Susceptibility of Cotton Aphid, *Aphis gossypii* Glover (Homoptera:Aphididae) Clones to Dichlorvos and its Relationship to Activity Levels of some Esterases

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Abstract: Susceptibility of cotton aphid, *Aphis gossypii* (Homoptera:Aphididae) clones to dichlorvos and its relationship to activity levels of some esterases were determined for a two-year period. While acetylcholinesterase and butyrylcholinesterase activities failed to reveal any definite trend concerning their quantitative involvement in susceptibility for the two years, carboxylesterase activities established a high degree of correlation ($r^2=0.97\pm0.01; P=0.001$) with LC$_{50}$ values, which in turn had a close host relation. A $p$-nitrophenyl acetate hydrolyzing esterase also showed activity differences with regards to varying degrees of susceptibility ($r^2=0.88\pm0.03; P=0.001$) and hence can be speculated to be a supporting factor in resistance. After 13 generations in the absence of insecticide application, LC$_{50}$ value fell to 44.50% of the starting level, while carboxylesterase activity fell to 72.60%. The implications of these in clarifying the status of resistance in the cotton aphid are discussed in this paper.

Key Words: Cotton aphid, Dichlorvos, Esterases, Resistance.

Introduction

The upsurge of diverse forms of the cotton aphid, *Aphis gossypii* Glover, has been a bother to researchers and the farming community. The insect exhibits seasonal biological variation, possesses differential rates of reproduction in both glasshouses and open fields, and is generally a serious pest of a number of horticultural crops. Reports concerning the failure of insecticides to yield desirable controls have been on the increase. Of late, dichlorvos which looked the most promising of all the organophosphorus and carbamate insecticides has also began recording field control failures in most farming areas in Japan and elsewhere. In this respect, it is envisaged that information about the possible resistance mechanism to this insecticide and other organophosphates should provide convincing facts to support the development of an effective resistance management strategy.

In the few reports available on the biochemical aspect of resistance in *A. gossypii*, 1-naphthyl acetate was used as the model and sole substrate to determine the activity of esterases. This substrate is said to be hydrolyzed by a wide range of enzymes including carboxylesterases, cholinesterases, lipases, amidases, proteinases, and thioesterases. Studies concerning the use of specific substrates to clarify the role of other esterases in resistance of *A. gossypii* are practically non-existent and information concerning the possible involvement of these esterases in resistance has only been speculative. Variation in
carboxylesterase activity in some insects as well as some other species of aphids has been noted to be solely responsible for organophosphate (OP) resistance\textsuperscript{1-9}. However, the true picture reflecting this trend in \textit{A. gossypii} still remains an issue for in-depth clarification. Against this background, an objective was set up to find out if any relationship(s) exist between susceptibility to dichlorvos and \textit{in vitro} enzymatic hydrolysis of some surrogate non-insecticidal substrates, in clones of cotton aphid.

\textbf{Materials and Methods}

\textbf{Aphids} Each clone was developed from a single aperous viviparous female picked from the field and reared on its original host in the insectary (23±2°C; 16L/8D h). Names were assigned to clones based on the host as well as order and year of collection. For example, the first clone developed on eggplant in 1992 was named E-1-92, and the second on cucumber in 1993 named C-2-93, etc.

\textbf{Chemicals} Chemicals used were of the highest grade commercially available. The following chemicals were purchased from Wako Chemical Industries Ltd. (Osaka, Japan): sodium anhydrous, potassium phosphate monobasic, disodium hydrogen phosphate, 1-naphthol, sodium dodecylsulphate (SDS), eserine sulphate, acetylthiocholine iodide (AcSCh), butyrylthiocholine iodide (BtSCh), and 5,5'-dithiobis-2-nitrobenzoic (DTNB). Azoic diazo component (FBS), 1-naphthyl acetate (1-NaA), 2-naphthyl acetate (2-NaA), \textit{p}-nitrophenyl acetate (\textit{p}-NpA), and polyethylene glycol (Triton X-100) were purchased from Tokyo Kasei Industrial Company, Tokyo, Japan. Dichlorvos 50EC was a gift from Tomono Chemical Industry, Tokyo, Japan.

\textbf{Toxicity test} The dipping method described by \textit{HAMA}\textsuperscript{10}, was adopted with some modifications. One end of a glass tube (30×20 mm, L/ID) was sealed with a piece of nylon cloth. Aphids were then introduced into the tube and the other end sealed with parafilm to prevent escape. The bottom end with the nylon cloth was then soaked with shaking for 30 sec in the various concentrations of the test insecticide dissolved in water, after the aphids had been made to fall to that end. Excess liquid was removed by blotting on a piece of filter paper. Aphids were then picked with a soft brush and placed in plastic cups containing excised leaves of their respective hosts, which were then left under a temperature of 20°C. Water was used as control. Number of dead insects was counted after 24 h and fifty as well as ninety-five percent lethal concentrations calculated using an NEC computer with a basic programme which corrected for control mortality using the formula of \textit{Asdor}\textsuperscript{11}.

\textbf{Enzyme preparations} Individual aphids were homogenized in 0.3 ml of phosphate buffer pH 7.0 using a hole slide glass, and the resultant solution was used as enzyme source for carboxylesterase assay. For cholinesterase and \textit{p}-nitrophenyl acetate hydrolyzing esterase determinations, 50 adult insects were homogenized in 1 ml of phosphate buffer containing 0.2% of Triton X-100, and centrifuged at 4°C for 10 min at 3,000 x g. The resultant supernatant fraction was then utilized for the assay.

\textbf{Carboxylesterase assay} Procedure for carboxylesterase assay was as previously described
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and consisted of 100 μl enzyme extract in phosphate buffer pH 7.0 incubated at 40 °C for 10 min with the substrate. Colour development after incubation was effected with a solution mixture of sodium dodecyl sulphate-fast blue salt (SDS-FBS) and read at 600 nm on a spectrophotometer against a control that lacked enzyme.

Cholinesterase assay Cholinesterase was assayed according to the method of Ellmen et al. with some modifications as described for Nephotettix cincticeps⁴⁴. A typical reaction mixture consisted of 1.8 ml of phosphate buffer pH 8.0, 50 μl of substrate (ASCh or BSCH), 0.1 ml of 0.01 M 5, 5'-dithiobis-2-nitrobenzoic (DTNB) and 100 μl of enzyme extract. After incubation of enzyme and substrate at 30°C for 5 min, DTNB was then added and allowed for an additional incubation time of 10 min. The reaction was stopped by the addition of 100 μl of 1×10^-3 M eserine sulphate. Activity was measured at 412 nm on a spectrophotometer.

p-NpE assay p-nitrophenyl acetate hydrolyzing esterase (p-NpE) was assayed by measuring the production of p-nitrophenol from p-nitrophenyl acetate. The assay was a modified form of that described by Krisch⁴⁵, and consisted of 2.1 ml of potassium phosphate buffer pH 7.0, 40 μl of 0.5 M p-NpA in acetone, 0.76 ml of 1% Triton X-100 and 100 μl of enzyme solution. The reaction mixture was incubated at 25°C (though this might not be the optimum temperature) for 10 min and later measured at 405 nm on a spectrophotometer against a control that lacked enzyme.

Susceptibility change in the absence of insecticide application A dichlorvos selected (E-D-R) strain kept on eggplant, was used for the study. Insects were kept in the insectary (23 ± 2°C; 16L/8D) without further insecticide selection. Carboxylesterase activity (1-naphthyl acetate as substrate) was measured for each generation while toxicity tests were carried out after every three generations.

Filter paper test This assay which was adopted for rapid determination of low and high form esterase-containing aphids was a modification of the method of Pasteur and Georgihiou⁴⁶. An aphid was homogenized in 20 μl of 0.06 M phosphate buffer containing 0.2% Triton X-100, pH 7.0. About 10 μl of enzyme solution was then transferred onto Toyo filter paper No 51A (20×400 mm, Toyo Roshi Kaisha Ltd., Japan), followed by a similar quantity of 30 mM 1-naphthyl acetate in ethanol. After enzyme-substrate reaction for about 2 min at room temperature, the filter paper was dipped into 0.2% fast blue salt for the development of blue spots. It was then washed with water and fixed in acetic acid in cold and dark for 12 h, after which it was transferred into a solution of 10% glycerol for same period of time. Both sides of the filter paper were then overlaid with cellophane and dried in dark at room temperature for at least three days. Comparisons were made against a standard which was graded with varying concentrations of 1-naphthol in ethanol and similarly developed with fast blue salt.

Results and Discussion

Susceptibility to dichlorvos by cotton aphid clones showed the unambiguous role of host association. In both 1992 and 1993, cucumber and watermelon host associated species proved
fairly tolerant to dichlorvos as compared with the others (Tables 1. and 2.). Concentration-mortality responses indicated significant differences between individuals of cucumber and watermelon on one hand, and eggplant and okra on the other, due to the failure of 95% FL to overlap. The gradual increase in resistance from April to October in a particular year, indicates resistance is selected as the days advance due to rigorous pesticide applications. Though farmers have been complaining about poor field control of cotton aphid by dichlorvos, it was realized from the studies that tolerance was not all that high, considering the fact that LCso values were all less than the producer’s recommended rate of 500 ppm.

Ambiguity of this sort is a common feature when it comes to relating results of laboratory assays to field resistance. This is because conclusions drawn from laboratory assay results do not always reflect the true field picture and thus tend to exaggerate the potential importance of resistance in a particular insect. However, since studies on pesticide resistance has to take into account the interest of the farming community, complaints of
farmers cannot be neglected for the sake of unreflective laboratory assay results. Variability in resistance ratio (RR) values and slopes of the probit regressions of the various clones indicates migration of susceptible or resistant individuals from one host or place to the other may be limited and that resistance is selected on a relatively small scale.

Table 3. Relationship between susceptibility to dichlorvos and esterase activities in 1992 clones of Aphis gossypii

<table>
<thead>
<tr>
<th>Clone</th>
<th>LC₅₀(ppm)</th>
<th>CarE activity</th>
<th>ChE activity</th>
<th>p-NpE activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-NaA</td>
<td>2-NaA</td>
<td>ASCh</td>
</tr>
<tr>
<td>E-1-92</td>
<td>39.15</td>
<td>1.88</td>
<td>1.25</td>
<td>0.23</td>
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<tr>
<td>O-1-92</td>
<td>76.51</td>
<td>7.06</td>
<td>3.53</td>
<td>0.16</td>
</tr>
<tr>
<td>E-2-92</td>
<td>79.62</td>
<td>11.25</td>
<td>5.00</td>
<td>0.12</td>
</tr>
<tr>
<td>O-2-92</td>
<td>83.06</td>
<td>10.85</td>
<td>5.00</td>
<td>0.10</td>
</tr>
<tr>
<td>T-1-92</td>
<td>118.00</td>
<td>12.22</td>
<td>12.22</td>
<td>0.05</td>
</tr>
<tr>
<td>W-1-92</td>
<td>342.32</td>
<td>27.33</td>
<td>20.00</td>
<td>0.22</td>
</tr>
<tr>
<td>C-1-92</td>
<td>457.15</td>
<td>44.11</td>
<td>23.53</td>
<td>0.38</td>
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<tr>
<td>C-2-92</td>
<td>487.16</td>
<td>44.71</td>
<td>22.50</td>
<td>0.36</td>
</tr>
</tbody>
</table>

\[ r^2(P=0.001) = 0.976 \quad 0.927 \quad 0.680 \quad 0.467 \quad 0.907 \]

___*Measurements not taken

Carboxylesterase (CarE) activity = nmol/10min/μg protein
Cholinesterase (ChE) & p-nitrophenyl acetate hydrolyzing esterase (p-NpE) activities = μmol/min/mg protein

Table 4. Relationship between susceptibility to dichlorvos and esterase activities in 1993 clones of cotton aphid

<table>
<thead>
<tr>
<th>Clone</th>
<th>LC₅₀(ppm)</th>
<th>CarE activity</th>
<th>ChE activity</th>
<th>p-NpE activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-NaA</td>
<td>2-NaA</td>
<td>ASCh</td>
</tr>
<tr>
<td>E-1-93</td>
<td>12.00</td>
<td>4.00</td>
<td>3.06</td>
<td>0.36</td>
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<tr>
<td>E-3-93</td>
<td>31.85</td>
<td>5.35</td>
<td>4.80</td>
<td>0.21</td>
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<tr>
<td>E-4-93</td>
<td>38.07</td>
<td>5.80</td>
<td>4.26</td>
<td>0.40</td>
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<tr>
<td>E-6-93</td>
<td>111.35</td>
<td>22.90</td>
<td>11.05</td>
<td>0.29</td>
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<td>C-1-93</td>
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<td>41.78</td>
<td>16.56</td>
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<tr>
<td>C-2-93</td>
<td>394.41</td>
<td>43.88</td>
<td>18.35</td>
<td>0.31</td>
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<tr>
<td>W-2-93</td>
<td>441.78</td>
<td>61.73</td>
<td>20.13</td>
<td>0.29</td>
</tr>
</tbody>
</table>

\[ r^2(P=0.001) = 0.962 \quad 0.963 \quad 0.013 \quad 0.179 \quad 0.855 \]

*Units of esterase activities are as stated in Table 3

Response to various forms of substrates (Tables 3. and 4.) gave a clear indication about
the trend of dichlorvos susceptibility and substrate utilization/hydrolysis by the associated enzymes from A. gossypii. Generally, naphthyl substrates with longer acid moiety, i.e. naphthyl laurate and myristate indicated a no-activity response, suggesting that the esterases involved lack lipase activity\textsuperscript{17}. On the other hand, the shorter naphthyl acetates, i.e. 1 and 2, proved to be the most reliable of the surrogate non-insecticidal naphthyl substrates. With the relatively high correlation between total carboxylesterase activity and LC\textsubscript{50} values ($r^2=0.97\pm0.01$; $p=0.001$), the involvement of this group of esterases in resistance of this insect cannot be overemphasized. Similar trends have already been reported qualitatively, using polyacrylamide gel electrophoretic technique\textsuperscript{10}. These results tend to support earlier reports on OP resistance studies of A. gossypii\textsuperscript{33,19}. Even though isolation of low and high forms of the enzyme from clones/colonies of the same host was not unlikely, generally the Curcubitaceae host associated species were of the high form type while the Solanaceae host associated ones were of the low form type. This shows that, the form of the naphthyl acetate hydrolyzing esterase might be host linked even though reasons for such are yet to be found.

Activity levels of the p-nitrophenyl hydrolyzing esterase were measured for both years and from the results, this enzyme seems to be a contributing factor in resistance of cotton aphid. This form of esterase has been implicated in insecticide resistance of some insects\textsuperscript{20,21}, but yet to be confirmed in aphids.

Activity levels of both acetylcholinesterase and butyrylcholinesterase in relation to dichlorvos susceptibility gave no definite pattern as regards their quantitative involvement in resistance. Results were erratic and incoherent. It is however likely that the roles of these enzymes in resistance of this aphid might manifest themselves through other mechanisms such as lowered sensitivity to insecticides. In China, studies have revealed a correlation of a combination of elevated carboxylesterase activity and lowered acetylcholinesterase sensitivity with organophosphate resistance\textsuperscript{30}. The possibility however, that the acetyl-and butyrylcholinesterase activities in this aphid may be linked to its host needs further evaluation. This is because host relation and dietary composition have been shown to have effect on acetylcholinesterase specific activity of cotton aphid and its inhibition by omethoate\textsuperscript{22}.

![Fig. 1. Trend of carboxylesterase activity and susceptibility to dichlorvos in the absence of further insecticide application over thirteen generations.](image-url)
Fig. 1. shows the trend in activity and tolerance to dichlorvos in the E-D-R strain upon rearing without further insecticide selection. While carboxylesterase activity fell to 93.22% in the third generation, tolerance to dichlorvos (in terms of LC₅₀) fell to 98.22%. However, at the end of the sixth generation, activity was 86.24% of the original while LC₅₀ had fallen to 62.93%. Less than fifty percent, i.e. 44.50% of the original tolerant level was retrieved at the end of the thirteenth generation, while activity remained at 72.60%. These show that while tolerance to dichlorvos decline sharply after cessation of insecticide application, carboxylesterase activity gradually and tend to be somehow stable between the sixth and tenth generations. Despite the relative fall in tolerance and carboxylesterase activity, no qualitative change in carboxylesterase banding pattern was observed electrophoretically up to the thirteenth generation. This suggests that dichlorvos resistance in cotton aphid declines quantitatively in the absence of insecticide but continues to persist qualitatively and possess the genetic potential to readdress itself to challenges when insecticide application resumes. Takada and Murakami, observed that a T-I type clone kept in the laboratory for at least two years, did not lose its high carboxylesterase activity on rearing without insecticide selection. In their work however, neither percentage activity nor level of insecticide tolerance relative to the original was calculated. Resistance in cotton aphid is likely to be genetically predetermined and thus qualitative reversion may not take place within a short period of discontinued insecticide application. From the results presented, it is most probable the aphid limits the production of carboxylesterases in the absence of an insecticide. However, upon resumption of insecticide application, it reactivates its genetic system to produce enough enzymes to fight against susceptibility.

Due to the need to carry out out-of-laboratory tests to have an idea about the level of resistance in the field before rigorous biochemical assessment, a handy filter paper

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**Fig. 2.** Filter paper test for discriminating organophosphate resistant strains of *A. gossypii* from susceptible ones.

"A" is the reference graded with various concentrations of 1-naphthol, and "B" reflects relationship between 1-naphthol production by carboxylesterase of *A. gossypii*, and level of susceptibility to dichlorvos (LC₅₀). See materials and methods for detailed description of procedure.
technique was developed (Fig. 2.). Though intensities of the blue coloured products were not quantified, a visual positive correlation with in vitro assay was closely observed. This afforded a clear picture of the degree of esterase involvement in resistance, whose path to an authentic and unambiguous judgment broadened out with time and after a series of trials. This technique should be of enormous assistance to workers and farmers in monitoring resistance in this aphid because of its rapidity, reliability and simplicity. It should also afford a second look at certain control practices whose ineffective implementations have led to either pests escaping contact with applied pesticides, or a high kill rate of pest’s natural enemies, that ensures a continuous crop infestation and/or higher resurgence rate. The diazonium coupled product is highly unstable and requires extremely careful procedures to minimize colour intensity loss during preservation. However, except for the purpose of documentation, such preservations may not be required in a routine monitoring work and thus, on-the-spot visual colour intensity should be a reliable and rapid indicator of the extent of organophosphate resistance or susceptibility.

References


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