. armigera*, we should be cautious, however, in tank-mixing with other esterase-inhibiting insecticides such as organophosphates and pyrethroids.

ACKNOWLEDGEMENTS

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Biological evaluation of spiromesifen against *Bemisia tabaci* and an assessment of resistance risks

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ABSTRACT

The efficacy of spiromesifen was evaluated against insecticide-resistant 'Q' biotypes of *Bemisia tabaci* from Israel and Spain. The compound was extremely effective against early instars (LC_{50}s of 0.1 – 6.2 ppm for 12-d-old nymphs). One Spanish 'Q' type was significantly less susceptible (15-fold). Further selection of that strain with spiromesifen however, did not select for increasing resistance. Spiromesifen was highly effective against pyriproxyfen- and imidacloprid-resistant whitefly. It also had pronounced transovariole effects on oviposition and egg hatch. Given the current difficulties experienced in the control of 'Q' biotype *B. tabaci* around the Mediterranean, it is likely that spiromesifen will be a valuable addition to the available chemical options. We consider that the efficacy of this compound is unlikely to be compromised by existing resistance mechanisms.

INTRODUCTION

Over the past ten years, the whitefly *Bemisia tabaci* (Homoptera: Aleyrodidae) has become a devastating insect pest of horticultural crops (especially tomatoes, curcubits and peppers) in southern Europe, North Africa and the Middle East, and it remains a considerable threat to protected horticulture in northern Europe. It can cause serious crop losses by direct feeding, but its pest status has increased dramatically due to the widespread occurrence of several whitefly-transmitted plant viruses (e.g. *tomato yellows leaf curl virus*; TYLCV).

The effective management of *B. tabaci* and its associated plant viruses poses a severe challenge to scientists and the horticultural and pest management industries. Although much work is being conducted to formulate management tactics, including cultural, physical and biological control methods, these are proving difficult to implement on a large scale. This is due in part to the fact that *B. tabaci* is an extremely efficient vector of some viruses (e.g TYLCV; Mehta *et al.*, 1994) and growers can tolerate only very low thresholds of insects. Insecticides therefore remain an integral component of *B. tabaci* control.

There is however, in this species, an alarming increase in resistance not only to more conventional insecticides but to novel and environmentally compatible compounds such as buprofezin, pyriproxyfen and the neonicotinoids (Horowitz & Ishaaya, 1992; Elbert & Nauen, 2000; Ishaaya & Horowitz, 1995), all of which are highly relevant to Southern European horticulture.

The current study was conducted to ascertain whether the novel compound spiromesifen (Nauen *et al.*, 2002) might be a useful addition and / or alternative to the chemical tools already available for whitefly control in the Mediterranean region. The test populations used were predominantly of the 'Q' biotype, as this is the biotype that is currently perceived as being the most problematic in the Mediterranean basin.

MATERIALS AND METHODS

All assays were conducted using formulated spiromesifen supplied by Bayer CropScience on strains of *B. tabaci* listed in Table 1.

Table 1. Biotype and response of strains of *Bemisia tabaci* to imidacloprid and pyriproxyfen

Strain	Year collected	Origin	Biotype	Imidacloprid / pyriproxyfen resistance[a]
SUDS	1978	Sudan	-	None
ALM1	1994	Spain	Q	Slight /none
ESP99	1999	Spain	Q	Medium/none
EL EJIDO	2000	Spain	Q	High/medium
HOF CARMEL	1998	Israel	Q	Medium/medium
PYRI-R	Lab. selected	Israel	Q	None/high

[a]unpublished data (Rothamsted Research)

Effects on second instars (12-d-old nymphs)

All larval-dip bioassay protocols were similar to those published by Cahill *et al.* (1996). All except three true leaves of a cotton plant were removed. The remaining three were cut to a size of c. 20 cm^2. Plants were infested with adult whiteflies in clip cages at c. 20 mass-reared females per leaf for 48 h. Afterwards the whiteflies were removed and, at the appropriate point (when the desired stage was predominant), the infested leaves were dipped in serial dilutions of insecticide for 10 s. Percentage mortality was scored by counting all dead, live, hatched or unhatched eggs and nymphs at day 21, once all survivors had progressed through to the pupal instars.

Selection experiments

On three occasions the population most tolerant of the spiromesifen compounds (ESP99) was further selected by dipping 12-d-old nymphs in 10 ppm spiromesifen solutions and assaying the F1 progeny of the survivors.

Transovariole effects on egg-hatch

Adults were exposed to different concentrations of material on leaf boxes for 72 h. Survivors were then clip-caged to whole plants for a further 48 h. Subsequent hatch of the resulting eggs

was monitored at day 10. This method is analogous to that used by Ishaaya & Horowitz (1992) for assessing the transovariole effects of pyriproxyfen.

Larger scale illustrations of spiromesifen efficacy against resistant *B. tabaci*

In order to illustrate the efficacy of spiromesifen on large, insecticide-resistant populations of mixed life stages under 'field conditions', sizable populations of *B. tabaci* were established on cotton plants in large population chambers (Rowland *et al.*, 1990).

Effect of spiromesifen on a pyriproxyfen-resistant population

An Israeli 'Q' biotype strain, resistant to imidacloprid and pyriproxyfen (Hof Carmel) was used for this illustration. Thirty-one days after infestation, with populations of >2000 insects spread over six cotton plants (5 – 6 node stage), these simulators were sprayed to near run-off with the recommended rates of spiromesifen (Bayer CropScience, 150 ppm a.i.) or pyriproxyfen (Sumitomo, Sumilarv; 40 ppm a.i.).

Effect of spiromesifen on an imidacloprid-resistant population

A Spanish, imidacloprid-resistant 'Q' biotype (ESP99) was used for this illustration. Pots containing cotton plants were treated with the recommended field rate of imidacloprid (Scotts, Intercept 70WG, 0.02 g/l of compost) or were left untreated. Two days after the treatment date all plants, whether imidacloprid-treated or not, were infested with whitefly. On day 12, when large numbers of nymphs were present on the plants, untreated plants were sprayed with the recommended rate of spiromesifen.

RESULTS AND DISCUSSION

Bioassays

The LC_{50}s for 12-d-old (2^{nd} instar) nymphs varied between 0.10 ppm (Pyri-R) and 6.16 ppm (ESP99) (Table 2). ESP99 was significantly more tolerant than the SUDS standard (15-fold), but this tolerance could not be further selected for. None of the tolerance patterns seen correlated with imidacloprid or pyriproxyfen resistance.

Effects on egg-lay and egg-hatch

The effects of pre-exposing adults to spiromesifen residues for 72 h were consistent (Fig. 1). At concentrations of 10 - 100 ppm, the mean number of eggs laid by females over a 72-h period was reduced dramatically. Moreover, the numbers of these eggs hatching was also affected. The combined effects of spiromesifen on egg-lay and egg-hatch clearly have the potential to severely limit fecundity in *B. tabaci*.

Figure 1. Effect of exposing adults of *Bemisia tabaci* to spiromesifen for 72 h on subsequent egg-lay over the next 72 h on a fresh, untreated surface (means and SEs).

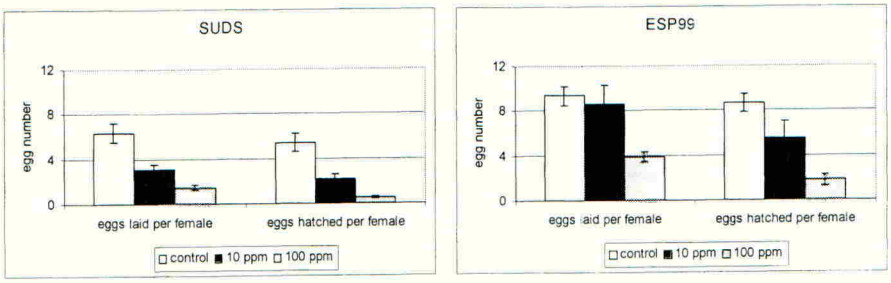


Table 2. Mortality of 12-d-old *Bemisia tabaci* nymphs, in response to spiromesifen treatment

Strain	n	LC_{50} (ppm)[a]	95% CLs	Slope	RF
SUDS	20528	0.42 b	0.25 to 0.64	0.96 ±0.02	-
PYRI-R	898	0.10 a	0.02 to 0.22	0.99 ±0.10	0.2
EL EJIDO	4744	0.36 ab	0.17 to 0.70	0.68 ±0.02	0.8
Hof Carmel	3385	0.90 bc	0.25 to 2.44	0.68 ±0.03	2.1
ALM1	3830	1.24 bc	0.56 to 2.35	0.92 ±0.04	3.0
ESP99[b]	7067	6.16 d	3.12 to 10.87	0.84 ±0.03	14.7
ESP99-sel (x2)[b]	3266	2.59 cd	1.05 to 5.03	1.00 ±0.05	6.2
ESP99-sel (x3)[b]	878	3.26 cd	2.30 to 4.32	1.62 ±0.20	7.7

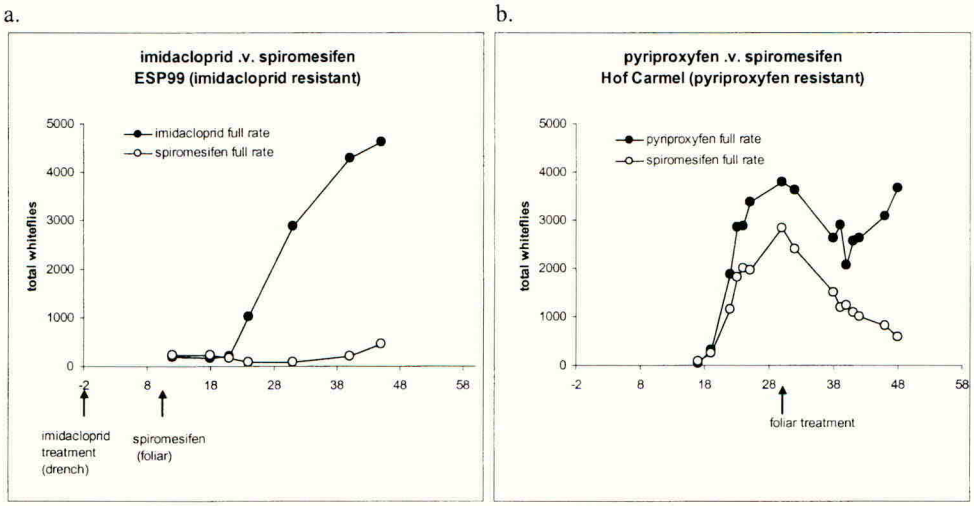
[a] Limits followed by different letters denote significant differences ($p<0.05$).
[b] Sub-populations of ESP99 were further selected by spiromesifen (10 ppm) to ascertain whether the variation in tolerance could be increased.

Laboratory bioassays, in which insects are treated topically or confined to treated leaf surfaces for prescribed periods, are convenient for screening the intrinsic toxicity of insecticides against different life stages. They are not necessarily very informative about efficacy under field conditions where deposition is usually non-uniform and where some stages may avoid exposure by inhabiting parts of the plants not reached by insecticide. Additional illustrations of the efficacy of the spiromesifen compound, on large populations of insecticide-resistant whitefly, were therefore considered desirable.

The compound was very effective against imidacloprid-resistant whitefly (Figure 2a). At 48 d (40 d after soil application of imidacloprid and 37 d after spraying with spiromesifen the full rate of spiromesifen had reduced numbers to just 5% of those that had been treated with the full rate of imidacloprid.

Spiromesifen was also extremely effective against pyriproxyfen-resistant whitefly (Figure 2b). Twenty-five days after spraying, the population sprayed with spiromesifen was still in decline (numbering < 500 individuals) whilst that sprayed with pyriproxyfen had returned to pre-spray levels (numbering > 4000 individuals).

Figure 2. The effect of spiromesifen on imidacloprid –resistant whitefly (a) and on pyriproxyfen-resistant whitefly (b).



CONCLUSION

Spiromesifen is clearly highly effective against *B. tabaci.* It is lethal to young instars at low concentrations and has additional effects on oviposition and on egg hatch. Its efficacy is unaffected by imidacloprid or pyriproxyfen resistance. It has considerable potential as a chemical control tool for this pest.

ACKNOWLEDGEMENTS

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Fluoxastrobin: risk assessment and anti-resistance management strategy for seed treatment application in winter wheat

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ABSTRACT

Fluoxastrobin is a novel broad-spectrum strobilurin (QoI) fungicide from the chemical class of dihydro-dioxazines. Applied as a seed treatment, fluoxastrobin shows very good efficacy at 5-10 g a.i./100 kg seed against seed- and soil-borne pathogens such as *Microdochium nivale* and *Tilletia caries* but no activity against wind-borne diseases. In the case of strobilurins, strains of several pathogens targeted by foliar application have developed field resistance to the QoI fungicides. As fluoxastrobin is the first QoI fungicide expected to be registered for application to cereal seeds in Europe, a very detailed risk assessment has been undertaken. Additionally, extensive base-line sensitivity studies and investigations on the chemodynamic behaviour have been carried out. This assessment identified a somewhat enhanced risk only in the case of *M. nivale* as this pathogen may be targeted by QoIs and by fluoxastrobin from both seed and foliar application within a crop/season. A robust resistance management strategy will therefore be implemented and fluoxastrobin will be marketed for both seed and foliar use only in combination with DMI-fungicides, particularly prothioconazole (which is also effective against *M. nivale*), providing a product with complementary and excellent efficacy against economically important seed- and soil-borne diseases whilst providing an appropriate anti-resistance management strategy in line with FRAC guidelines.

INTRODUCTION

Fluoxastrobin (code name: HEC 5725) belongs to the chemical class of the methoxyimino-dihydro-dioxazines. This fungicide is a strobilurin analogue and binds to the Q_o–ubiqinone binding-site of the cytochrome bc_1 subunit of complex III (Becker *et al.*, 1981). Fluoxastrobin shows cross-resistance to other QoI fungicides. Fluoxastrobin controls economically important pathogens from all fungal classes by inhibition of mitochondrial respiration. Targeted as foliar application in combination with a DMI (prothioconazole), diseases controlled by fluoxastrobin include: septoria leaf spot and glume blotch, brown and stripe rust, tan spot, scald and net blotch (Dutzmann *et al.*, 2002). As fluoxastrobin is the first QoI fungicide expected to be registered not only for foliar application but also for treatment of winter wheat seeds in Europe, an extensive risk assessment has been undertaken. This analysis was based on studies including field trials, base-line sensitivity assessment, chemodynamic behaviour and quantification of residues.

MATERIALS AND METHODS

Assessment of spectrum of efficacy under field conditions

Numerous field trials were carried out in compliance with approved guidelines from 1997 to 2001 in order to establish the optimum use pattern for fluoxastrobin and to characterize the effect of a seed treatment containing fluoxastrobin on wind–borne diseases such as powdery mildew or septoria leaf spot (*Septoria tritici*). As wheat is normally sown relatively late in autumn, the incidence of mildew infection tends to be low. Furthermore, the appearance of resistance to QoI fungicides in winter wheat since 1999 did not allow a trial series to be made over several years. For this reason, characterization of activity on powdery mildew was performed on barley, as barley is sown some weeks earlier and is more regularly prone to mildew infections.

Sensitivity profile of *Microdochium nivale* populations

To obtain isolates of *Microdochium nivale*, kernels originating from grain samples taken from different cereal-growing areas in Europe were surface-sterilized with sodium hypochlorite immediately after harvest and placed in Petri dishes on potato dextrose agar. After incubation under near-UV radiation at 18°C for 6 days, mycelia and conidiospores could be microscopically identified and single spore isolates generated on the same media. For determination of baseline sensitivity, a microtitre test system was used. For this, potato dextrose broth was treated with different concentrations of fluoxastrobin a.i. (0.0003, 0.001, 0.003, 0.01, 0.03, 0.1 and 0.3 mg/l) and used to fill wells of a 96-well microtitre plate (two wells per concentration). Then, each well was inoculated with 200 conidiospores of a single-spore isolate and incubated for 6 days at 20°C and 150 rpm in darkness. Two untreated wells inoculated with the same isolate served as a control. After incubation, the mycelial growth was measured photometrically and EC_{50} values were calculated on the basis of different light transmission depending on mycelial density.

Systemicity studies with radio-labeled active ingredients

In controlled systemicity studies, wheat seeds (cv. Kanzler) were treated with ^{14}C-labelled fluoxastrobin, as FS-formulation, at 15 g a.i./100 kg of seed. Seeds were planted into an artificial substrate (LECA) and maintained at 18°C and 70% relative humidity under hydroponic cultivation conditions. Artificial light was given for 12 h/day. At the end of the experiment, plants were harvested and dried. The distribution of radioactivity was determined with the aid of an image analyser (Fuji X BAS 2000); illumination period, 24 h. The false colour processing was done with the software TINA.

Determination of fungicide concentrations in the second and third leaf

To investigate residue levels in the growing crop, green-mass samples (untreated, fluoxastrobin 10 g a.i./dt seed, fluoxastrobin + azole 7.5 + 7.5 g a.i./dt seed) were taken from a field trial at the 2-leaf and 3-leaf stages. With standard residue methods (method 00649), the concentration of fungicidally active ingredients in the whole shoot was determined.

RESULTS AND DISCUSSION

Field efficacy of fluoxastrobin

Results of field trials show that fluoxastrobin, when applied as a seed treatment at the proposed dosage of <5-10 g/dt seed, had no significant systemic effect on the leaf pathogens *Blumeria graminis* (barley powdery mildew) or *Septoria tritici* (Table 1). Therefore, seed treatment is not expected to cause significant selection pressure on leaf, stem and ear pathogens such as *Blumeria graminis* or *Septoria tritici*.

According to the efficacy spectrum (Table 2), *Microdochium nivale* is the only important pathogen which may be targeted by QoI fungicides and by fluoxastrobin from both seed and foliar application within a crop/season. Therefore, *M. nivale* is the only important pathogen for which, theoretically, there could be an enhanced risk of resistance.

Table 1. *In-vivo* activity of fluoxastrobin against wind-borne fungi after seed treatment in trials in Germany

Pathogen	Dose of fluoxastrobin (g a.i./dt)	% disease control[a]	
		Trial 1	Trial 2
Blumeria graminis f.sp. *hordei*	untreated	(26.7%)	(5.7%)
	10	0	7[ab]
Septoria tritici	untreated	(7%)	(7%)
	5	0	0
	10	0	0
	15	0	0

[a] % infection in untreated wheat is shown in brackets.
[b] Statistically not significant using t-test at $p < 0.05$.

Table 2. Summary of *in-vivo* spectrum of activity of fluoxastrobin against different fungi after seed and foliar application in wheat

Pathogen (disease)	Efficacy level of fluoxastrobin under field conditions	
	Seed treatment (≤ 10 g/dt seed)	Foliar application (recommended rate)
Blumeria graminis f.sp. *tritici* (barley mildew)	-	+/+++
Septoria tritici (septoria leaf spot)	-	+++
Ustilago spp. (rusts)	++	-
Tilletia caries (bunt)	+++	-
Fusarium culmorum; F. avenaceum; Gibberella zeae (fusarium ear blight)	+	-/+
Microdochium nivale (seedling blight; snow mould)	+++	++/+++
Pseudocercosporella herpotrichoides (eyespot)	-	+/++

-, no activity; +, low activity; ++, moderate activity; +++, high activity.

Base-line sensitivity monitoring of *Microdochium nivale* towards fluoxastrobin

The sensitivity profile of *Microdochium nivale*, established on the basis of 308 single spore isolates originating from France, Great Britain, Germany and the Netherlands shows that mean EC_{50} values (in mg/l) varied between 0.0016 and 0.0049 at the maximum over all regions, indicating very small differences in sensitivity towards fluoxastrobin (Table 3). Moreover, the standard deviation in all regions was low and the factor mEC_{50}max / mEC_{50} min over all tested isolates is very small (value: 3.1). This narrow sensitivity profile shows that the population of *M. nivale* is very homogeneous and allows the assumption that this fungal population has, up to now, not been exposed to a significant selection pressure by QoI fungicides.

Table 3. Sensitivity base-line of *Microdochium nivale* towards fluoxastrobin (EC_{50} in mg/l) from different regions of Europe in the year 2000

Country: region or department	n	mEC_{50} value	Standard deviation
France:			
Nord Pas de Calais, Picardie	77	0.0023	0.0011
Bretagne. Loire Atlantique	11	0.0020	0.0006
Bourgogne	2	0.0024	0.0012
Centre	13	0.0020	0.0009
Pays de la Loire. Poitou-Charentes	3	0.0027	0.0011
Cher. Nievre	7	0.0022	0.0004
Aude. Bouches du Rhone	2	0.0026	0.0004
Gers	6	0.0017	0.0006
Indre et Loire. Sarthe	10	0.0022	0.0007
Alsace	11	0.0021	0.0008
Champagne-Ardennes	14	0.0022	0.0006
Haute Garonne	4	0.0019	0.0003
Aisne. Alpes maritimes	6	0.0021	0.0004
Maignelay	3	0.0020	0.0005
Aube. Yonne	10	0.0021	0.0006
Haute Normandie	1	0.0049	
Ile de France	3	0.0016	0.0006
Rougemontier	8	0.0025	0.0007
others	15	0.0020	0.0005
UK:			
Weymouth	2	0.0016	0.0004
Germany:			
Burscheid	7	0.0017	0.0004
Weikersheim	18	0.0020	0.0006
Haidenhofen	2	0.0018	0.0002
Netherlands:			
Moerstraten. Fijnaart	32	0.0022	0.0009
Others	41	0.0024	0.0008
Mean		0.0022	0.0006
Total	308		

Systemicity studies with radio-labelled active ingredients

It is a general experience that uptake rates in hydroponic systems are significantly greater than under practical conditions in the field. Therefore, the relative distribution of a fungicide within the plant can be determined, but not the absolute uptake rate.

In these systemicity studies, 84% of the applied label stayed within the kernel 8 days after sowing (Table 4). About 16% of the applied radioactivity was detected in the coleoptile and roots, whereas no measurable radioactivity was found in the first emerging leaf. After 11 days, about 3% and after 15 d about 4% of the total applied radioactivity per kernel was in the first leaf. In the second emerging leaf, less than 1% of the total radioactivity was found after 15 d and, in the third emerging leaf, the translocated amount of fluoxastrobin was below quantitative measurement. Fluoxastrobin showed low systemicity in seed-treated wheat plants but demonstrated good properties against seed and soil-borne diseases from its strong presence in the kernel.

Table 4. Distribution of ^{14}C fluoxastrobin in wheat plants after seed treatment

Period after planting (d)	% of applied activity in kernels	% of applied activity in root and coleoptile	% of applied activity first leaf	% of applied activity second leaf	% of applied activity third leaf
8	84	16	0	-	-
11	73	23	3	0	-
15	70	>25	4	<1	0

Determination of fungicide concentrations in the second and third emerging leaves

Table 5. Concentrations in young wheat leaves after seed treatment with fluoxastrobin (field trial)

Growth stage at analysis	Treatment [g a.i./dt]	Residue (mg/kg)		
		fluoxastrobin (E-)	fluoxastrobin (Z-)	total fluoxastrobin
2 leaf stage	untreated	<0.045	<0.005	<0.05
	fluoxastrobin (10 g)	<0.045	<0.005	<0.05
	fluoxastrobin & azole (7.5 g & 7.5 g)	<0.045	<0.005	<0.05
3 leaf stage	untreated	<0.045	<0.005	<0.05
	fluoxastrobin (10 g)	<0.045	<0.005	<0.05
	fluoxastrobin & azole (7.5 & 7.5 g)	<0.045	<0.005	<0.05

Method used:
fluoxastrobin: method 00649. LoQ = 0.05 mg/kg (total); 0.045 mg/kg (E-isomer); 0.005 mg/kg (Z-isomer).

Results of the field residue study (Table 5) show that, within the detection limits, no measurable residues of fluoxastrobin applied at 10 g a.i./dt could be detected in wheat plants

harvested at the 2- or the 3-leaf stages. In addition, the combination with an azole did not modify the systemic behaviour of fluoxastrobin as again no measurable residues could be detected. These findings strongly support the results that have been observed in the biological studies, showing that fluoxastrobin does not generate a significant selection pressure on wind-borne diseases.

CONCLUSIONS

Biological field trial results, supported by controlled systemicity studies and field residue analysis, showed that fluoxastrobin applied as a seed treatment at low doses (≤ 10 g a.i./100 kg seeds) does not cause a significant selection pressure on wind-borne diseases such as powdery mildew and septoria leaf spot.

The risk assessment identified a somewhat enhanced risk in the case of *Microdochium nivale* since this pathogen may be targeted by QoIs and by fluoxastrobin from both seed and foliar application within a crop/season. Therefore, a preventive anti-resistance strategy is desirable in order to minimise this risk. In order to lower the resistance risk of the QoI fungicide fluoxastrobin as a seed treatment, it will be marketed exclusively in mixture with an effective DMI partner such as prothioconazole (Products: ®Bariton, ®Scenic), which shows, at the application rates used, a good activity against seed- and soil-borne *M. nivale*. Although no likely risk can be identified, this mixture concept will also help to decrease any resistance risk for the DMI partner. Moreover, monitoring studies will continue after commercialization of the product.

In conclusion, extensive studies have demonstrated that the use of fluoxastrobin as a seed treatment in wheat will not lead to significant selection pressure for any wind-borne diseases. Furthermore, commercial use will only be in mixture with a robust and effective disease control partner, thus further minimising any potential resistance risk concerns for seed/soil borne diseases.

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The response of *Echinochloa colona* populations from Nigeria to oxadiazon, propanil and pendimethalin

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ABSTRACT

The responses of suspected resistant populations of jungle rice (*Echinochloa colona*) from Nigeria were compared with susceptible and resistant reference populations in glasshouse dose-response and field simulation experiments using oxadiazon, propanil and pendimethalin. Results indicated that some Nigerian populations (from Miri, Gubi and Inkil) showed varying degrees of partial tolerance of oxadiazon and one (Miri) of pendimethalin. However, all the Nigerian populations were susceptible to propanil. The level of control achieved with pendimethalin, usually a pre-emergence herbicide, was generally lower than that of oxadiazon or propanil. If the results obtained are indicative of what is occurring in Nigeria, then the evolution of herbicide resistance to oxadiazon and pendimethalin may be occurring and could be a threat. This threat is more acute if current control practices remain unchanged.

INTRODUCTION

The development of herbicide resistant weeds is a serious worldwide threat to agricultural production. Since its first detection in the 1960s, herbicide resistance has been reported in 276 weed biotypes in 59 countries (Heap, 2003). Jungle rice *(Echinochloa colona* (L.) Link) is one of the serious grass weeds of the tropical world and its resistance to different classes of herbicides has been reported in several countries including, in Central and South America, Mexico, Costa Rica, Colombia and Venezuela (Heap, 2003). *E. colona* is also the most important grass weed in the rice (*Oryza sativa*)-growing areas of the scrub savannah region of Nigeria where rice weed management now largely depends on the use of herbicides.

Oxadiazon, propanil and pendimethalin are amongst the most widely and most frequently used herbicides for the control of grass weeds in rice, including *E. colona* (Akobundu, 1987). When introduced in the early 1980s, these herbicides provided adequate control of *E. colona* and other grasses. Recently, however, farmers in Nigeria have reported cases of poor control such that higher doses and more frequent applications together with hand weeding are required for adequate management of *E. colona.* Repeated use of herbicides could result in the build up of herbicide-resistant biotypes and resistance was suspected as the possible cause of the recent observed herbicide failures. Determination of the herbicide resistance status of *E. colona* in the rice growing areas of Nigeria is required for developing appropriate management strategies. Thus, this study was conducted at Rothamsted

Research to determine if selection for resistance to oxadiazon, propanil and pendimethalin has occurred in the *E. colona* populations from rice fields of Nigeria.

MATERIALS AND METHODS

Seeds

Seeds were collected in October and November 2000, from four rice fields (at Miri, Gubi, Inkil and Fulani) in Nigeria in which farmers had reported cases of herbicide control problems. Three standard populations were used in addition, for reference purposes: two susceptibles, Herbiseed-S (from UK) and LARS-S (susceptible to propanil, originating from Columbia) and one resistant LARS-R (resistant to propanil, originating from Costa Rica).

Glasshouse dose-response experiment

Seeds of all seven populations were pre-germinated in 9 cm Petri dishes containing four filter papers moistened with 7 ml of 2 g/l KNO_3 under a 14 h photoperiod at 30°C/20°C (day/night) controlled environment regime. Seedlings were then transplanted into 5 cm square pots containing Kettering loam (4% organic matter) at one seedling per pot. All plants were kept in glasshouse conditions of 14 h photoperiod of 300 μm m^{-2} s^{-1} light intensity at 30°C/20°C (day/night) and 80% relative humidity for 2 weeks until they attained the four-leaf stage. Plants were sprayed with commercial formulations of oxadiazon (an inhibitor of protoporphyrinogen oxidase) (250 g/l), propanil (an inhibitor of Hill's reaction at photosystem II) (800 g/kg) and pendimethalin (an inhibitor of microtubules assembly) (400 g/l), each with eight different doses in a set of serial dilutions in the range: 62.5 – 8,000 g oxadiazon a.i./ha; 240 – 30,400 g propanil a.i./ha and 82.5 – 10,560 g pendimethalin a.i./ha. Treatments were applied using a laboratory sprayer equipped with a flat-fan nozzle ('Teejet' 110015VK) delivering 262 litres spray solution/ha at a spray pressure of 210 kPa. Sprayed plants were returned to the glasshouse and, after 24 h, watered from above as necessary. The experiment was a complete randomised design with 12 replicate plants per herbicide dose. There were 40 untreated pots per population. Plants were harvested 14 d after spraying and foliage fresh weights determined per pot as a measure of herbicide activity.

Container experiment (field simulation conditions)

An experiment was set up to validate the response of three populations of *E. colona* (LARS-R, Miri and LARS-S) from the glasshouse dose-response assay. Seeds were pre-germinated as in the previous experiment. Seedlings were then transplanted into plastic containers (27 x 18 x 10 cm deep) containing Kettering loam at 12 plants per container. All plants were kept in glasshouse conditions of 14 h photoperiod of 300 μm m^{-2} s^{-1} light intensity at 30°C/20°C (day/night) and 80% relative humidity for 2 weeks until they attained the three-leaf stage. Herbicide treatments comprised the recommended and double the recommended rates of oxadiazon (1 and 2 kg a.i./ha), propanil (4.8 and 9.6 kg a.i./ha) and pendimethalin (3.3 and 6.6 kg a.i./ha). Treatments were applied 3 weeks after transplanting, at the four-leaf stage, using a laboratory sprayer with a single flat-fan nozzle ('Teejet' 110015VK) delivering 271 litres spray solution/ha at 210kPa. The experiment

was a randomised complete block design with three replicates per treatment. There were three untreated containers for each population. Plants were returned to the same glasshouse conditions. Herbicide activity was determined by assessing reduction in foliage fresh weight 4 weeks after spraying.

Statistical analysis

Foliage fresh weight data from the glasshouse dose-response experiment were analysed using Maximum Likelihood Programme (Ross, 1987). The concentration of each herbicide required to reduce foliage fresh weight by 50 % relative to untreated controls (ED_{50}) and $\log_{10}$ ED_{50} was calculated for each population. Foliage fresh weight data from the container experiment underwent an analysis of variance using Genstat Version 5.0 (Payne *et al.*, 1997) to determine SEDs.

RESULTS

Glasshouse dose-response experiment

Foliage fresh weight of all populations was reduced with increasing doses of oxadiazon up to 8000 g a.i./ha, but mainly at doses in excess of 500 g a.i./ha. The ED_{50} values (concentration of herbicide required to reduce foliage fresh weight by 50%) for oxadiazon treatments ranged from 821to1521 g a.i./ha (Table 1). These values were high, with only the susceptible standard (Herbiseed-S) and one Nigerian population (Fulani) recording ED_{50} values below the recommended rate of oxadiazon (1000 g a.i./ha). The Miri population from Nigeria had the highest ED_{50} value (1521 g a.i./ha) and those of Gubi and Inkil were also significantly higher than the susceptible standard Herbiseed-S. Resistance indices ranged from 1.0 to 1.9, with the population Miri recording the highest value, indicating that all populations except Fulani showed slight tolerance of oxadiazon. No populations showed a high degree of resistance to oxadiazon.

Table 1. Responses of seven populations of *Echinochloa colona* to oxadiazon, propanil and pendimethalin in a glasshouse dose-response experiment

	Oxadiazon			Propanil			Pendimethalin		
Population	ED_{50}	RI	Log_{10} ED_{50}	ED_{50}	RI	Log_{10} ED_{50}	ED_{50}	RI	Log_{10} ED_{50}
Herbiseed-S	821	1.0	2.91	5283	1.0	3.72	1622	1.0	3.21
LARS-S	1162	1.4	3.07	6302	1.2	3.80	2031	1.3	3.31
LARS-R	1340	1.6	3.13	43981	8.3	4.64	4275	2.6	3.63
Inkil	1227	1.5	3.09	3160	0.6	3.50	2439	1.5	3.39
Fulani	942	1.1	2.97	2942	0.6	3.47	1183	0.7	3.07
Gubi	1320	1.6	3.12	3803	0.7	3.58	2100	1.3	3.32
Miri	1521	1.9	3.18	3019	0.6	3.48	4409	2.7	3.64
SED			0.068			0.108			0.121

ED_{50} is the dose required (g/ai/ha) to reduce foliage fresh weight by 50% relative to untreated controls of the same population. Resistance index (RI) is the ratio of ED_{50} value relative to that of the susceptible standard, Herbiseed-S

The standard resistant population, LARS-R, demonstrated a high level of resistance to propanil, with over 70 % of plants surviving 38,400 g a.i./ha, eight times the recommended rate. The four populations from Nigeria and two susceptible standards did not vary greatly in their sensitivity to propanil. The values for ED_{50} and for RI were significantly higher in LARS-R (ED_{50}= 43981 g a.i./ha, RI= 8.3) (Table 1). Other populations recorded ED_{50} and RI ranging from 2942 to 6302 kg a.i./ha and 0.6 to 1.2 respectively. All populations from Nigeria had ED_{50} values below the recommended rate of propanil and RI below 1, indicating they were susceptible to propanil.

Two populations (LARS-R and Miri) showed some evidence of tolerance of pendimethalin. Reductions in foliage fresh weight (%) with increase in pendimethalin dose for these populations were slightly less than those for the other five populations at the three highest doses The ED_{50} values for these two populations were significantly ($P<0.05$) higher than both the Herbiseed and LARS-S standards (Miri, 4409 g a.i./ha; LARS-R, 4275 g a.i./ha; Herbiseed 1622 g a.i./ha) (Table 1) The highest level of pendimethalin sensitivity was recorded in the Fulani population, with the lowest ED_{50} of 1183 g a.i./ha. Resistance indices ranged from 0.7 to 2.7, with the Fulani and Miri populations recording the lowest and highest values, respectively. Of the Nigerian populations, Miri showed the greatest tolerance of pendimethalin. Comparing the three herbicides, the results indicate a degree of cross-tolerance of oxadiazon and pendimethalin in the Miri population, which was the least sensitive to both herbicides. The Inkil and Gubi showed slightly reduced sensitivity to oxadiazon.

Container experiment

Results confirm that the susceptible standard population LARS-S was sensitive to oxadiazon at the recommended rate of 1 kg a.i./ha, while LARS-R and Miri showed slightly reduced susceptibility at this rate (Table 2). However, all three populations were effectively controlled with 2 kg oxadiazon/ha.

Table 2. Percentage reduction in foliage fresh weight in three populations of *Echinochloa colona*, relative to untreated controls of the same populations, by two doses of oxadiazon, propanil and pendimethalin in a container experiment.

	Oxadiazon (kg a.i./ha)		Propanil (kg a.i./ha)		Pendimethalin (kg a.i./ha)	
Population	1	2	4.8	9.6	1.32	2.64
LARS-S	87.9	96.7	73.8	87.9	51.9	76.7
LARS-R	77.5	95.4	-21.9	7.4	35.0	77.3
MIRI	74.2	91.5	82.0	96.8	22.6	62.1
SED			5.22			

Neither rate of propanil gave control of the resistant LARS-R population, confirming its high resistance status. Moderate control of Miri and LARS-S was achieved at the recommended rate (4.8 kg a.i./ha) and good control at the higher rate (9.6 kg a.i./ha), confirming susceptibility. The overall level of control achieved with pendimethalin was generally lower than that of oxadiazon and propanil. All populations were poorly

controlled at the recommended rate of 1.32 kg a.i./ha and control at the higher rate of 2.64 kg a.i./ha was mediocre (62 - 77%).

DISCUSSION

This study indicated that three populations of *E. colona* from Nigeria (Inkil, Gubi and Miri) showed slight insensitivity to oxadiazon and one population (Miri) showed tolerance of pendimethalin, but all were susceptible to propanil. In the glasshouse experiment, most populations had ED_{50} values above the recommended rate of oxadiazon (1000 g a.i./ha). Oxadiazon is the most widely used herbicide in this region because of its past history of effectiveness. Selection pressure through continuous use may have shifted the three *E. colona* populations, especially the Miri population, from susceptibility to partial tolerance, thus potentially creating a resistance threat. Studies have shown that continuous use of a given herbicide or chemically related herbicides promotes the build-up of resistance in *Echinochloa* spp. However, differences between populations in terms of resistance indices were too low to demonstrate categorically that any of the Nigerian populations were resistant to oxadiazon. As a general rule, tested populations can be considered resistant only when resistance indices (RI) values are greater than two (Valverde *et al.,* 2000). Resistance can not be assigned purely on the basis of resistance indices and interpreting RI values below three in terms of the impact of herbicide performance in the field should be done with caution.

The low levels of resistance in these experiments do not accord with reports of poor control from the field in Nigeria. Therefore, factors other than herbicide resistance may have contributed to the reported high cases of oxadiazon failures in this region. Herbicide choice, dose, timing, adverse environmental conditions, inaccurate calibration, incorrect mixing, worn out equipment and failure to read and understand the product label are all possible causes of herbicide failure.

There was good agreement between the results for the glasshouse and container experiments with all three herbicides at the recommended doses. The marginal difference between the oxadiazon and pendimethalin responses in the Miri population from Nigeria recorded for in the dose-response studies was supported by the container experiment. Miri was sensitive to propanil in the dose-response experiment, and was the best-controlled population in containers. These results indicate that glasshouse dose-response assays and the container experiments are capable of detecting marginal differences in tolerance between populations. The advantage of containers, compared with field trials, is that they allow comparison of herbicide performance on several populations under identical soil and environmental conditions.

Multiple mechanisms of resistance to propanil and some graminicides are known to have evolved in *E. colona* around the world (Heap, 2003; Gressel, 2000). The levels of herbicide tolerance, albeit slight, exhibited by populations from the scrub savannah region of Nigeria in this study indicate that resistance may be a risk. The experiments conducted here cannot confirm whether the small differences in tolerance recorded in the Nigerian populations are a consequence of selection pressure by herbicides, or merely reflect innate inter-population variation. Hence, there is a need to monitor the response to herbicides in Nigerian populations such as Miri, to determine whether resistance is developing and, if so, how fast

and what mechanisms are responsible. To achieve effective control of these populations in the field, alternative herbicides with better efficacy may have to be introduced. These results agree with findings of Valverde *et al.* (2000), which suggested that herbicide alternation regimes may serve as a useful tool in controlling herbicide-resistant *E colona* biotypes. It will also be important to determine which other factors, unrelated to resistance, are responsible for poor control by herbicides, and to develop weed management strategies based on rotation, cultural and biological methods as a means of reducing the dependence on herbicides.

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A mutation in the C domain of the acetolactate synthase (ALS) gene of *Bidens pilosa* confers resistance to imazethapyr

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ABSTRACT

A biotype of *Bidens pilosa* resistant to imazethapyr has been isolated and characterized. A preliminary set of whole plant and *in vitro* ALS assays in the presence of this herbicide strongly suggested that resistance was due to an alteration in target site by mutation. This was confirmed when the C, A, D, B and E conserved domains of the ALS were amplified. This was done by PCR using a set of universal and degenerate oligonucleotides useful for the cloning of these sequences in all plants tested to date. Several amino acid replacements were found. Some of them are either out of the five conserved domains from where mutations can confer resistance to herbicides, or are located in non-conserved regions of the gene. This suggests that these substitutions are not responsible for the observed resistance. However, a M_{18} to T substitution, located in the D domain, was found in the resistant plants. This mutation has only been reported in resistant plants isolated in the laboratory. This is the first case of a resistant plant isolated from the wild with this same mutation.

INTRODUCTION

Acetolactate synthase (ALS), which catalyses the first common step in the biosynthesis of the branched-chain amino acids, is the target of five herbicide groups: sulfonylurea, imidazolinone, triazolopyrimidine, pyrimidinylthiobenzoate, and sulfonylamino-carbonyl-triazolinone. Resistance to ALS-inhibiting herbicides was first described in 1987, 5 years after the commercial introduction of chlorsulfuron (Mallory-Smith *et al.*, 1990). Since then, the number of ALS-resistant weed populations has been increasing at an exponential rate, and over 80 cases have been reported by June 2003 (Heap, 2003).

Most cases of resistance are due to the presence of nuclear-inherited dominant mutations in the DNA sequence coding for this enzyme (Devine & Shukla, 2001). These mutations have been found to be located in at least five different conserved domains where the herbicides bind to the enzyme (Tranel & Wright, 2002). One of these cases is *Bidens pilosa.* This weed is a parasite of soybean crops, and was the first to develop resistance to acetolactate synthase herbicides in Brazil in 1993. No previous molecular studies have been done on *B. pilosa,* making it difficult to clone the ALS gene.

The aims of this study were to determine the molecular basis conferring resistance of the *B. pilosa* biotype, and to study the degree of resistance to the herbicide imazethapyr.

MATERIALS AND METHODS

Plant material

Resistant (R) and susceptible (S) biotypes of *B. pilosa* were collected from infested soybean fields in Mato Grosso do Sul, Brazil. Seeds from the resistant population were from fields that had been treated with imidazolinones and sulfonylurea herbicides for 8 years before they were collected.

Seeds were germinated in Petri dishes in a growth chamber under controlled conditions (28/25°C day/night temperature, with a 16 h photoperiod of 350 µmol/m radiation and 70% relative humidity). Plants of uniform size were selected when the cotyledon leaves had emerged and expanded and were then transplanted to plastic pots containing a 50% perlite-turf mixture. Plants were grown in a greenhouse under controlled conditions.

For the molecular studies, seeds were surface-sterilized for 5 min in a 30% hypochlorite solution and then washed twice with sterile water. Sterilized seeds were sown in Petri dishes with 1.5% agar supplemented with 0.02% KNO_3 and 0.02% gibberellin GA3 under the same conditions described above. DNA was isolated from individual resistant plants germinated in selective medium containing 10^{-4} M of technical grade imazethapyr.

Chemicals

Technical grade and commercially formulated imazethapyr (Pursuit ®) were provided by BASF.

Whole plant assays

The herbicide was sprayed once at the four- to five-leaf stage of development using a laboratory track sprayer delivering 200 l/ha at 200 kPa. Two plants per pot were sprayed. Plants were harvested and analysed 21 d after treatment in the dose-response experiment. The dose experiment had a randomized block design with three replications per dose. The imazethapyr doses were 100, 200, 400, 800, 1000, 1600 and 2000 g a.i./ha. The herbicide dose that caused 50% reduction of shoot fresh weight (ED_{50}) was calculated for imazethapyr as previously described (Menendez *et al.*, 1994). The R/S ratio was calculated as $ED_{50}(R)/ED_{50}(S)$.

ALS activity assay

The ALS response to herbicide was determined *in vitro* using crude extracts. ALS enzyme was isolated from R and S seedlings. The extraction and assay methods have been described elsewhere (Osuna & De Prado, 2003). The herbicide concentration that caused 50% reduction in ALS activity (I_{50}) was calculated for imazethapyr and the R/S ratio was calculated as $I_{50}(R)/I_{50}(S)$.

DNA extraction

DNA was extracted from plants at the four-leaf stage using a CTAB-based extraction method with a Macherey–Nagel kit using 250 mg of plant material and following the kit's instructions.

PCR amplification of conserved domains

Two sets of degenerated universal primer pairs were used to amplify the regions containing the conserved domains C, A and D (primers ALS-U-295 and ALS-L1170) and domains B and E (primers ALS-U-1580 and ALS-L-2160), according to the method developed previously (Duran, unpublished).

Cloning and sequencing of PCR products

PCR products were visualized in agarose gels and purified using GeneClean. Extracted bands were treated with 2 U of Klenow, adding 5 µl of dNTPs 0.125 mM and incubating for 15 min at 37ºC and were cloned into the *Eco*RV site of pBluescript KS II+. Recombinant plasmids were introduced into competent *E. coli* DH5α. Plasmids with inserts were isolated using a Macherey Nagel Plasmid Isolation Kit, according the manufacturer's protocol. Inserts were sequenced by the central services at the University of Cordoba using M13 forward and M13 reverse primers with an ABI PRISM™ 310 sequencer and ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit.

RESULTS AND DISCUSSION

Dose-response assays

Putative resistant and susceptible populations were treated with imazethapyr. The ED_{50} value for the R biotype was 13 times greater than the susceptible biotype (Table 1).

Table 1. ED_{50}, resistance factor [ED_{50} (R) / ED_{50} (S)], I_{50}, and resistance factor [I_{50} (R) / I_{50} (S)] values for ALS from resistant and susceptible *B. pilosa* biotypes

Biotype	ED_{50} (g a i ha^{-1})	Resistance Factor [ED_{50} (R) / ED_{50} (S)]	I_{50} (M)	Resistance Factor [I_{50} (R) / I_{50} (S)]
R	1365	> 13	> 100	> 21
S	< 100	-	4.68	-

ALS activity assays

The imazethapyr concentration required to reduce the ALS activity by 50% (I_{50}) was greater than 100 µM when extracts were obtained from the biotype R, and 4.68 µM for the S biotype (Table 1). These ALS assays are consistent with resistance to imazethapyr of *B. pilosa* being mediated by a target-site modification, and are similar to other resistance reports such as in *Amaranthus quitensis* (Tuesca & Nisensohn, 2001), *Amaranthus rudis* (Hinz & Owen, 1997) and *Bidens pilosa* (Christoffoleti & Foloni, 1999). In all these cases, the ALS enzyme changed its structure, diminishing or even preventing the binding of the herbicides without loss of enzymatic activity (Saari *et al.*, 1994).

Molecular studies

PCR reactions were performed using the degenerated and universal oligo primers previously described (Duran, unpublished). Two different PCR fragments were obtained. The first one, coding for 237 amino acids, contained the conserved C, A and D domains of the ALS. The second one coded for a 71 amino acid fragments containing the B and E conserved domains. No differences were observed in the nucleotide sequences coding the B and E domains among resistant and susceptible plants. However, a detailed comparison of the sequence coding the C, A and D domains first revealed the presence of two different ALS isoenzymes. The first one, called isoenzyme 1, has 76% similarity to *Arabidopsis thaliana* sequence, while the second, called isoenzyme 2, has 81% similarity (Figure 1).

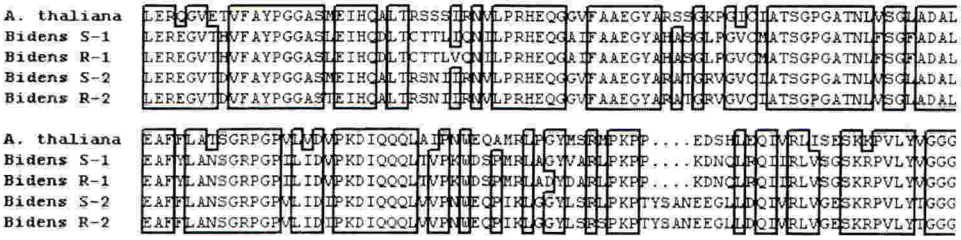

Figure 1. Partial alignment of the sequences coding for the C, A and D conserved ALS domains. Conserved regions are in boxes. S stands for susceptible plants, and R for resistant biotypes. The numbers stand for the two types of ALS isoenzymes found. Notice the gap found in sequence of isoenzyme 1 that helps in recognizing these two isoenzymes.

Both isoenzymes were found in resistant and susceptible plants. This means that resistance is not due to the presence of a particular isoenzyme in the resistant plant. The comparison revealed several changes in the amino acid sequences. The first one is an I_{30} to V substitution in isoenzyme 1 (Figure 2). We also noticed a G_{169} to D and a V_{171} to D substitutions. Although all these residues are conserved in most ALS cloned to date, they are not located in any of the five described conserved domains, presumably involved in the binding of the herbicide to the enzyme. Their involvement in resistance could therefore initially be ruled out. These amino acid changes can be due to the existence of a natural polymorphism in this plant. It is also consistent with the tetraploid nature of this plant.

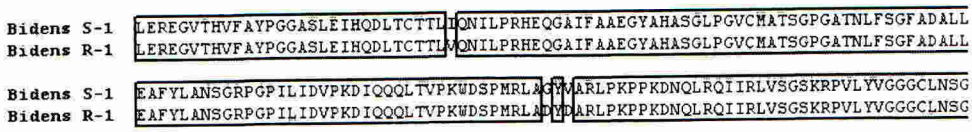

Figure 2. Partial alignment of the *B. pilosa* fragment of isoenzyme 1. The letter S stands for susceptible and R for imazethapyr resistant biotypes. The number stands for the type of isoenzyme considered in the alignment.

In the isoenzyme 2 (Figure 3), also two more changes are found between susceptible and resistant sequences. The first and the most important is a M_{18} to T substitution located in the D domain.

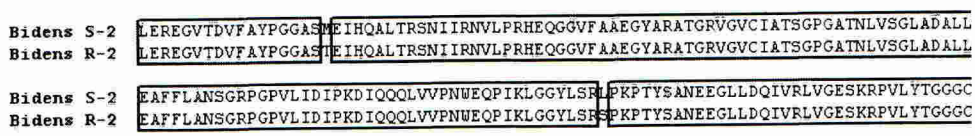

Figure 3. Partial alignment of the isoenzyme 2 fragment of *B. pilosa*. The letter S stands for susceptible and R for imazethapyr resistant biotypes.

This mutation has been described previously only in mutants isolated in the laboratory (Ott *et al.*, 1996). This is the first case reported of a resistant plant isolated from the wild with this mutation. This suggests that the fitness of this resistant plant in nature is adequate enough to allow the plants to survive, and that a decreased fitness does not explain the lack of plants isolated with this particular mutation from nature. In this isoenzyme, a L_{174} to S replacement has also occurred. This portion of the sequence, however, is not conserved at all among the acetolactate synthase and so it remains unlikely that this mutation could be involved in the observed resistance. This work represents our attempt to develop a rapid and universal method for the molecular diagnostic of the resistance to herbicides in weeds.

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A new mutation site in the acetolactate synthase (ALS) gene in *Amaranthus quitensis* resistant to imazethapyr

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ABSTRACT

An *Amaranthus quitensis* population (R7) resistant to imazethapyr was found in a soybean field in Cordoba, Argentina. Greenhouse and laboratory experiments determined resistance to imazethapyr and imazapyr in this population. Based on whole-plant experiments, the resistant population required over 10 times more imazethapyr than a susceptible population to reduce growth by 50%. Cross-resistance to several imidazolinones was also detected. Based on *in-vitro* enzyme activity assays, ALS in the R7 population was 12-fold less sensitive than ALS in those susceptible to imazethapyr. Polymerase chain reaction amplification and sequencing of two regions of the ALS gene containing the domains B/E and C/A/D, respectively, were performed. The resistant population had a mutation that caused a Gly by Cys substitution in the domain E, adjacent to one where mutations in the ALS gene have been documented in other resistant weed species.

INTRODUCTION

Pigweed [*Amaranthus quitensis* (HBK)] is one of the most important weeds of soybean crops in Argentina causing important losses in yield (Leguizamon *et al.*, 1994). Weed control is largely based on herbicide use, since *A. quitensis* is susceptible to many selective herbicides. Herbicides belonging to the ALS inhibitor group have usually been used in soybean fields in Argentina. The first ALS inhibitor-resistant *A. quitensis* population was reported in 2001 in Argentina (Tuesca & Nisensohn, 2001).

Resistance to ALS-inhibiting herbicides is mostly conferred by alterations of the ALS enzyme (Saari *et al.*, 1994; Devine & Eberlein, 1997). Target-site resistance to ALS inhibitors in all weed biotypes thus far has been due to a substitution of one of five conserved amino acids (Tranel & Wright, 2002). Three of these amino acids (Ala_{122}, Pro_{197} and Ala_{205}) are located near the amino-terminal end of ALS and the other two (Trp_{574} and Ser_{653}) are located near the carboxy-terminal end. In domain E, a mutation coding for Ser_{653} Thr substitution has recently been described in several *Amaranthus* populations (McNaughton *et al.*, 2001; Patzoldt & Tranel, 2002; Diebold *et al.*, 2003). It usually results in imidazolinone, but not sulfonylurea, resistance. An *A. quitensis* population resistant to imazethapyr was identified in a soybean field in Cordoba, Argentina. The objectives of this research were to asses the magnitude of

resistance in this *A. quitensis* population to imazethapyr both at the whole plant level and by ALS assays *in vitro*, and to determine the molecular basis for this resistance.

MATERIALS AND METHODS

Plant material

Seeds from a resistant *A. quitensis* population (named R7) were collected from different soybean fields in the province of Cordoba (Argentina) that had been intensively treated for several years with imazethapyr. Seeds from the susceptible population were collected from nearby areas never treated with herbicides.

Seeds of both populations were placed in a tray with a peat+soil mixture (1+1) and allowed to germinate in a growth chamber (28/25°C day/night 16-h photoperiod photon-fluxdensity m^{-2} s^{-1} under 350 µmol m^{-2} s^{-1} and 80% relative humidity). Pre-germinated seeds were then transplanted (four plants per pot) into 7 x 7 cm pots filled with a peat+soil mixture (1+1) and grown under the above conditions.

Whole plant assays

R7 and sensitive (S) *A. quitensis* plants were treated with imazethapyr at the three- to four- leaf stage. The herbicide application rates ranged from 0 to 10 g a.i./ha for the S population and from 0 to 30 g a.i./ha for the R7 population. Herbicide applications were made using a laboratory track sprayer delivering 200 l/ha at 200 kPa. Treatments were replicated three times and shoot fresh weight per plant was determined 21 days after treatment and expressed as percent of the untreated control. Dose-response curves were plotted and herbicide rate that caused 50% shoot growth inhibition (ED_{50}) was established for each population (Osuna *et al.*, 2002).

ALS activity assays

ALS extraction and activity was carried out following the protocol described in Osuna & De Prado (2003). Three experiments, each with a separate tissue extract from different plant material, were conducted per population and each sample at each herbicide concentration was assayed per triplicate. Imazethapyr and imazapyr concentrations that caused 50% ALS activity reduction (I_{50}) were established for each population.

DNA analysis

Genomic DNA was extracted from young leaves of each sampled plant from the different populations. The extraction procedure was performed according the recommendations of the kit used (Dneasy plant mini kit, Qiagen GmbH, Hilden, Germany). DNA content and quality after extraction was checked by gel electrophoresis.

Based on the published sequence of the ALS gene from *Amaranthus* sp. (Woodworth *et al.*, 1996), the following primers were designed to amplify the region containing the domains B and E: primers UpDomB (5′GGGGCTATGGGGTTTGGTCTA3′) and Low Dom E (5′GCCCTTCTTCCATCACCCTCTG3′). A second set of primers, Amf1

(5′TACCGATGTTTTTGCTTACC3′) and Amr1 (5′TGCTTATTCTTCCGGATTTCA3′), were designed according to Michel (2000) to amplify the region containing the domains C, A and D. The primers were synthesized using the software DNAstar. The PCR-purified products were sequenced using the Thermo Sequenase™ Cy5™ Dye terminator kit (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). The sequences of the resistant and susceptible populations were compared for the identification of mutations.

RESULTS

Whole plant assays

Dose response analysis confirmed that population R7 had a high level of imazethapyr resistance. For the R7 population 21.1 g a.i./ha imazethapyr was required to achieve 50% growth reduction; the ED_{50} of the standard susceptible population was 2 g a.i./ha, being the resistance factor 10.8. (Table 1)

The R7 population of *A. quitensis* was cross-resistant to imazapyr (Table 1) as well as imazamethabenz and imazaquin but not to imazamox (data not shown).

Table 1. ED_{50} and I_{50} values of imazethapyr-resistant and –susceptible biotypes of *Amaranthus quitensis* populations

	Whole plant assay		Enzyme assay	
	ED_{50} (g a.i./ha)	Resistance factor [ED_{50}(R)/ ED_{50}(S)]	I_{50} (µM)	Resistance factor [I_{50}(R)/ I_{50}(S)]
Imazethapyr				
S	2.0 ± 0.11		0.9 ± 0.12	
R7	21.7 ± 1.98	10.80	11.7 ± 1.68	12.48
Imazapyr				
S	2.7 ± 0.24		1.3 ± 0.95	
R7	18.3 ± 0.99	6.78	11.7 ± 1.68	9.38

ALS activity assays

In order to elucidate the mechanism of resistance, *in-vitro* studies were carried out using a crude extract of the ALS enzyme. The resistance level of ALS isolated from the R7 population was more than 12-fold higher for the imazethapyr than that found in the S population (Table 1). The same pattern of cross resistance was found as at the whole plant level. These data are consistent with the results found for the whole-plant studies and support the hypothesis that the resistance mechanism is based on an altered target site.

DNA analysis

In order to elucidate the molecular basis of the observed target site, PCR amplification of the ALS gene with the primers pairs Up DomB/Low DomE, as well as Amf1/Amr1, was conducted. A single band of the expected length of 476 bp for the region containing the

domains B and E and 894 bp for the region containig the domains C, A and D was produced. Sequencing of both fragments and comparison of the sequences enabled the identification of the mutations.

No mutations were identified in population R7 that coded for amino acid substitutions in the domains A, B, C and D (data not shown). There was, however, a G to T mutation in domain E of ALS gene resulting in a glycine by cysteine substitution (Figure 1).

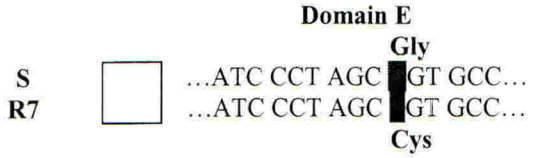

Figure 1. Nucleotide and deduced amino acid sequences in domain E region of R7 and S populations of *Amaranthus quitensis*. The shaded box indicates the nucleotide and substituted amino acid.

DISCUSSION

These results confirmed resistance to imazethapyr in the R7 population of *A. quitensis* at the whole plant as well as ALS enzyme activity level. The ED_{50} for most populations was less than the field-recommended doses (1500 and 100 g a.i./ha for imazapyr and imazethapyr, respectively); previous studies in *A. quitensis* showed that the effective doses for the control of this weed with imazethapyr were less than the field-recommended dose (Vita *et al.*, 2000). Cross-resistance to several imidazolinones was found in the R7 population, but not to imazamox (unpublished data). Cross-resistance to ALS-inhibiting herbicides is a common phenomenon but the pattern of cross-resistance is unpredictable (Saari *et al.*, 1994). The wide variation in target-site cross-resistance between biotypes and among diffferent ALS inhibitors results from a number of different functional mutations of the ALS gene. In studies on *Amaranthus hybridus,* Manley *et al.* (1999) found cross-resistance both to imazethapyr and imazaquin but not to the sulfonylureas studied. Lack of cross-resistance to some imidazolinones but not to others, as found in the present study, has been reported previously (Kudsk *et al.*, 1995).

Sequencing of the ALS gene enabled the identification of a point mutation leading to substitution of Gly in domain E by Cys. Because no other mutation was found in the ALS of population R7, we conclude that the $Gly_{654}Cys$ substitution is the most likely cause of resistance to imazethapyr in this population. To our knowledge, this is the first report of a weed species developing resistance due to this mutation after selection in field. It has not been described in laboratory selections either. The only mutation identified in domain E conferring resistance to ALS inhibitor codes for a Ser_{653} Thr change in the ALS protein in several *Amaranthus* populations. In each case it conferred resistance to imidazolinones but not to sulfonylureas (Diebold *et al.*, 2003). The mutation described in this work is located adjacent to that in which mutations in the ALS gene has been documented in other resistant weed species.

Here, cross resistance to some other imidazolinones was found, but studies on sulfonylurea have been not performed.

Futher studies need to be carried out in order to confirm that the $Gly_{654}Cys$ substitution is responsible for the resistance to ALS-inhibiting herbicides found in the R7 population of *A. quitensis*. DNA sequences changes associated with resistance can be characterized in some mutants by using mutagenesis, tissue culture selection, transformation and/or back crossing (Devine & Eberlein, 1997).

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