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3. PRACTICAL SECTION FOR GROWERS

Commercial Benefits of Project

The HDC Salads R&D Group identified effective aphid control as their top priority for research and development. The present work will result in improved crop protection strategies by providing a scientific basis to assist with the choice of insecticides that in turn should prevent any further increase in chemical usage, especially that prompted by resistance, and may even reduce the number of treatments applied. This in turn will improve the environmental acceptability of control strategies and improve product quality.

Background and Objectives

Owing to their outstanding efficiency, insecticides exert intense selection for insect pests genetically adapted to tolerate greater exposure than others. This may arise from an improved ability to detoxify insecticides, or differences in the sensitivity of insecticide target sites. Under selection, such individuals increase gradually in frequency in populations to a point at which control treatments are less effective than before and eventually fail. Indeed, reports by growers and advisors of insecticide treatments losing efficiency, or requiring shorter intervals between applications, often provide the first suspicions of resistance.

Resistance to insecticides in the currant-lettuce aphid, *Nasonovia ribisnigri*, has previously been confirmed in laboratory assays of samples from the UK (FV 210). This follows its detection in southern France and Spain (Rufingier *et al*., 1997) though no further research on its incidence or management is being done in southern Europe (N. Pasteur pers. comm.). The implications of these laboratory results for the control of this pest in the field require urgent attention.

The peach-potato aphid, *Myzus persicae*, also occurs commonly on lettuce, as well as a number of other crops, and has been known for many years to be resistant to insecticides both in the UK and elsewhere.

Lettuce crops receive on average five applications of insecticide (MAFF Pesticide Usage Survey for 1995), though those grown in mid-summer, or where the recently introduced seed treatments of imidacloprid are used are likely to have different treatment regimes. There is currently a very limited number of insecticides available for the control of aphids on lettuce, and this could conceivably diminish further as a consequence of a current review on the use of organophosphate (OP) insecticides in UK agriculture and horticulture. At present, however, the outcome of this review is uncertain.

The unrestrained use of insecticides without taking account of the levels and mechanisms of resistance present within aphid populations could accelerate the development of resistance leading to the complete loss of effective chemicals, including the recently introduced imidacloprid seed treatment. There are also few new insecticides likely to become available for aphid control on lettuce in the near future. Two potential candidates, acetamaprid (Aventis; a foliar rather than systemic insecticide) and thiamethoxam (Syngenta), act at the same target site as

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imidacloprid, raising the possibility of cross-resistance between all three products. Excessive reliance on imidacloprid as a seed treatment could therefore threaten the efficacy of these chemicals also. Further escalation of resistance in *N. ribisnigri* should be avoided at all costs, through management of resistance to insecticides both available currently and those that may be introduced in the future, if growers are to continue to produce quality lettuce free of aphids that meets the demands of processors and retailers.

In response to these concerns, HDC commissioned a new three year project (FV 210a) to investigate further the incidence, implications and management of insecticide resistance in UK populations of *N. ribisnigri*. In the first year (1999) of this project, varied levels of resistance to both pirimicarb and cypermethrin were identified in field populations, appearing to be the result of two independent resistance mechanisms. There was little or no evidence of resistance to the OP, heptenophos, or the neonicotinoid, imidacloprid. Biochemical tests confirmed the correlation between pirimicarb resistance and an increase in esterase activity but attempts to create a rapid diagnostic by measuring total esterase content in aphids failed to distinguish categorically between susceptible (S) and resistant (R) individuals.

Field experiments conducted at Wellesbourne showed pirimicarb to be less effective against the pirimicarb resistant clone (Nr2A) six days post treatment. Similarly, residues of pirimicarb were less effective against resistant than susceptible aphids one and two days after the insecticide had been applied, suggesting that the relatively low levels of pirimicarb resistance detected in *N. ribisnigri* populations reduce the residual life of pirimicarb rather than its initial efficacy as a foliar spray.

Owing to the reduced use of OPs in lettuce protection, these compounds have since been removed from screening and field experiments to be replaced with more work on pyrethroid resistance. This report summarises results achieved during the second phase of FV 210a and outlines work planned during the remainder of the project. Laboratory work was conducted at IACR-Rothamsted and field experiments at HRI Wellesbourne.

Summary of Results and Conclusions

Laboratory experiments

In the laboratory, bioassays were conducted on three *N. ribisnigri* clones (Nr4A, Nr8A and Nr10A), established from field populations sampled in 1999, and five mixed field populations of *N. ribisnigri* sent to Rothamsted from around the UK during 2000. An additional population originated from Northern France. The main bioassay used against *N. ribisnigri* entailed placing winged adults on leaf discs cut from lettuce that had previously been dipped into insecticide solutions and allowed to dry. Mortality was scored three days later. The compounds tested were cypermethrin, deltamethrin, lambda-cyhalothrin, pirimicarb, imidacloprid and nicotine. As in the first year of the project, the Nr1A clone of *N. ribisnigri* was used as a fully susceptible reference strain and the Nr2A clone, shown to exhibit *c*.10-fold resistance to pirimicarb in project FV 210, was used as a pirimicarb resistant reference strain.

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Bioassays using the three new clones, Nr4A, Nr8A and Nr10A were conducted in order to investigate cross-resistance patterns within and between insecticide classes. Results demonstrated three phenotypic responses to all three pyrethroids tested: full susceptibility (Nr8A), resistance (up to *c*. 50-fold) (Nr4A) and intermediate resistance (Nr10A). The mechanism for pyrethroid resistance appeared to be independent of that for pirimicarb as the response of Nr4A did not deviate significantly from that of the susceptible Nr1A to the latter compound. There was a close correlation in responses to cypermethrin, deltamethrin and lambda-cyhalothrin, implying cross-resistance to the pyrethroid class as a whole.

Bioassays using mixed field populations from 2000 showed a similar pattern to the clonal results reported above with responses to pyrethroids ranging from susceptible to *c.*50-fold resistance. Again, these responses appear to be independent of the levels of pirimicarb resistance, which also ranged from fully susceptible to *c.* 4-fold resistance.

Field population responses to nicotine were varied while all populations appeared more susceptible to imidacloprid than Nr1A.

None of the field strains examined showed evidence of the altered target-site mechanism, modified acetylcholinesterase (MACE), that has been reported for *N. ribisnigri* from southern Europe, and which is currently spreading through the UK in *M. persicae*. This mechanism has the potential to confer total immunity to pirimicarb and triazamate.

Resistance to pirimicarb in field populations continued to be associated with an enhanced esterase band, E0.34, on PAGE gels as described in the 2000 report. E0.34 was subsequently purified from the Nr2A clone allowing the role of this enzyme in insecticide detoxification to be studied in more detail. Inhibition studies with the purified E0.34 showed that there is little or no recovery of enzyme activity after incubation with pirimicarb. This indicates that E0.34 is not rapidly breaking down the insecticide but probably sequestering it, thus making both the insecticide and enzyme inactive before the insecticide reaches its target site. This enzyme inhibiting process would account for the relatively low levels of resistance seen in the bioassays. Radiolabel studies have shown the increase in esterase activity to be caused by an increase in production of E0.34 rather than the presence of a modified, more active form.

Although the rapid total esterase assay has successfully been refined since the previous report to allow the inhibition work described above to be conducted, it still did not provide the clarity required to distinguish unambiguously between pirimicarb S and R individuals. Therefore, the purified E0.34 is also being used to raise polyclonal antibodies for use in a diagnostic immunoassay of the type successfully used to diagnose levels of esterase-based resistance in *M. persicae*.

The mechanism of pyrethroid resistance has still to be elucidated. The responses of pyrethroid resistant populations demonstrate a high enough level of resistance to suggest a knock-down resistance (*kdr*) mechanism is responsible. *Kdr* is a target-site mechanism which confers resistance to pyrethroids and DDT. It is caused by specific gene mutations that have previously been identified in resistant *M. persicae* populations. Using molecular diagnostics, pyrethroid S and R *N. ribisnigri* clones

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have been screened for the same mutation but no differences have been found. It is possible that other mutations are present in the pyrethroid resistant *N. ribisnigri* clones that have caused the *kdr* response, but further evidence for this type of mechanism is required before a more exhaustive screen for mutations is conducted.

Field experiments
The implications of resistance to pirimicarb and pyrethroids in *N. ribisnigri* for the field performance of insecticides were investigated in three experiments at HRI Wellesbourne. In the first experiment, the effects of pirimicarb and deltamethrin on susceptible (Nr1A), pirimicarb-resistant (Nr2A) and pyrethroid-resistant (Nr4A) aphids were studied in the field. In the second experiment the effect of pirimicarb and deltamethrin residues on these clones was studied in the glasshouse. In the third experiment the importance of plant age as a factor affecting field control of these aphids was examined.

The results of the first experiment showed similar and significant reductions in Nr2A and Nr4A *N. ribisnigri* two days after pirimicarb application, although greater numbers of Nr2A small nymphs were found compared to Nr1A nymphs six days after treatment. This differed from results obtained on deltamethrin-treated plants as lower mortality of Nr4A *N. ribisnigri* was seen compared to Nr2A *N. ribisnigri* two days after application. The mortality of Nr2A *N. ribisnigri* was similar on both pirimicarb-treated and deltamethrin-treated plants.

Use of a leaf dip technique produced more repeatable results than experiments in 1999, with high mortality of Nr1A up to four days after treatment. This compared with high mortality of Nr2A and Nr4A aphids up to two days after treatment. More Nr4A aphids survived on plants with 1 day old deltamethrin residues than on plants with 1 day old pirimicarb residues, but Nr2A aphids were killed equally by 1 day old residues of both insecticides. This mortality level was comparable to the mortality of Nr4A aphids on pirimicarb-treated plants.

There was no control of the susceptible aphids after insecticide applications in the third experiment, so no useful information about the effect of plant age on aphid control was obtained. Despite this, data collected from the untreated plants indicated that there may be an effect of plant age on the capacity of *N. ribisnigri* to increase in numbers. Additionally, numbers of Nr4A increased more rapidly than Nr1A on untreated old and young plants in this experiment.

In general, results of field experiments conformed to expectations showing resistance detected in laboratory bioassays to impair the extent and duration of control efficacy of the same compounds under field conditions.

Future plans

Major objectives for the next twelve months are as follows:

- Further characterisation of resistance patterns in *N. ribisnigri* samples collected in the UK, especially from sites experiencing control difficulties

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- Assuming the final steps to raise an antiserum to esterase E0.34 are successful, a diagnostic immunoassay for esterase-based resistance to pirimicarb will be developed and validated using field-caught aphids

- Work to investigate possible causes of pyrethroid resistance in *N. ribisnigri* will be continued, as will that to detect a MACE-type mechanism of resistance to pirimicarb as early as possible

- Depending on advice we receive from project sponsors, new insecticides (e.g. pymetrozine) and/or combinations of existing ones (e.g. pirimicarb + lambda-cyhalothrin; 'Dovetail') will be incorporated into laboratory and field experiments

- Field experiments will focus on comparing the relative efficacy and persistence of different pyrethroids against susceptible and resistant *N. ribisnigri*.

- Articles summarising our findings so far and the resulting practical recommendations will be published in trade journals

Action Points for Growers

Based on results to date and experience with other pests, we can advance the following action points and tentative recommendations.

- Growers should be aware that difficulties with controlling aphids on lettuce may relate to resistance to insecticides in *M. persicae, N. ribisnigri* or (conceivably) both species

- Suspected cases of resistance should be reported immediately to advisors or technicians, who should in turn seek specialist advice if deemed necessary. If *M. persicae* is implicated, insects can be sent to IACR-Rothamsted for rapid characterisation of resistance status. Within project FV210a, IACR-Rothamsted can accommodate limited testing of *N. ribisnigri* as well.

- Make every effort to apply insecticides according to agreed recommendations, ensuring correct timing and maximum coverage of plants. Without specialist advice, do not deviate from recommended application rates as this will not assist with defeating resistance and could exacerbate the problem.

- Never follow up a suspected control failure with a repeated application of the same product. At present there is no evidence for a consistent association between resistance to pirimicarb and pyrethroids in *N. ribisnigri*. Thus, for the time being it appears possible to alternate these insecticide classes. However, owing to broad cross-resistance between pyrethroids, the apparent failure of one pyrethroid should not be followed by application of another. OPs appear less strongly resisted than pirimicarb but may be affected by the same mechanism, ie. there is a danger that OP use will select for pirimicarb resistance. If so, it would be preferable to use OPs after rather than prior to the use of pirimicarb.

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- Growers should be aware that the use of pyrethroids for caterpillar control will select for aphid resistance and as such steps should be taken towards implementation of IPM for caterpillar control where possible.

- Use of pirimicarb only compromises the length of the control period rather than the level of initial kill. As a result pirimicarb applications should be optimised to ensure maximum kill of both susceptible and resistant aphids during and immediately after spraying. The appearance of a MACE-type mechanism, leading to potential immunity from pirimicarb, remains a distinct threat to the future of this insecticide. Cases of pirimicarb apparently having no effect on numbers of *N. ribisnigri* should therefore be reported to advisors or the manufacturer, with a view to further investigation of the causes at IACR-Rothamsted.

- Imidacloprid still appears unaffected by resistance but, due its high persistence and extent of usage, constitutes a major resistance risk in both *N. ribisnigri* and *M. persicae*. Suspicions of imidacloprid failing should be reported immediately to advisors or the manufacturer. Rothamsted has the facilities to confirm or refute resistance to imidacloprid in either species.

Anticipated Practical and Financial Benefits from Study

The cost-benefits of this research are considerable. The value of the UK lettuce crop is £64.3 M (MAFF Basic Horticultural Statistics for the UK). The widespread development of resistance to insecticides could make it non-viable to grow lettuce in the UK that meet the quality standards of freedom from aphid infestation demanded by processors and retailers, leading to partial or even total collapse of the industry. However, through knowledge and management of resistance, the life of available insecticides will be prolonged and time will be provided for the development of alternative control strategies (aphid resistant varieties, biological control, aphid behavior modifying chemicals etc.).

The industry will benefit from more detailed knowledge of the extent and level of resistance to insecticides in aphid populations that infest lettuce in the UK and so be better informed when selecting chemical control agents to ensure efficacy and minimise the problems of insecticide resistance. Such knowledge should also assist with identifying needs and opportunities for introducing new insecticides that could contribute to resistance management strategies.

Growers and agrochemical producers will also benefit from the development of tools for rapid detection of resistance to insecticides in aphid populations and hence guide the selection of control agents and the preservation of existing products. These monitoring techniques could be implemented at a central location, or could equally be conducted by trained personnel (eg. pest management advisors) in regional laboratories.

Improved attention to resistance management will avoid the environmental impact of applying ineffective products.

Consumers will benefit by having quality produce to which the minimum of insecticides has been applied to achieve effective aphid control.

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4. SCIENCE SECTION

4.1. INTRODUCTION

The effective control of aphids on the foliage of outdoor lettuce is essential to ensure the marketability of this crop. In the UK, lettuce is colonised by a complex of aphid pests including the currant-lettuce aphid, *Nasonovia ribisnigri* (Mosley), the peach-potato aphid, *Myzus persicae* (Sulzer), and the potato aphid, *Macrosiphum euphorbiae* (Thomas). *N. ribisnigri* is often the major pest and is much more specific to lettuce than the other two species, which attack several other crop and non-crop plants (Ellis *et al.*, 1995). On ecological grounds, *N. ribisnigri* is therefore a primary candidate for the selection of resistance to insecticides, which remain the mainstay of its control in the UK and elsewhere in Europe.

Published bioassay data from project FV210 relating to UK field populations of *N. ribisnigri* showed widespread but varied levels of resistance to pirimicarb and lower, varied levels of resistance to pyrethroids and organophosphates (Barber *et al*., 1999). Resistance was correlated with an intensely-staining esterase band disclosed by polyacrylamide gel electrophoresis (PAGE) but no direct link was established. Bioassays conducted in France with field strains originating from southern France and Spain exhibited a maximum of 12-fold resistance at LC_{50} to the organophosphate (OP) acephate and 660-fold to the cyclodiene endosulfan (Rufingier *et al.*, 1997). Maximum levels of resistance to the pyrethroid deltamethrin (28-fold) and the carbamate pirimicarb (19-fold) were intermediate to these extremes. Laboratory selection experiments using French field populations of *N. ribisnigri* have since shown that endosulfan resistance can result from glutathione-S-transferase detoxification, and pirimicarb resistance from modified acetylcholinesterase (MACE) (Rufingier *et al.*, 1999).

The first year of the current project, FV210a, identified four distinct modes of resistance in the field populations collected during 1999: (1) fully susceptible populations; (2) pirimicarb resistant populations; (3) pyrethroid resistant populations and (4) populations resistant to both pirimicarb and pyrethroids. These results suggested that two independent resistance mechanisms were present in UK populations of *N. ribisnigri*. Pirimicarb resistance in these populations was consistently associated with an intensely staining esterase band which had been identified in project FV210. However, a rapid total esterase diagnostic assay did not categorically distinguish between susceptible (S) and resistant (R) individuals.

Three clones derived from the 1999 field populations have since been used to clarify cross-resistance patterns within and between insecticide classes. In addition, new field populations received during 2000 have been screened for resistance to a range of compounds. Owing to their restricted use in the protection of lettuce, OPs have been replaced in this study by a broader range of pyrethroids and alternative compounds. Field populations were also screened for elevated esterase activity, which has been correlated with pirimicarb resistance, and modified acetylcholinesterase (AChE, the target site of OPs and carbamates). Purification of the esterase of interest, E0.34, also allowed the function of the esterase in detoxification to be studied in detail.

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Field experiments conducted at Wellesbourne during 1999 showed pirimicarb to be less effective against the pirimicarb resistant clone (Nr2A) six days post treatment. Similarly, residues of pirimicarb were less effective against resistant than susceptible aphids one and two days after the insecticide had been applied, suggesting that the relatively low levels of pirimicarb resistance detected in *N. ribisnigri* populations reduce the residual life of pirimicarb rather than its initial efficacy as a foliar spray.

Results from 1999 were used to refine the experimental treatments used in field and glasshouse experiments during 2000. The discovery of a pyrethroid-resistant clone of *N. ribisnigri* and the loss of heptenophos as an active ingredient to the industry were taken into account when planning these experiments. As a result three experiments were done in 2000. In the first experiment, the effects of pirimicarb and deltamethrin on susceptible (Nr1A), pirimicarb-resistant (Nr2A) and pyrethroid-resistant (Nr4A) aphids were studied in the field. In the second experiment the effect of chemical residue on these differently susceptible clones was studied in the glasshouse. In the third experiment the importance of plant age as a factor affecting field control of these aphids was examined.

4.2. MATERIALS AND METHODS

4.2.1 Laboratory Experiments

Aphid strains and rearing methods
The five laboratory 'standard' clones maintained in culture throughout the study are: Nr1A, a clone derived from a long-standing susceptible strain initially established at HRI Wellesbourne in 1994 and transferred to Rothamsted in 1995; Nr2A, a clone collected in 1997 from a site in Kent experiencing control problems with pirimicarb (Barber *et al*., 1999); Nr4A, a clone originating from a 1999 field sample from Chichester found to be resistant to cypermethrin; Nr8A, a clone from a 1999 Yorkshire field sample exhibiting responses similar to those of Nr1A; and Nr10A, a clone exhibiting an intermediate response to both pirimicarb and cypermethrin, originating from Suffolk.

Six field strains collected during 2000 from the north (1 strain), west (1 strain), south (1 strain) and east (2 strains) of England and one from Northern France were tested with the pyrethroids, cypermethrin, deltamethrin and lambda-cyhalothrin; the carbamate, pirimicarb; the neonicotinoid, imidacloprid; and nicotine.

All strains of *N. ribisnigri* were reared parthenogenetically in the laboratory on whole plants of *Lactuca sativa* cv. 'Webb's Wonderful', without exposure to insecticides, at 21°C with a 16:8h (L:D) photoperiod. Plants were changed regularly and new ones re-infested to avoid host plant deterioration and excessive crowding of aphids.

Insecticides
Formulated insecticides used for leaf-dip bioassays were cypermethrin ('Cythrin', 100g/l EC) (emulsifiable concentrate); deltamethrin ('Decis', 25g/l EC); lambda-cyhalothrin ('Hallmark', 50g/l EC); pirimicarb ('Aphox', 500g/kg SG) (soluble granules) and nicotine ('XL-All Nicotine 95%, 950g/l LI) (liquid). For leaf-dipping, all formulations were diluted to the required concentration in distilled water

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containing 0.01% 'Agral' (Zeneca Agrochemicals), a non-ionic surfactant added to improve leaf-wetting and to compensate for the loss of formulant at low insecticide concentrations. Imidacloprid was applied topically as technical material (>99% purity; Promochem Ltd.) diluted to the required concentrations in acetone.

Bioassays

Leaf-dip bioassays   Leaf discs (35mm diameter) cut from lettuce (*Lactuca sativa* cv. 'Webb's Wonderful') were dipped in insecticide solution for 20s, placed upside down on an agar bed (25mm in depth) in disposable plastic containers (30mm high), and allowed to air-dry. Alate adult *N. ribisnigri* of the required strain (10 per container) were placed on the treated leaf surface and confined by applying a ring of fluon to the exposed lip of the container. Leaf discs dipped in water plus Agral were used as controls. Bioassay containers were covered with a fine mesh lid and stored upright in a constant environment facility at 20°C under ambient daylight conditions.

Topical application bioassay   For bioassays with imidacloprid, alate adults were placed on untreated leaf-discs in containers (10 per container) as described above, and dosed individually with a 0.25µg droplet of insecticide in acetone, with acetone alone used as a control. Treated aphids were stored as described above.

Design and analysis of bioassays   Dose-response bioassays against the five standard clones were conducted using at least two batches of 10 alate aphids per concentration (i.e. 20 insects) at at least three insecticide concentrations. Adults incapable of co-ordinated movement of legs (after gentle prodding if necessary) were scored as dead. All bioassays were scored at intervals 48h and 72h following initial exposure to insecticide. Field strains collected during 2000 were tested at least once over 3 - 5 concentrations with two batches of 10 alate adults per concentration. Owing to this low number of insects and the possibility of genetic heterogeneity within strains, no attempt was made to fit probit lines to these data.

Polyacrylamide gel electrophoresis (PAGE)

Electrophoretic patterns of non-specific esterases in individual aphids after homogenisation in sucrose/Triton X-100 (5%/1.6%) were analysed using Ornstein-Davis, 7.5% polyacrylamide gel slabs containing 0.2% Triton X-100 and a discontinuous buffer system (Davis, 1964) run at 250V for 2h. Gels were rinsed in 0.2M phosphate buffer, pH 6.0 for 30min then stained in the same buffer containing 5mM Fast Blue RR, 1% acetone and 0.6 mM 1-naphthyl acetate. Gels were fixed and stored in 7% acetic acid.

Radiolabelling   10 aphids of Nr1A and Nr2A were homogenised in 25µl sucrose/Triton X-100. The mass homogenate was centrifuged at 1100g for 10 s and the supernatant taken. 10µl of each supernatant was run on an Ornstein-Davis gel as described above. 2µl diisopropylfluorophosphate (DFP) was added to the remaining 15µl of each supernatant and centrifuged at 1100g for 10 s. After a 30 min incubation at 24°C, 10µl of each supernatant was run on an Ornstein-Davis gel as described above. After rinsing in distilled water for 30 min, the gel was soaked in 1M sodium salicylate solution for 30 min. The gel was then dried for 3hrs and placed next to X-ray film (Fuji medical X-ray film) at -80°C for 5 weeks before developing.

AChE assays

Assays characterising the sensitivity of AChE to pirimicarb were based on the methods of Moores *et al*. (1988) for rapid diagnosis of AChE sensitivity in single insects from field populations. Single aphids (8 per field strain) were homogenised in 20µl phosphate/Triton buffer (pH 7.5, containing 0.1M phosphate and 0.1% Triton X-100) in separate wells of a 96-well microplate, and left for 30 min at 4ºC to enhance AChE solubilisation. Buffer (100µl), homogenate (50µl) and DTNB (50µl) were equilibrated in a fresh microplate, using duplicate samples of homogenate to give an uninhibited control synchronised with one inhibition reaction. Assays were started by the addition of ATChI in buffer (100µl) with and without a diagnostic concentration of pirimicarb, to give a final substrate concentration of 0.5mM, a final DTNB concentration of 15µM and a final pirimicarb concentration of 10^{-5}M. Assays were monitored for 20 min by a Thermomax microplate reader (Molecular Devices), utilising SOFTmax software that subsequently fitted linear regressions to successive absorbence readings taken at 405 nm from each well.

Esterase Purification

The E0.34 protein was purified by gel filtration, polyacrylamide gel electrophoresis and electro-elution. Nr2A aphids (1g) were homogenised at 0ºC in 5ml phosphate buffer (pH 7.0, 0.02M, containing 0.1% Triton X-100) and centrifuged at 1100g for 5min. The supernatant was taken and low molecular weight material was removed from the crude homogenate by passing it through a column (2.5cmX25cm) of Sephadex G-25. The 15ml (approx.) sample recovered from the column was concentrated to a 4ml volume using a Millipore 'Miniplus' concentrator. Sucrose (5%) was added to the concentrate before loading the sample in equal volumes onto four Ornstein-Davis gels (described above) which were run for 2h at 250V. Borders (1cm wide) were crinkle cut from the gels, rinsed in 0.2M phosphate buffer, pH 6.0 for 30min then stained in the same buffer containing 5mM Fast Blue RR, 1% acetone and 0.6mM 1-naphthyl acetate for 10min. The stained borders were realigned with the respective gels and the band of interest was cut from the unstained sections. The slices were diced and electro-eluted in 0.6% Tris-base/0.15% glacial acetic acid at room temperature for 4h. The eluted esterase was recovered and stored at -20ºC until required. This process was repeated until a sufficient amount protein had been purified.

Total esterase assays

Total esterase assays, used to study the purified esterase, were initially based on the methods of Grant *et al*. (1989). Single aphids were homogenised in 20µl phosphate buffer (pH 7.0, 0.02M, containing 0.1% Triton X-100) in individual wells of a 96-well microplate. A separate sample of 0.2M phosphate buffer, pH 6.0 containing 1.5mM Fast Blue RR salt was filtered, and 1-naphthyl acetate in acetone was added to give a final substrate concentration of 1mM. 200µl of this was added to each homogenate and mixed. Reactions were monitored for 10 min at a wavelength of 450nm using a Molecular Devices Thermomax kinetic plate reader. Owing to discrepancies between results seen using PAGE and those using total esterase assay, subsequent assays included 0.1% Triton X-100 in the pH6.0 staining solution.

Esterase Reactivation Studies

Recovery of E0.34 after incubation with pirimicarb was monitored over time using the total esterase assay described above. Purified E0.34 , with an uninhibited total esterase value of >100 mOD/min^{-1}, was incubated in 5ml phosphate buffer (pH 7.0, 0.02M, containing 0.1% Triton X-100) containing pirimicarb (10^{-3}M) and bovine

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serum albumin (0.25%), included to maintain enzyme stability, until esterase inhibition was >90%. The 5ml sample was loaded onto a column (2.5 X 25cm) containing Spephadex G-25. Previous studies using radiolabelled DFP established that the insecticide bound esterase came off the column in the 40-45ml fraction, 10ml before the unbound insecticide was released. Sub-samples (80µl) of the 40-45ml fraction were then screened for esterase activity over the course of 6h using the total esterase assay described above.

Isolation and sequencing of sodium channel gene

Total RNA was extracted from ~ 50mg of Nr1A or Nr4A *N. ribisnigri* as described by Martinez-Torres *et al.* (1997). First strand cDNA was synthesised from total RNA using Superscript II reverse transcriptase and oligo dT primer (200ng). The cDNA was used as template for two rounds of PCR using degenerate sodium channel primers (1° reaction with D1 and Dg2 primers; 2° reaction with D2 and D5). Primer sequences are shown in table 1. All primers were bought commercially from MWG-Biotech. Reaction conditions were as described by Matinez-Torres *et al.* (1997). The sodium channel fragments amplified were direct PCR sequenced using the internal aphid primer aph3. Sequence files were analysed using vector NTI and Wisconsin GCG software packages.

Table 1 Oligonucleotide primers

Name	Sequence
D1	AARYTNGCNAARTCNTGGCC
Dg2	GCDATYTTRTTNGTNTCRTTRTC
D5	GCNAARTCNTGGCCNAC
aph3	TTGGTTCTCCGACGTG

Degenerate primers:
R=A=G, Y=C+T, D=A=G=T, W=A+T, S=G+C, M=A+C, N=A+C+G+T

4.2.2 Field Experiments

Aphid strains and rearing methods

The three clones of *N. ribisnigri* susceptible (Nr1A), pirimicarb resistant (Nr2A) and pyrethroid resistant (Nr4A), whose esterase banding integrity had been confirmed previously by IACR Rothamsted, were maintained in separate glasshouses on lettuce cv. Saladin.

Insecticide treatment of plants

Formulated insecticides used for field experiments were pirimicarb ('Pirimor') at a rate of 0.5g/L @ 600L/ha and deltamethrin ('Decis') at a rate of 250ml/ha @ 600L/ha, applied by hand held 1.5m boon at a height of 1m above the crop. In the glasshouse experiment individual lettuce plants were dipped into a pirimicarb solution of 0.5g/ L or a deltamethrin solution of 0.42ml/ L (equivalent to 250ml/ ha @ 600L/ ha).

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Effect of insecticides on pyrethroid- and pirimicarb-resistant N. ribisnigri (1st experiment)

The first field experiment tested the effectiveness of pirimicarb and deltamethrin for controlling three clones of *N. ribisnigri* (Nr1A, Nr2A and Nr4A) as compared to untreated control plants. Lettuce cv. Saladin were grown for three weeks in an insect proof cage in a glasshouse until planting on 9 May into individual plots. Each plot was planted with two rows of ten lettuce. Plant spacing was 30cm within rows and 45cm between rows. Each plot of 20 plants was covered with an insect proof mesh cage the following day. Each of the nine treatment combinations (three aphid clones X three treatments) were assigned randomly to a single plot in each of six blocks, giving six replicates of each of the nine treatment combinations (54 plots in total).

Once established, each lettuce was inoculated with approximately ten individuals of *N. ribisnigri* of the appropriate clone by placing a piece of leaf from the bulked-up aphid cultures with approximately ten aphids on it in the middle of each plant. Of the 20 plants in each cage, six were cut, individually bagged and taken to the laboratory on each sampling occasion. The number of aphids at each life stage was recorded, as was the total number of aphids per plant. Owing to poor weather, inoculated *N. ribisnigri* were allowed to establish for 17 days before the first pre-treatment sample was taken. The following day plants were treated with either, pirimicarb (0.5g/L @ 600L/ha) or deltamethrin (250ml/ha @ 600L/ha) or left untreated (control). Plants were then sampled two and six days after treatment. A subset of each *N. ribisnigri* clone from each untreated cage was sent to IACR-Rothamsted to confirm their resistant status. No cross contamination was observed.

Analysis of aphid numbers per plot (summed across the six sampled plants per occasion) was done within a generalised linear model framework, using a log-linear model assuming Poisson distributed data. Over-dispersion of the counts was allowed for in the analysis. Analyses included the total pre-treatment count (after $\log_e$ transformation) as a covariate to adjust for plot differences in the numbers prior to treatment. Treatment effects estimated in the model are re-expressed in terms of the percentage mortality for each chemical treatment relative to the untreated control.

Effect of pirimicarb or deltamethrin residues on survival and reproduction of *N. ribisnigri* (2nd experiment)

Individual marked leaves of five week old lettuce cv. Saladin were dipped in solutions of pirimicarb (0.5g/ L) or deltamethrin (0.42ml/ L) or were left untreated. Degradation of the chemicals was allowed between 8 and 15 May 2000 outdoors. Plants were taken to the glasshouse zero, one, two, four and seven days after treatment to conduct experiments. On each occasion pirimicarb-treated, deltamethrin-treated and untreated plants were infested with Nr1A, Nr2A and Nr4A *N. ribisnigri*. Plants were arranged in 5 blocks outdoors to allow even degradation. From each block three plants per treatment were inoculated with either susceptible (Nr1A), pirimicarb-resistant (Nr2A) or pyrethroid-resistant (Nr4A) *N. ribisnigri*. This meant that all nine treatment combinations were replicated five times, as there were five blocks, giving 45 plants per experimental occasion. Each plant was inoculated with ten winged *N. ribisnigri* between 10-12 and 14-16 days old in two clip cages (five per cage). Mortality, measured as dead and moribund individuals, and reproduction were recorded after 48h.

Data for percentage mortality and reproduction rate per recovered adult were subjected to analysis of variance for each sampling occasion separately. Percentage mortality data were arcsine transformed, and reproductive rate data to a $\log_e$ transformation, prior to analysis to stabilise sample variances. Back-transformed means were calculated following each analysis.

Effect of plant age on effectiveness of control (3rd experiment)
This field experiment tested any additional effect of plant age on the effectiveness of pirimicarb and deltamethrin in controlling each of the three clones of *N. ribisnigri* (Nr1A, Nr2A and Nr4A) when compared to untreated control plants. Two different plant ages were superimposed onto the structure of the first field experiment so that this experiment had 18 treatment combinations (three clones x three treatments x two plant ages). Specific plots were planted with three week old lettuce (cv. Saladin) on either 21 June (old plants) or 5 July (young plants). Each plot was planted with two rows of ten lettuce. Plant spacing was 30cm within rows and 45cm between rows. Each plot of 20 plants was covered immediately with an insect proof mesh cage. Each of the 18 treatment combinations was randomly applied to a single plot in each of three blocks.

Each lettuce was inoculated with approximately ten *N. ribisnigri* of the appropriate clone by placing a piece of leaf from the bulked-up aphid cultures with approximately ten aphids in the middle of each plant. Of the 20 plants in each cage, six were cut, individually bagged and taken to the laboratory on each sampling occasion. The number of aphids at each life stage was recorded, as was the total number of aphids per plant. In this experiment the gap between initial aphid inoculation and sampling prior to treatment application was 11, not 17 days.

Analysis of aphid numbers per plot (summed across the six sampled plants per occasion) was done within a generalised linear model framework, using a log-linear model assuming Poisson distributed data. Over-dispersion of the counts was allowed for in the analysis. Analyses included the total pre-treatment count (after $\log_e$ transformation) as a covariate to adjust for plot differences in the numbers prior to treatment. Treatment effects estimated in the model are re-expressed in terms of the percentage mortality for each chemical treatment relative to the untreated control. In an additional analysis the numbers of aphids per untreated plot was subjected to analysis of variance, following a $\log_e$ transformation. Back-transformed means were calculated following each analysis.

4.3 RESULTS

4.3.1 Laboratory Experiments

Clonal bioassays
Bioassays using cypermethrin, deltamethrin and lambda-cyhalothrin showed a consistent cross-resistance pattern to pyrethroid insecticides (figure 1a-1c). The clone shown previously to exhibit strongest resistance to cypermethrin (Nr4A) was also the most resistant to deltamethrin and lambda-cyhalothrin. Responses to pirimicarb showed Nr4A to be susceptible (figure 1d) thus suggesting that the pyrethroid resistance mechanism is independent of the elevated esterase activity related to pirimicarb resistance. Responses of Nr8A were very similar to those of

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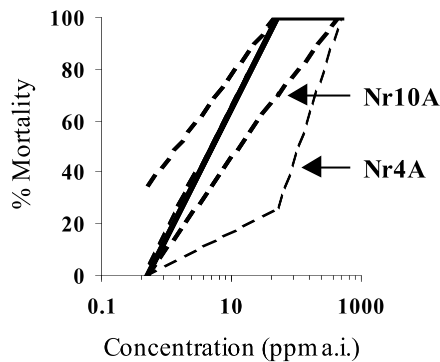
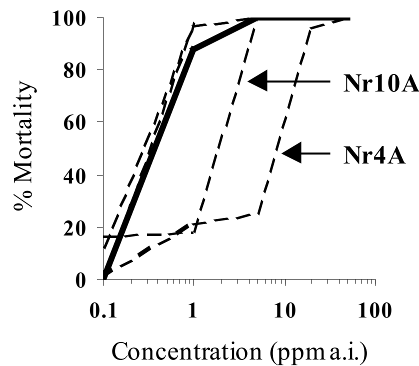
Nr1A thus demonstrating full susceptibility. Nr2A was fully susceptible to all three pyrethroids but has maintained the resistance to pirimicarb, although not to as greater level as that reported in FV210. The responses of Nr10a appear to lie between the susceptible and most resistant response for all four compounds.

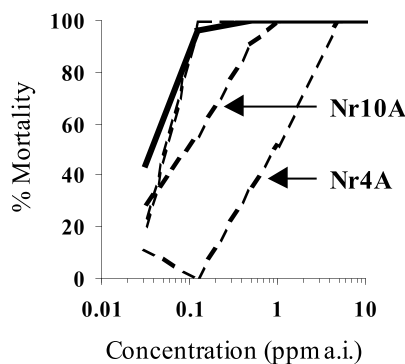
(a) Cypermethrin



(b) Deltamethrin



(c) Lambda Cyhalothrin



(d) Pirimicarb

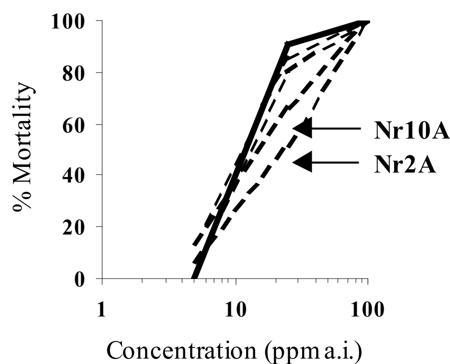
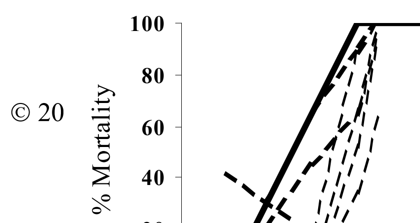


Figure 1a-d. Response of five *N. ribisnigri* clones to four insecticides. Clones of interest are indicated on individual graphs. Solid lines show response of the susceptible Nr1A clone.

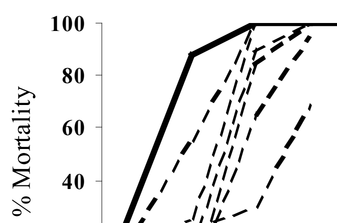
Screening of field strains

Bioassays Responses of the field strains collected in 2000 were very similar to those reported for the 1999 field samples. Tests with pyrethroids showed three broad categories of resistance corresponding to those of clones Nr8A, susceptible; Nr10A, intermediate; and Nr4A, resistant (figure 2a-2c). Responses to pirimicarb could also broadly be classed as S (those populations with a similar response to Nr1A), or R (those with a similar response to Nr2A) (figure 2d). Again, resistance mechanisms to pirimicarb or pyrethroids appeared to occur independently of each other, i.e. some of the most pyrethroid resistant populations were fully susceptible to pirimicarb and *vice versa*.

(a) Cypermethrin



(b) Deltamethrin



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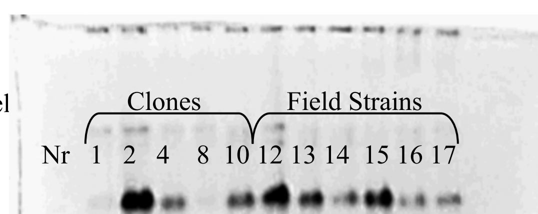
Responses to nicotine were varied and difficult to interpret without further replication (figure 2e) while all populations were at least as susceptible to imidacloprid than Nr1A (figure 2f).

AChE screening As with standard strains, a discriminating concentration of 10^{-5}M pirimicarb disclosed no evidence of MACE-type resistance to the chemical in individuals from field strains.

Esterase banding Field strains exhibited a variety of stain intensities on gels when treated with 1-naphthyl acetate. Comparison with the five clones shows a similar mix of high, medium and low intensity staining (figure 3). The increase in E0.34 band intensity was found to correlate well with the reduced mortality levels of field populations at a diagnostic concentration of pirimicarb (10ppm) (figure 4).



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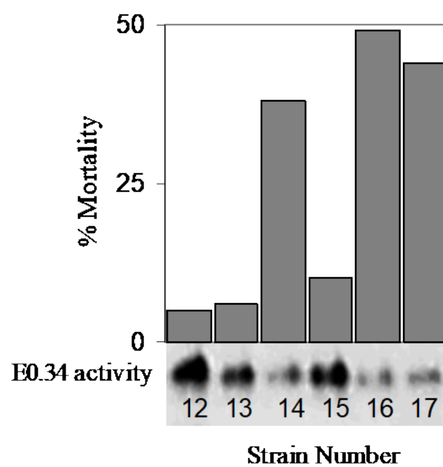
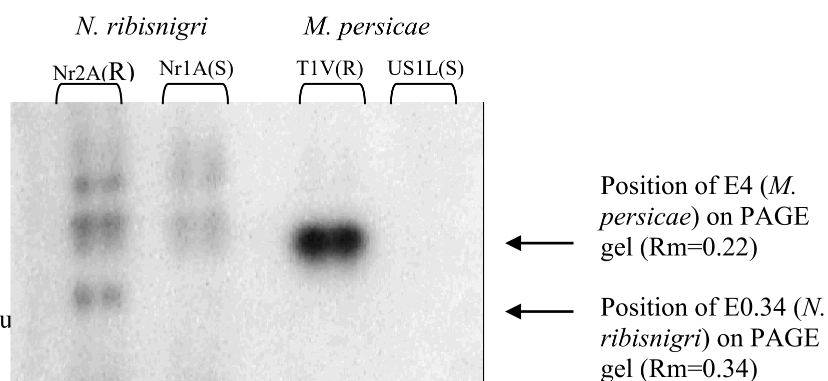


Figure 4. Comparison of E0.34 stain intensity in *N. ribisnigri* field populations and response to a single concentration (10ppm) of pirimicarb at 72hrs.

Esterase analysis

Radiolabelling Radiolabel binding studies showed an increase in the amount of ^{3}H DFP at the E0.34 position in Nr2A compared with Nr1A. This suggested that, as with *M. persicae* E4, the elevation in esterase activity is caused by overproduction of the esterase rather than the presence of a more active form (figure 5).



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Esterase purification The esterase, E0.34, associated with resistance to pirimicarb was successfully purified by electro-elution (figure 6). *Circa* 1mg of purified protein was recovered from approximately 20g of Nr2A. The pure sample was used for the production of anti-serum for use in an immunoassay, and for reactivation studies described below.

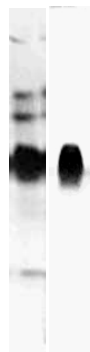

Figure 6. Comparison of banding patterns of crude Nr2A homogenates (left gel strip) and purified E0.34 (right gel strip).

Reactiviation studies After removal of excess inhibitor, insecticide bound E0.34 was shown to recover activity extremely slowly over the course of six hours (an increase of $2mODmin^{-1}hr^{-1}$) suggesting that the major role of E0.34 in insecticide detoxification is by sequestration rather than rapid hydrolysis of the insecticide.

Isolation and sequencing of sodium channel gene
No difference in gene sequence was found between Nr1A and Nr4A at the location of the *M. persicae kdr* mutation site.

4.3.1 Field Experiments

Effect of insecticides on pyrethroid-and pirimicarb-resistant *N. ribisnigri* (1st experiment)
Aphid mortality on pirimicarb and deltamethrin treated plants is summarised in Tables 2a and b. The results are discussed in relation to pirimicarb-treated plants and then in relation to deltamethrin-treated plants.

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A significant reduction in total aphid numbers for the susceptible clone (Nr1A) was observed on pirimicarb-treated plants two and six days after treatment (Table 2a). This reduction in total numbers was statistically the same for both the pirimicarb-resistant (Nr2A) and pyrethroid-resistant (Nr4A) clone when compared to the Nr1A on pirimicarb-treated plants two and six days after treatment. However, there were significantly more ($P < 0.1$) Nr2A young nymphs per plant than Nr1A young nymphs on pirimicarb-treated plants 6 days after treatment (Table 2b).

In contrast, there was a significant difference in aphid numbers between clones on deltamethrin-treated plants two days after treatment. No significant difference in total numbers of Nr1A was seen between deltamethrin-treated plants and untreated controls two days after treatment. However, differences were seen in the response of Nr2A and Nr4A to deltamethrin-treated plants. Significantly greater mortality of adult and large nymphs ($P < 0.1$) and adult ($P < 0.05$) Nr2A and significantly lower mortality of adult and large nymphs'($P < 0.1$) and adult ($P < 0.1$) Nr4A *N. ribisnigri* were seen compared to Nr1A on deltamethrin-treated plants two days after treatment (Table 2a). A significant reduction in total numbers of Nr1A *N. ribisnigri* was seen on deltamethrin-treated plants compared to untreated controls 6 days after treatment with similar reductions being seen for both Nr2A and Nr4A on deltamethrin-treated plants 6 days after treatment. Lower mortality ($P <0.1$) of small nymphs of Nr4A *N. ribisnigri* was seen 6 days after treatment when compared to Nr1A *N. ribisnigri*.

Effect of pirimicarb and deltamethrin residues on survival and reproduction of *N. ribisnigri* (2nd experiment)

The use of dip tests at field doses gave much more repeatable results than those achieved from spraying plants had achieved in 1999. In this experiment, high mortality of susceptible (Nr1A) *N. ribisnigri* was seen on pirimicarb- and deltamethrin-treated plants (Table 3a). The initially high mortality of pirimicarb-resistant (Nr2A) and pyrethroid-resistant (Nr4A) *N. ribisnigri* on treated plants immediately after treatment declined dramatically one day after treatment (Table 3a).

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Table 2 *The percentage mortality of three clones of N. ribisnigri compared to untreated controls, two and six days after treatment with pirimicarb or deltamethrin **

a. two days

Aphid development stage	Insecticide	Aphid clone		
		Nr1A	Nr2A	Nr4A
Total aphids	pirimicarb	59.8	58.0	74.7
	deltamethrin	31.7	56.1	4.2
Adults and large nymphs	pirimicarb	57.7	51.8	60.8
	deltamethrin	15.8	57.7	-33.5
Adults only	pirimicarb	64.8	58.4	64.8
	deltamethrin	30.2	63.4	-18.2
Small nymphs	pirimicarb	60.6	60.5	77.7
	deltamethrin	35.7	55.7	12.1

b. six days

Aphid development stage	Insecticide	Aphid clone		
		Nr1A	Nr2A	Nr4A
Total aphids	pirimicarb	54.8	2.0	49.3
	deltamethrin	49.0	38.9	19.7
Adults and large nymphs	pirimicarb	35.6	15.0	51.0
	deltamethrin	25.8	21.0	15.2
Adults only	pirimicarb	42.1	15.6	52.6
	deltamethrin	34.9	36.8	8.3
Small nymphs	pirimicarb	61.1	21.7	48.9
	deltamethrin	56.8	44.7	21.0

* Negative values represent an increase in aphid numbers compared to untreated control plants

Mortality of Nr2A *N. ribisnigri* was comparable on pirimicarb and deltamethrin-treated plants one day after treatment. However, this was not the case for Nr4A *N. ribisnigri*, as significantly higher mortality was observed on pirimicarb-treated plants compared to deltamethrin-treated plants one day after treatment (Table 3a). Mortality on pirimicarb and deltamethrin treated plants was significantly reduced for Nr2A and Nr4A compared to the susceptible *N. ribisnigri* clone two and four days after treatment. Low mortality levels on all treatment combinations were observed seven days after treatment.

Reproduction of Nr2A and Nr4A *N. ribisnigri* on deltamethrin treated plants was significantly greater than that of Nr1A zero, one and two days after treatment

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(Table 3b). This was not the case on pirimicarb treated plants where significantly greater reproduction of Nr2A and Nr4A compared to Nr1A was observed only one and two days after treatment (Table 3b).

Table 3a *Percentage mortality (transformed value in brackets) of N. ribisnigri clones Nr1A, Nr2A and Nr4A on plants 0,1,2,4,and 7 days after no treatment or treatment with deltamethrin or pirimicarb.**

Treatment Factor	treatment factor	Number of days after treatment 0	1	2	4	7
Clone						
Nr1A		73.2 (58.8)	76.8 (61.2)	56.4 (48.7)	75.0 (60)	60.6 (51.1)
Nr2A		81.7 (64.7)	55.1 (47.9)	17.7 (24.9)	29.5 (32.9)	4.8 (12.7)
Nr4A		80.2 (63.6)	37.6 (37.8)	30.3 (33.4)	42.0 (40.4)	19.6 (26.3)
LSD		(6.77)	(10.56)	(13.94)	(12.37)	(7.59)
Plant treatment						
Untreated		3.1 (10.2)	10.7 (19.1)	14.4 (22.3)	29.0 (32.6)	18.7 (25.6)
Pirimicarb		99.7 (86.9)	86.7 (68.6)	25.5 (30.3)	65.9 (54.3)	5.2 (13.2)
Deltamethrin		100 (90)	73.8 (59.2)	66.1 (54.4)	52.3 (46.3)	7.1 (15.4)
LSD		(6.77)	(10.56)	(13.94)	(12.37)	(7.59)
Clone	*plant treatment*					
Nr1A	Untreated	0 (0)	0.4 (3.7)	4.2 (11.8)	16.5 (23.9)	4.8 (12.7)
Nr1A	Pirimicarb	99.5 (86.3)	100 (90)	59.9 (50.7)	95.2 (77.4)	2.7 (9.5)
Nr1A	Deltamethrin	100 (90)	100 (90)	98.7 (83.5)	96.1 (78.6)	15.4 (23.1)
Nr2A	Untreated	6.0 (14.2)	22.6 (28.4)	6.1 (14.3)	22.5 (28.3)	5.0 (12.9)
Nr2A	Pirimicarb	100 (90)	78.2 (62.2)	2.6 (9.3)	41.1 (39.9)	0.7 (4.8)
Nr2A	Deltamethrin	100 (90)	63.8 (53.0)	60.3 (51)	25.8 (30.5)	12.3 (20.5)
Nr4A	Untreated	7.8 (16.3)	18.3 (25.3)	42.9 (40.9)	51.0 (45.6)	12.3 (20.5)
Nr4A	Pirimicarb	99 (84.4)	64.8 (53.6)	26.5 (31)	51.2 (45.7)	18.4 (25.4)
Nr4A	Deltamethrin	100 (90)	32.2 (34.6)	22.8 (28.5)	24.7 (29.8)	29.8 (33.1)
LSD		(11.73)	(18.3)	(24.14)	(21.43)	(13.15)

Table 3b *Reproduction per recovered adult (transformed values in brackets) of N. ribisnigri clones Nr1A, Nr2A and Nr4A on plants 0,1,2,4,and 7 days after no treatment or treatment with deltamethrin or pirimicarb.**

Treatment Factor	treatment factor	Number of days after treatment 0	1	2	4	7
Clone						
Nr1A		0.16 (-1.86)	0.11 (-2.10)	0.40 (-0.70)	1.03 (0.03)	4.34 (1.49)
Nr2A		0.58 (-0.54)	0.54 (1.62)	3.83 (1.37)	0.60 (-0.51)	0.03 (-3.50)
Nr4A		0.97 (-0.03)	1.54 (4.56)	5.37 (1.70)	4.66 (1.56)	5.95 (1.80)
LSD		(0.82)	(0.82)	(0.67)	(1.25)	(0.86)
Plant treatment						

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Untreated		4.99 (1.61)	4.34 (1.49)	4.65 (1.56)	2.04 (0.76)	0.84 (-0.06)
Pirimicarb		0.01 (-4.26)	0.18 (-1.28)	1.23 (0.29)	0.75 (-0.16)	1.12 (0.20)
Deltamethrin		1.16 (0.23)	.069 (-0.23)	1.68 (0.52)	1.53 (0.49)	0.60 (-0.35)
LSD		(0.82)	(0.82)	(0.67)	(1.25)	(0.86)
Clone	*plant treatment*					
Nr1A	Untreated	3.31 (1.2)	3.24 (1.18)	3.28 (1.19)	4.05 (1.40)	4.57 (1.52)
Nr1A	Pirimicarb	0.00 (-4.61)	0.01 (-4.11)	0.15 (-1.85)	0.43 (-0.85)	5.25 (1.66)
Nr1A	Deltamethrin	0.11 (-2.16)	0.03 (-3.38)	0.24 (-1.42)	0.62 (-0.47)	3.63 (1.29)
Nr2A	Untreated	5.74 (1.75)	3.50 (1.28)	5.58 (1.72)	0.41 (-0.90)	0.02 (-3.59)
Nr2A	Pirimicarb	0.00 (-4.61)	0.57 (-0.56)	3.19 (1.16)	0.52 (-0.64)	0.06 (-2.79)
Nr2A	Deltamethrin	3.48 (1.25)	2.43 (0.89)	3.37 (1.22)	1.02 (0.02)	0.01 (-4.13)
Nr4A	Untreated	6.54 (1.88)	7.37 (2.00)	5.81 (1.76)	5.81 (1.76)	6.55 (1.88)
Nr4A	Pirimicarb	0.02 (-3.57)	2.29 (0.83)	4.85 (1.58)	2.74 (1.01)	5.52 (1.71)
Nr4A	Deltamethrin	4.99 (1.61)	5.92 (1.76)	5.75 (1.75)	6.75 (1.91)	6.04 (1.8)
LSD		(1.43)	(1.54)	(1.15)	(2.17)	(1.48)

* For each experimental occasion, mortality (Table 3a) and reproduction (Table 3b) for the individual factors
'plant treatment' (i.e. effect of particular treatment across all clones) and 'clone' (mortality of that clone on all plants) are
given separately followed by mortality (Table 3a) and reproduction (Table 3b) for each of the nine
treatment interactions (I.e. combinations of plant treatment and aphid clone).
Significant differences between these values for each individual factor for each set of interaction values are made by
by comparing them with the least squares deviation (LSD) value (in brackets) at the bottom of each column

Effect of plant age on effectiveness of control (3rd experiment)

There was no significant difference in mortality of susceptible (Nr1A) *N. ribisnigri* after treatment with pirimicarb or deltamethrin in comparison to untreated plants two or six days after treatment. As a result, it would be unwise to draw any conclusions about the effect of plant age on control of insecticide-resistant *N. ribisnigri* (Tables 4a and 4b). Such low levels of mortality in the positive control indicate that insecticide application was not effective in this experiment. Despite this, results from untreated plants suggest that the assumptions behind the experimental design may be invalid. In doing this experiment it was assumed that young and old plants were equally acceptable for the three clones of *N. ribisnigri*, and that each clone would increase in numbers at a similar rate on plants of the same age. Data from the untreated plants in this experiment suggest that this was not the case. Significantly more aphids were found on young plants compared to old plants across all clones one day before and two days after treatment (Table 5). Also there were significantly more pyrethroid-resistant (Nr4A) *N. ribisnigri* compared to susceptible *N. ribisnigri* across both plant ages for all sampling dates (Table 5).

Table 4a *The percentage mortality of three clones of N. ribisnigri compared to untreated controls, two days after treatment with pirimicarb or deltamethrin*

Aphid development stage	Insecticide	plant age	Aphid clone Nr1A	Nr2A	Nr4A
Total aphids	pirimicarb	old	25.1	3.7	3
		young	2.7	-21.1	29.5
	deltamethrin	old	80.2	-13.6	-45.6
		young	39.1	-118	-62.3

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Table 4b *The percentage mortality of three clones of N. ribisnigri compared to untreated controls, six days after treatment with pirimicarb or deltamethrin*

Aphid development stage	Insecticide	plant age	Aphid clone Nr1A	Nr2A	Nr4A
Total aphids	pirimicarb	old	65.2	42.3	27.7
		young	33.7	-154.4	-60.1
	deltamethrin	old	84.9	-4.9	2.6
		young	72.2	-349.6	-241.3

Table 5 *Log transformed number of three clones of N. ribisnigri per plant on young and old untreated lettuce in the field **

No. of days before and after treatment	age	Aphid clone Nr1A	Nr2A	Nr4A	Plant age	LSD	Factor
-1	old	2.29	3.27	4.24	*3.27*	*0.36*	Plant age
	young	4	4.44	5.08	*4.51*	**0.45**	clone
	clone	**3.15**	**3.86**	**4.66**		0.63	interaction
+2	old	2.69	4.38	4.35	*3.81*	*0.42*	Plant age
	young	4.33	4.46	5.72	*4.84*	**0.34**	clone
	all plants	**3.52**	**4.42**	**5.04**		0.59	interaction
+6	old	3.04	4.29	5.06	*4.13*	*NS*	Plant age
	young	2.93	3.54	5.44	*3.97*	**0.49**	clone
	all plants	**2.99**	**3.91**	**5.25**		0.84	interaction

* Number of aphids per plant for individual factors are separately identified (plant age in italics, clone in bold) for each sampling occasion.
The number of aphids per plant for specific treatment combinations (i.e. interactions of plant age and clone) are shown in standard type.
Significant differences between values for individual factors (plant age in italics, clone in bold and treatment interactions in standard type) are given for each sampling occasion.

4.4 DISCUSSION

Bioassays conducted on clones from 1999 samples and mixed field populations received during 2000, provide a clear-cut picture of the current resistance status of UK populations of *N. ribisnigri*. As described last year, populations can still be classed into one of four phenotypic modes: (1) susceptible to all insecticides tested (e.g. Nr8A); (2) resistant to pirimicarb (e.g. Nr2A); (3) resistant to all pyrethroids tested (e.g. Nr4A); (4) resistant to pirimicarb and pyrethroids (e.g. Nr10A). However, maximum resistance factors range from around only *c.*4-fold to pirimicarb to *c.*50-fold to cypermethrin. The field population received from France exhibited responses similar to those of Nr10A, i.e. intermediate resistance to both

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pirimicarb and pyrethroids. The confirmation of 50-fold pyrethroid resistance is of great concern and future field samples need to be monitored closely for any further change in pyrethroid resistance status. Initial findings show that if this resistance is a result of a *kdr* mechanism, it is not caused by the same mutation as found in *M. persicae*.

The continued susceptibility of all populations to imidacloprid is encouraging and probably reflects the limited exposure *N. ribisnigri* receives to this compound (seed treatment only). Initial bioassays with nicotine showed varied responses between field populations. These results will be used as baseline data for comparison with results collected over 2001. In addition, preliminary bioassays with the new compound, pymetrozine, will be conducted in case this compound is approved for use on lettuce.

The screening of field strains found no modified acetylcholinesterase (MACE) which is consistent with the low levels of resistance seen in the bioassays. If a MACE population is found in the UK or is acquired from abroad, further AChE characterisation will be undertaken to optimise conditions for detecting it in field populations.

The continued correlation between pirimicarb resistance in bioassays and enhancement of the esterase band, E0.34, supported the need to purify this enzyme and conduct further biochemical characterisation in order to understand how the enzyme is conferring resistance. While electro-elution was not the most efficient method of purification (using 20g of aphids to produce only 1mg of protein), without knowledge of the biochemical properties of the esterase, this was the fastest, most accurate method to use. Indeed, biochemical studies after purification demonstrated some unusual properties of E0.34 compared to E4 found in *M. persicae*, which would have impeded the more usual method of column purification. The purified E0.34 is now being used to develop a rapid *in vitro* immunoassay for diagnosing pirimicarb resistance in field populations.

Purification of E0.34 also allowed the interaction between the enzyme and pirimicarb to be studied. Previously reactivation work was hindered, as the role of E0.34 in detoxification could not be distinguished from the activity of other esterases, present in equal quantities in all populations. Incubation of purified E0.34 with pirimicarb showed the role of the esterase in detoxification is mainly one of sequestration rather than significant hydrolysis of the insecticidal ester. This is supported by the confirmation of last years finding that the elevation in esterase activity is due to over-production of the esterase rather than the presence of a more active form. The finite reserve of detoxifying esterase coupled with the action of sequestration probably accounts for the low-level resistance to pirimicarb observed in bioassays. Although resistance levels recorded for pirimicarb appear somewhat low to account for control problems, difficulties with relating laboratory bioassay data to field performance are well recognised (e.g. Sawicki, 1987). There are examples of substantially increased tolerance in bioassays causing little or no reduction in field control efficacy (Farnham *et al.*, 1984) and, conversely, examples of resistance barely detectable in bioassays having a major impact in the field (Dennehy & Granett, 1984). However, field experiments in the project have again supported the findings of laboratory work. The results of the first field experiment suggest that pirimicarb-resistant (Nr2A) *N. ribisnigri* are resistant due to a reduced

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period over which pirimicarb killed this clone effectively. Thus after comparable initial kill for pirimicarb-resistant and susceptible *N. ribisnigri,* surviving Nr2A reproduce earlier leading to a more rapid population recovery than susceptible (Nr1A) *N. ribisnigri*, a result consistent with the findings of field experiments in 1999. This contrasts with the effect of deltamethrin on pyrethroid-resistant (Nr4A) and pirimicarb-resistant (Nr2A) *N. ribisnigri*. In this case there was significantly lower initial mortality of Nr4A *N. ribisnigri* compared to Nr2A *N. ribisnigri*. However, there was no significant difference in mortality between Nr4A and the susceptible clone (Nr1A) although an increase in the number of adults and large nymphs and adults only was seen for Nr4A compared to Nr1A at a significance level of 10%.

The results of the second experiment, in the glasshouse, showed similar mortality of pirimicarb-resistant *N. ribisnigri* on both pirimicarb- and deltamethrin-treated plants that was in contrast to the results for pyrethroid-resistant *N. ribisnigri*, which showed significantly greater mortality 1 day after treatment on pirimicarb-treated plants compared to deltamethrin-treated plants. Additionally, Nr2A reproduction on pirimicarb-treated plants was greater that that of Nr1A on pirimicarb-treated plants one and two days after treatment.

The level of control in the plant age experiment was disappointing, but does allow attention to concentrate on two unexpected results from untreated plants. The different suitability to *N. ribisnigri* of young and old lettuce was marked in this experiment, and contrasted to previous work that gave no indication of an effect of plant age on suitability of lettuce for *N. ribisnigri*. It should be noted that previous data on the effect of plant age on aphid suitability were collected from plots that had natural aphid infestation (Tatchell *et al*., 1998). This may be accounted for by looking at differences between the experiments. The most obvious difference between the two sets of experiments is the time at which first inoculation occurred was controlled in the experiments reported here. We would speculate that early infestation may change suitability of the plant in the long term with initial infestation at a younger growth stage causing changes in the plant that are favorable to the aphid.

In the experiments conducted as part of this project aphids inoculated onto previously uninfested older plants showed a slower increase in numbers, compared to those on younger plants. It may be that younger lettuce are better hosts than older lettuce, but this would be expected to have become apparent in previous work (Tatchell *et al*., 1998). It is possible that infestation of young plants by *N. ribisnigri* causes significant change in subsequent nutritional status due to the injection of aphid saliva, acting to provide a positive feedback. If so, then this would explain why previously uninfested older plants in this experiment are lower quality hosts than younger plants but plants that have possibly been infested when young in open plots show no reduction in quality as a host for *N. ribisnigri* as they get older.

Another additional result was the significantly higher numbers of Nr4A, the pyrethroid-resistant clone, when compared to Nr1A particularly. These results have been obtained with single clones, and it may be that the greater capacity for increase in Nr4A is unrelated to the insecticide resistant character of the clone. Similarly, it may be that Nr1A is a clone that shows a relatively slow rate of increase, and that other susceptible clones may show a rate of increase more

comparable to Nr4A than Nr1A. This can only be determined by further investigations into the capacity for increase of more than 1 clone for each insecticide resistance characteristic, so that a range of data can be obtained, and how this then relates to insecticide resistant status can be more accurately ascertained.

4.5 FUTURE PLANS

Major objectives for the next twelve months are as follows:

- Further characterisation of resistance patterns in *N. ribisnigri* samples collected in the UK, especially from sites experiencing control difficulties

- Assuming the final steps to raise an antiserum to esterase E0.34 are successful, a diagnostic immunoassay for esterase-based resistance to pirimicarb will be developed and validated using field-caught aphids

- Work to investigate possible causes of pyrethroid resistance in *N. ribisnigri* will be continued, as will that to detect a MACE-type mechanism of resistance to pirimicarb as early as possible

- Depending on advice we receive from project sponsors, new insecticides (e.g. pymetrozine) and/or combinations of existing ones (e.g. pirimicarb + lambda-cyhalothrin; 'Dovetail') will be incorporated into laboratory and field experiments

- Field experiments will focus on comparing the relative efficacy and persistence of different pyrethroids against susceptible and resistant *N. ribisnigri*.

- Articles summarising our findings so far and the resulting practical recommendations will be published in trade journals

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