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Selection for resistance to azadirachtin in the green peach aphid, *Myzus persicae*

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Abstract. Two lines of *Myzus persicae* of the same origin were treated repeatedly with pure azadirachtin (aza), or a refined neem seed extract (NSE), at the equivalent concentration of aza. After 40 generations, the aza-selected line had developed 9-fold resistance to aza compared to a non-selected control line, whereas the NSE-selected line did not. These results suggest that a blend of active constituents in a botanical insecticide such as neem might diffuse the selection process, mitigating the development of resistance compared to that expected with a single active ingredient.
Key words. Green peach aphid; *Myzus persicae*; pesticide resistance; selection; neem; azadirachtin.

Much effort has been devoted to the discovery and development of plant extracts and phytochemicals as alternatives to synthetic insecticides for pest management. Among the most promising of the natural products investigated to date are those derived from the Indian neem tree, *Azadiracta indica* (Meliaceae). Azadirachtin (aza), a tetranortriterpenoid, is the most active constituent in neem-based products. It has antifeedant, growth disrupting and reproductive inhibiting bioactivities against over 200 species of insects[1]. The extremely low mammalian toxicity, selective activity against pest insects, and systemic action in a variety of important crop plants are further attractive properties of aza-containing products[2].

As with any novel pesticide, one major concern is whether target insects will develop resistance to components of neem following repeated exposure. To our knowledge, only one study to date[3] has addressed this question. The diamondback moth, *Plutella xylostella*, did not show any sign of resistance to a neem seed kernel extract after 42 generations of selection. In contrast, two lines of *P. xylostella* selected with deltamethrin showed 20- and 35-fold resistance to this synthetic pyrethroid in the same study.

Neem-based insecticides, like Margosan-O (W.R. Grace Co, Columbia, Maryland, USA) and Azatin (Agridyne Technologies Inc., Salt Lake City, Utah, USA), have been registered in the United States for use in greenhouses, commercial nurseries, home and garden applications and recently have been approved for use on food crops[1,4]. Other neem-based products are under review for registration in other countries[5]. Additional studies on the potential for the development of resistance to azadirachtin or neem extract are necessary to develop resistance management strategies should they be required.

The green peach aphid, *Myzus persicae* (Sulzer), is a serious pest and vector of a multitude of viral diseases of field crops, vegetables, ornamentals, and greenhouse crops worldwide. It has displayed a remarkable ability to establish resistance to almost every insecticide with which it has been treated[6]. Azadirachtin and neem-based insecticides can be effective in controlling *M. persicae* and other aphid species[4,7-9]. However, no studies have been done on the potential for *M. persicae* to develop resistance to neem extracts or aza. We report here the results of a selection experiment with *M. persicae* utilizing a refined neem seed kernel extract and pure azadirachtin, respectively, as the selecting agents. Our working hypothesis was that resistance, if acquired, would be higher in the aphid line selected with pure aza than in the line selected with neem extract.

Materials and methods

A colony of *M. persicae* was maintained on mustard cabbage, *Brassica chinensis* L., cv Pakchoi, plants in the laboratory without any insecticidal pressure for more than six months prior to the selection experiments. The parental *M. persicae* colony originated from a greenhouse on the U.B.C. campus. It had never been exposed to neem products before, as neem-based pesticides have yet to be registered in Canada for any use.

The parental colony was divided into three lines. One was used in the selection with pure azadirachtin (98% pure, supplied by J.T. Arnason, University of Ottawa, Ottawa, Canada), another was used in the selection with neem seed extract (NSE), and the third one was assigned as a control without any chemical selection. The neem extract used was an oil-free, refined seed kernel extract, containing 25% azadirachtin, 5% salannin and 3% nimbin (V.V.L.N. Prasad, Hyderabad, India). This material serves as a technical grade concentrate used for the manufacture of neem insecticides.

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The lines were reared in 31 × 29 × 36 cm cages, each containing four mustard cabbage plants (4-fully expanded leaf stage) grown in a mixture of sandy loam soil and peatmoss (4:1) in plastic pots (10 cm diameter). All three lines were maintained in an insectary at room temperature (25 ± 2 °C) and supplemented with constant fluorescent light. At these rearing conditions, the average generation time was 6 days (from birth to reproduction).

Neem and azadirachtin have no, or very low, direct contact toxicity, and require ingestion to be fully effective[4,8,10]. In our experiment, selection was achieved by exposing aphids to treated plants on a weekly basis. This method had a couple of advantages: 1) it reduced the risk of aphids from different lines mixing because the aphids did not have to be removed from their cages to be treated; and 2) the aphids were exposed to the treatment materials by both contact and feeding. The concentration used was 2.4 ppm aza equivalents, a baseline LC_{50} value, derived from three bioassay results with the parental aphid colony at the beginning of the selection experiment. Azadirachtin solution was made from a 1000 ppm acetone stock, dissolved in 100 ml of 0.125% Triton X-100 solution. Neem seed kernel extract, 0.96 mg, was first dissolved in 0.5 ml acetone and then dissolved in 100 ml 0.125% Triton X-100 solution giving a final concentration of 2.4 ppm aza. A mustard cabbage plant was sprayed from all sides with a hand-held sprayer to the point of runoff (70–80 ml) and the remaining solution (20–30 ml) was poured into each respective pot. A treated plant was provided to each selected line on the first day of each week and an untreated plant was provided on the last day of each week. Nymphal aphids taken from the untreated plants were used in LC_{50} assays; these were offspring of aphids exposed to aza- or neem extract-treated plants. As such, these nymphs were not directly exposed to aza, except as embryos. In this way, the confounding effects of residual aza from systemic action in the treated mustard plants was minimized.

Before the selection experiment began, bioassays were done on the parental generation of *M. persicae*. From the third month after the selection experiment was initiated, the LC_{50} for azadirachtin was assayed every month for the aza-selected, neem-selected and control aphid lines, respectively using the method of Lowery and Isman[8]. Test concentrations ranged from 0 to 8 ppm (0, 1, 2, 4, 6 and 8) with 0.125% Triton X-100 as the carrier. This concentration range was adjusted accordingly, for the aza-selected line, based on its LC_{50} value in the previous month, so that the highest concentration would cause approximately 80% mortality. Leaf discs (2 cm diameter) cut from fully expanded mustard cabbage leaves were dipped into each solution for 10 seconds and allowed to air dry. Two leaf discs were placed on top of a rubber piece in the center of a plastic petri dish (0.7 × 5 cm diameter), so that aphids had access to the undersurface of the discs. Ten 2nd instar nymphs were carefully introduced with a fine brush onto the leaf discs (5/disc) and the dish covered with a tight-fitting lid. There were five replicates for each concentration. The petri dishes were placed in a covered 26 × 37 × 5.5 cm clear plastic tray and placed into a growth chamber maintained at 16 ± 1 °C with constant fluorescent light. Three days later, the treated leaf discs were replaced with two new untreated mustard cabbage leaf discs and surviving nymphs were transferred onto the new leaf discs. These untreated leaf discs were replaced with new ones after a further three days. On the tenth day from the beginning of the bioassay, final mortality was recorded and used to estimate probit regressions[8].

Results and discussion

Bioassay results for the three lines (two selected and one control) are presented in the table. After two months of selection (ca. 10 generations), the LC_{50} value for the aza-treated line was significantly higher than that of the control line (based on the criteria of non-overlapping 95% CI, table). This trend continued through eight months (ca. 40 generations) of selection. Although the LC_{50} values for the NSE-treated line were always slightly higher than those of the control line, at no point did they differ significantly. The figure shows the responses of the three lines to treatment with a discriminating concentration of aza (8 ppm). When a time factor was included in the regression analysis, the data

Table. LC_{50} (95% CI) values of azadirachtin (in ppm) for control, NSE-selected and aza-selected *M. persicae* lines.

Month	Control	NSE-selected	RF	Aza-selected	RF
February	2.42 (1.76–3.28)	2.42 (1.76–3.28)	1.00	2.42 (1.76–3.28)	1.00
April	2.26 (1.73–2.96)	4.91 (2.83–8.49)	2.17	5.46 (3.52–8.47)	2.42
May	1.95 (1.29–2.94)	3.20 (2.42–4.24)	1.64	ND	
June	2.70 (2.00–3.64)	4.02 (3.09–5.23)	1.49	5.42 (3.79–7.76)	2.01
July	1.64 (0.90–2.97)	ND		8.95 (4.76–16.80)	5.46
August	2.49 (1.77–3.51)	3.29 (2.65–4.09)	1.32	ND	
September	2.65 (2.00–3.52)	3.59 (2.45–5.28)	1.35	6.17 (4.30–8.86)	2.33
October	1.47 (0.98–2.19)	2.16 (1.47–3.18)	1.47	13.45 (9.90–18.27)	9.15

RF, resistance factor = LC_{50} in selected lines/LC_{50} in control line.
ND, not determined (insufficient aphids).

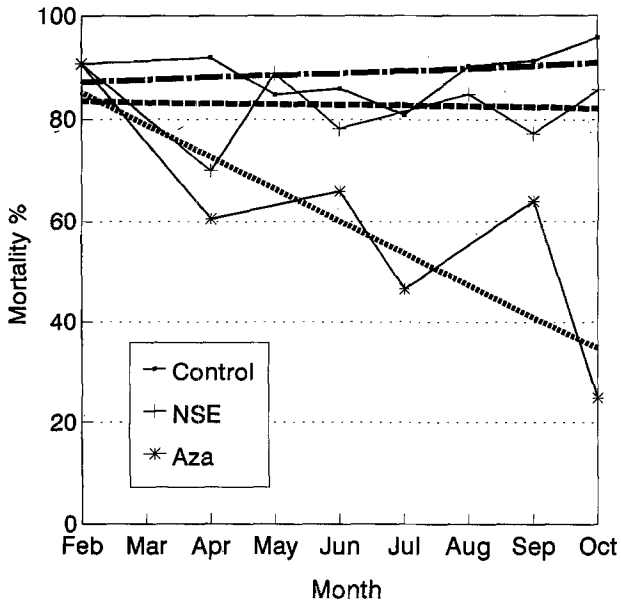

Figure. Susceptibility to a discriminating concentration of azadirachtin (8 ppm) in control, NSE-selected and aza-selected *M. persicae* lines in leaf disc assays. Dashed lines represent regression lines for mortality data versus date of bioassay.

clearly demonstrate that the aza-selected aphids became less susceptible, whereas the NSE-selected aphids did not (Aza-selected, $R^2 = 0.66$, $p = 0.0104$; NSE-selected, $R^2 = 0.02$, $p = 0.5390$; Control, $R^2 = 0.0358$, $p = 0.8228$).

There are numerous literature reports of insecticide resistance in *M. persicae* worldwide. Reported resistance levels vary considerably depending on the strains and insecticides tested; from >400 fold resistance to disulphoton sulphone to around 2 to DDT, diazoxon and pirimicarb[11–13]. Within each strain, resistance was generally least to carbamates, greater to organophosphorus compounds and greatest to the pyrethroids[14]. In our experiment, after 40 generations of selection the aza-selected line of *M. persicae* showed a resistance factor of 9, a low or moderate level of resistance compared to those reported in the literature. However, most reports of aphid resistance are based on populations occurring in the greenhouse or field, where the selection regime is much different from that of the laboratory. In greenhouse and field pest management, the concentrations of insecticides applied are usually greater than LC_{90}, thus, the selection pressure is much higher than that exerted in our study, although in the field influx of non-selected aphids can alleviate the selection pressure. Limited genetic variation in our parental laboratory colony compared to that of large field or greenhouse populations might have also contributed to the modest development of resistance to aza in our study. The reason we choose the LC_{50} level for selection was that in our laboratory, the populations were relatively small. Had we used LC_{90} for selection, we may well have had difficulties maintaining the lines.

The NSE-selected line did not show any evidence of resistance, corroborating the previous selection study with the diamondback moth, which similarly showed no sign of resistance in feeding and fecundity tests following 42 generations of selection with neem seed kernel extract[3]. Our ability to select for resistance in *M. persicae* with aza, but not with the neem extract containing aza, supports the hypothesis that a blend of active constituents in a botanical insecticide such as neem might diffuse the selection process, mitigating the development of resistance compared to that expected with a single active ingredient. One explanation is that different constituents in the mixture (viz. azadirachtin, salannin, nimbin and their respective analogues) might have different modes-of-action or different target sites in the aphid. Another reason might be that some ingredients other than azadirachtin inhibit detoxification enzymes that normally degrade aza. Our investigations of another botanical insecticide, from *Melia toosendan*, suggest this point (unpubl. data). The refined extract from this tree inhibits midgut esterase activity in *Spodoptera litura* larvae, whereas pure toosendanin, the principal active ingredient, does not.

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