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## Diagnostic Assays Based on Esterase-Mediated Resistance Mechanisms in Western Corn Rootworms (Coleoptera: Chrysomelidae)

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**ABSTRACT** Resistance to methyl-parathion among Nebraska western corn rootworm, *Diabrotica virgifera virgifera* LeConte, populations is associated with increased hydrolytic metabolism of an organophosphate insecticide substrate. An electrophoretic method to identify resistant individuals based on the staining intensity of esterase isozymes on nondenaturing polyacrylamide gels was developed. Three groups of esterases (I, II, and III) were visible on the gels, but only group II esterase isozymes were intensified in resistant populations. A total of 26 and 31 field populations of western corn rootworms from Nebraska (in 1998 and 1999, respectively) were assessed with nondenaturing polyacrylamide gel electrophoresis (PAGE) assays and diagnostic concentration bioassays. Significant correlations were observed between the two diagnostic assays. Group II esterase isozymes provide a reliable biochemical marker for detection of methyl-parathion resistance in individual western corn rootworms and a tool for monitoring the frequency of resistant individuals in field populations.

**KEY WORDS** esterase, insecticide resistance, biochemical marker, *Diabrotica virgifera virgifera*

THE WESTERN CORN rootworm, *Diabrotica virgifera virgifera* LeConte, is one of the most serious insect pests of corn, *Zea mays* (L.), in the Midwestern United States. Most of the economic loss attributed to this species is caused by larval injury to corn roots (Davis 1994, Gray and Steffey 1998, Urias-Lopez and Meinke 2001). Combined crop losses and management costs from *Diabrotica* species have been estimated to exceed US\$1 billion annually (Metcalf 1986).

Two chemical control strategies have been implemented to manage *D. v. virgifera* populations in continuous corn. Soil insecticides can be applied at planting time or first cultivation to reduce larval densities and protect roots (Mayo and Peters 1978). Aerial application of insecticides can be used to suppress adult rootworm populations and reduce oviposition thereby, decreasing larval densities the following season (Pruess et al. 1974). Chlorinated hydrocarbons were the first insecticides to be used in Nebraska for larval control (Hill et al. 1948). Continuous corn production and repeated use of cyclodiene insecticides, such as aldrin and dieldrin, in the same fields led to control failures by 1960 (Ball and Weekman 1962).

Organophosphate, carbamate, and pyrethroid insecticides have gradually replaced organochlorine insecticides, and have been widely used as soil insecticides across the Corn Belt. In localized areas of south-central Nebraska, adult management was adopted after the evolution of larval resistance to soil applied cyclodiene insecticides (Meinke 1995). This practice gradually expanded over large geographic areas as the number of aerial applicators and crop consultants increased. Historically, carbaryl formulations were initially used, but were eventually replaced by widespread use of methyl-parathion (especially PennCap-M) in adult management programs (Meinke et al. 1998). By the early 1990s, complaints about product performance had become common, especially in areas where beetle spraying was used consistently for multiple years (Wright et al. 1996, Meinke et al. 1998). Field surveys of adult susceptibility conducted from 1994–1995 confirmed that adult *D. v. virgifera* evolved a significant level of resistance to methyl-parathion and carbaryl in areas where adult management was the dominant control strategy for multiple growing seasons (Meinke et al. 1998).

In vivo metabolism experiments and in vitro enzyme activity assays indicated that the combined effects of oxidative and hydrolytic metabolisms in certain populations mediated *D. v. virgifera* resistance. Increased hydrolytic metabolism was observed in all populations examined (Miota et al. 1998, and Zhou and Siegfried, unpublished data). Synergism studies using the esterase inhibitor S,S,S-tributyl phosphorotrithio-

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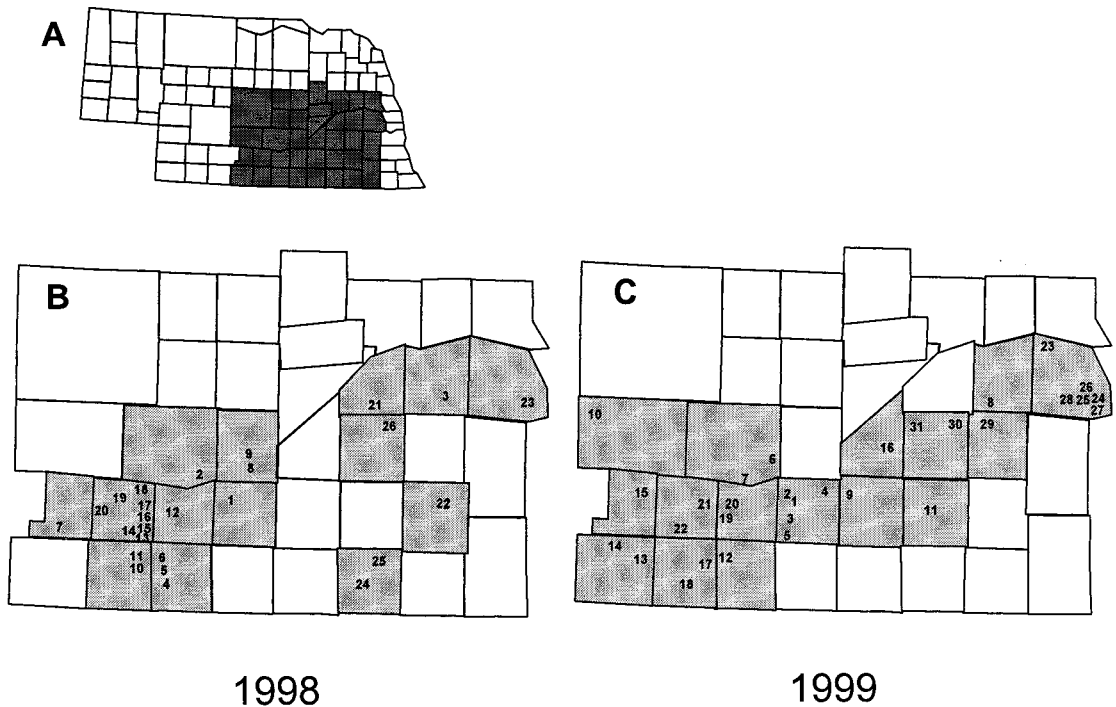


Fig. 1. *D. v. virgifera* collection sites across Nebraska in 1998 and 1999. The shaded area in the Nebraska State map (A) is enlarged below to show specific collection sites (designated by numerical numbers) within the shaded counties for 1998 (B) and 1999 (C). Each number represents a specific collection site within each year.

ate (DEF) further revealed a common involvement of esterase in both methyl-parathion (Miota et al. 1998) and carbaryl resistance (Scharf et al. 1999, 1999b).

Reliable detection of insecticide resistance is a key element of insect resistance management. Historically, conventional bioassays have been the primary diagnostic tools. However, rapid and sensitive biochemical techniques have been adopted to monitor the intensity and frequency of resistance among field populations of certain species (French-Constant and Roush 1991). Model substrates have been used successfully to identify a number of economically important insects with enhanced hydrolytic activity as part of their resistance mechanisms (e.g., Devonshire and Moores 1982, Byrne and Devonshire 1991, Rose et al. 1995).

The objectives of this study were to select a reliable resistance-associated marker in *D. v. virgifera* and develop a prototype biochemical monitoring technique for quantifying the frequency of resistant individuals among field populations.

#### Materials and Methods

***D. v. virgifera* Populations.** Field populations of adult *D. v. virgifera* were collected across Nebraska (Fig. 1) during late July and August of 1998 and 1999, respectively. Collection sites were selected to represent the major corn production areas of the state and corresponded to areas previously identified as having

variable levels of methyl-parathion susceptibility. All populations were collected before adult-targeted insecticide application had been initiated. Beetles were maintained in the laboratory at ambient temperature on a standard diet of head lettuce, and sweet corn, *Zea mays* (L.), for 1 to 5 d before use in the diagnostic concentration bioassays or before being frozen at  $-80^{\circ}\text{C}$  for later use in the nondenaturing PAGE assays.

**Chemicals.** Technical grade methyl-parathion (99% AI) was obtained from Chem-Services (West Chester, PA). Electrophoresis reagents were purchased from Bio-Rad Laboratories (Hercules, CA). Protein assay reagents and bovine serum albumin were purchased from Pierce (Rockford, IL). All other chemicals and solvents used were reagent grade or better.

**Diagnostic Concentration Bioassays.** Methyl-parathion diagnostic bioassays were conducted within 20 ml, insecticide-coated glass scintillation vials (Meinke et al. 1997, Scharf et al. 1999b). Vials were treated with  $0.5\ \mu\text{g}/\text{vial}$  methyl-parathion in  $0.5\ \text{ml}$  acetone solution and rolled until dry in a fume hood. This diagnostic concentration was selected based on the dose-response curves of a representative resistant and susceptible population and falls within the susceptible  $\text{LC}_{99}$  95% CI at 4-h exposure. The diagnostic concentration kills 100% of beetles bioassayed from very susceptible field populations and <5% of beetles from the most resistant Nebraska populations. We used 10 replicate vials for each population. Insects were immobilized on ice, and 10 unsexed adults were placed in

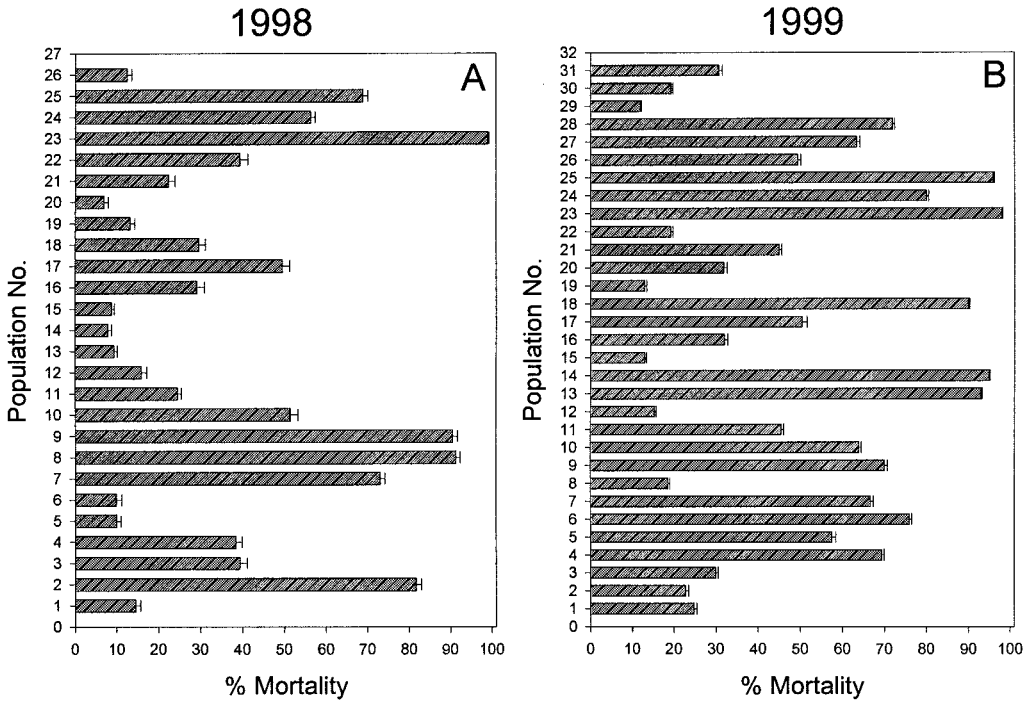


Fig. 2. Methyl-parathion diagnostic concentration bioassay results from 1998 (A) and 1999 (B). Within each year, population numbers correspond to collection sites presented in Fig. 1.

each vial. Vials were held at 22°C for 4 h and mortality was assessed as the inability to move when agitated.

**Esterase Enzyme Preparations.** *Mass homogenates.* Abdomens of 10 adult rootworms were homogenized in 0.5 ml ice-cold 0.1 M sodium phosphate buffer (pH 7.8, 0.1% Triton X-100) with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 × g and 4°C for 20 min. The resulting 10,000 × g supernatant was ultracentrifuged at 100,000 × g and 4°C for 60 min. The 100,000 × g supernatant was used as the enzyme source and was held on ice before electrophoresis.

*Single beetle homogenates.* Forty-eight adult abdomens were placed individually into the wells of a 96-well flat bottom microplate (353912 FALCON, Biosciences Discovery Labware, Franklin Lakes, NJ) containing 100 µl ice-cold homogenization buffer. Abdomens were homogenized by a combination of vertical and circular movement with a 96-spoke inoculating manifold (MC96, Dan-Kar, Reading, MA) for 30 s. The homogenate (10 µl) from each beetle was then transferred with a multi-channel pipettor to a new microplate and stored on the ice for subsequent nondenaturing PAGE assays.

**Single Beetle Nondenaturing PAGE Assays.** Nondenaturing PAGE assays were carried out in a vertical electrophoresis apparatus (Bio-Rad Mini Protean II, Richmond, CA) using a 7.5% separating gel and 4% stacking gel with a continuous Tris-glycine running buffer system (50 mM, pH 8.3). Samples (10 µl) of individual beetle homogenates were diluted 1:1 with

2 × sample buffer [20% sucrose (wt:vol), 0.1% bromophenol blue in 50 mM Tris-glycine running buffer (pH 8.3)] before loading. Gels were run at 120 V constant voltage for 75 min at 4°C. Esterase bands were visualized by incubating the gels in 100 ml 0.02 M sodium phosphate buffer (pH 7.0) with 2% (by volume) 30 mM β-naphthyl acetate dissolved in acetone at 30°C for 15 min before adding 0.04% fast blue BN salt (tetrazotized O-dianisidine) (wt/vol). After staining, the PAGE gels were examined for the presence or absence of group II esterase isozymes.

**Protein and Esterase Activity Assays.** Protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as the standard. General esterase activity was assayed by colorimetric measurement of β-naphthol formation. For these assays, the approach of van Asperen (1962) was adapted to a 96-well microplate format.

**Statistical Analyses.** All regression analyses were determined by SAS PROC REG (SAS Institute 2001).

**Results**

**Diagnostic Concentration Bioassays.** Collection sites of the *D. v. virgifera* field populations used in the 1998 and 1999 diagnostic assays are identified in Fig. 1. Collection sites ranged from south central to east central Nebraska, and extended over an area of ≈41,600 km<sup>2</sup>. The selected populations represented a wide range of methyl-parathion susceptibility (Fig. 2). The



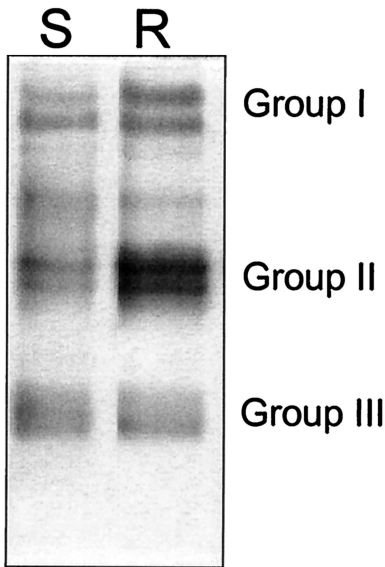


Fig. 3. Comparison of esterase isozymes between susceptible and resistant *D. v. virgifera* field populations separated by nondenaturing PAGE (S, susceptible population; R, resistant population).

percentage mortalities observed at the diagnostic concentration ranged from 7.0% (highly resistant population) to 99.0% (highly susceptible), with a generally continuous distribution of mortality in between.

**Nondenaturing PAGE Assays.** *Mass homogenates.* Three distinct esterase isozyme groups (designated as group I, II, and III) were clearly visible in the nondenaturing PAGE gels (Fig. 3) of both resistant and susceptible *D. v. virgifera* strains. However, the group II esterase isozymes from resistant populations showed consistently higher staining intensity relative to susceptible populations. Similar gel patterns of es-

terase isozymes from resistant and susceptible western corn rootworms were reported by Miota et al. (1998) and Scharf et al. (1999a).

There was no relationship between protein concentrations and general esterase activities in individual beetles ( $r^2 = 0.008$ ,  $P = 0.76$ ,  $n = 14$ , in resistant population;  $r^2 = 0.070$ ,  $P = 0.46$ ,  $n = 10$ , in susceptible population). Therefore, staining intensity should not be influenced by slight differences in total protein concentration among individual beetle homogenates, and it was determined that standardization of protein concentration was not necessary for the single beetle nondenaturing PAGE assays.

*Single Beetle Assays.* The group II esterase isozymes were clearly identified from homogenates of individual rootworm abdomens in resistant populations, but were almost undetectable in susceptible populations (Fig. 4). In 1998 and 1999, susceptibility as determined by diagnostic concentration bioassays explained 90 and 92%, respectively, of the variability in elevated group II esterase isozymes determined by nondenaturing PAGE (Fig. 5). These results provide further support for the involvement of hydrolytic enzymes in the mechanism of resistance to methyl-parathion.

**Discussion**

Results from this investigation indicate existence of a range of methyl-parathion susceptibilities among western corn rootworm populations in Nebraska. Susceptibility is highly correlated with specific activity patterns of general esterase isozymes. Three esterase isozyme groups are visible on nondenaturing polyacrylamide gels. Of these, group II esterases in resistant populations showed elevated activity toward the model substrate,  $\beta$ -naphthyl acetate.

Because of the esterase polymorphism in insects, electrophoresis provides a useful tool to categorize individual insects as resistant or susceptible. Nonden-

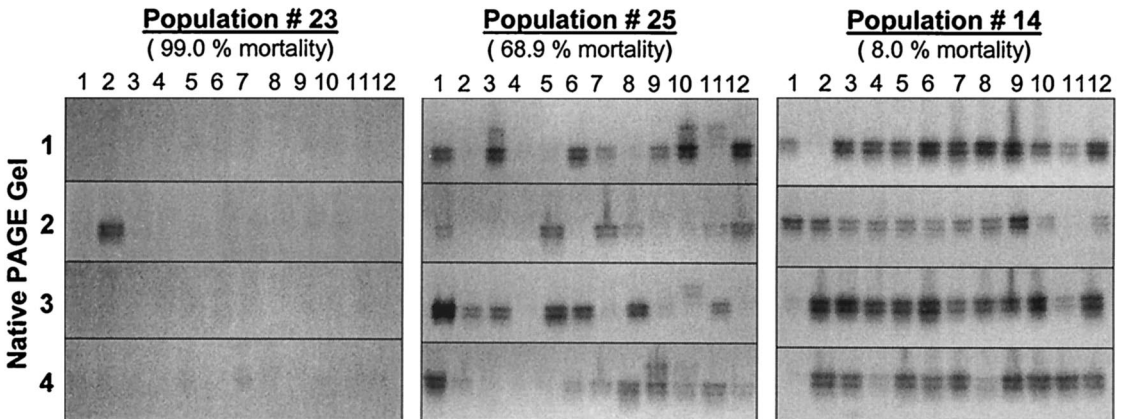


Fig. 4. Single beetle nondenaturing PAGE assays from three representative populations of the 1998 field survey with high (#23; 99% mortality), intermediate (#25; 68% mortality), and low (#14; 8% mortality) levels of susceptibility to methyl-parathion based on diagnostic concentration bioassay results. Number 1-4 (positioned vertically along gels) represents four separate gels per population. Numbers positioned horizontally above gels represents 12 beetles (one per lane) per gel ( $n = 48$  beetles per population).

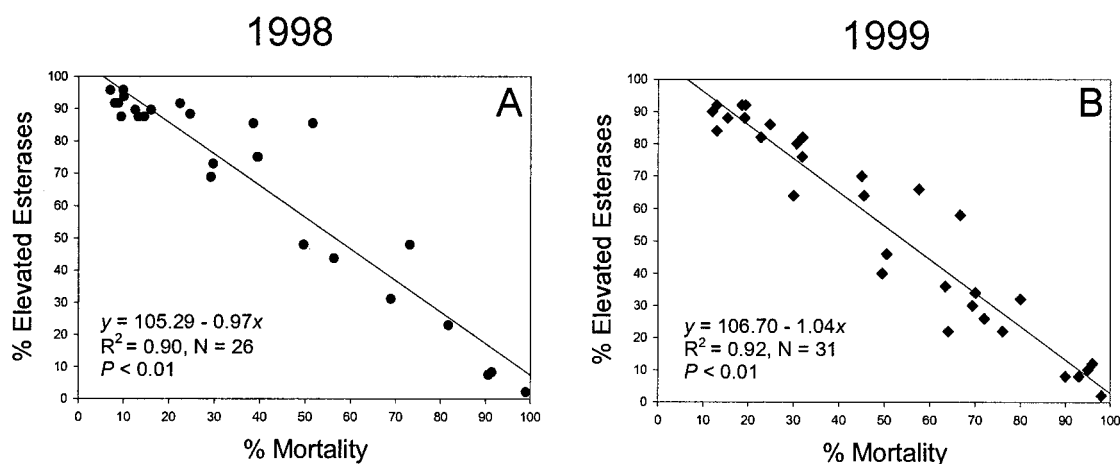


Fig. 5. Relationship and linear regression between *D. v. virgifera* mortality determined by the methyl-parathion diagnostic concentration bioassays and the frequency of elevated esterase isozymes estimated by the single beetle nondenaturing PAGE assays conducted in 1998 (A) and 1999 (B). Each point represents one of the 26 and 31 (respectively) populations that are shown in Fig. 2.

naturing PAGE detection of esterase isozymes associated with insecticide resistance has been used extensively as a diagnostic tool. For example, elevated esterase banding patterns have been found in resistant populations of *Schizaphis graminum* (Rondani) (Shufran et al. 1996, 1997), *Myzus persicae* Sulzer (Devonshire 1989), *Bemisia tabaci* (Genn.) (Byrne and Devonshire 1991, 1993), and *Anopheles albimanus* Wiedemann (Brogdon and Barber 1990).

The reliability of using electrophoresis for large-scale resistance monitoring has been questioned because of the subjectivity of assessing the intensity of esterase bands on the nondenaturing polyacrylamide gels. For example, from 1991 to 1995,  $\approx 14,000$  *Schizaphis graminum* from Kansas, Texas, Oklahoma, and Colorado were examined with nondenaturing PAGE assays. During the process, cost and under-estimation of resistant individuals hindered the ability to effectively monitor the resistance gene frequency in field populations by PAGE (Shufran et al. 1997). In this study, as expected, esterase isozyme patterns from single beetle homogenates were similar to those of mass homogenates, although single beetle gel patterns suggested greater diagnostic potential. When individual *D. v. virgifera* were homogenized and separated on nondenaturing PAGE gels, only elevated group II esterase isozymes from resistant individuals were clearly visible. This was a substantial improvement over the three-esterase groups observed on the mass homogenate gels. This distinctive esterase pattern from individual beetles made the determination of resistance status more efficient and much more precise.

Nondenaturing PAGE assays cannot only detect the existence of insecticide resistance in this case, but can also estimate the magnitude of resistance ratios. Resistance ratios estimated by the quantitative staining intensity of group II esterase isozymes (Quantity One Quantitation Software, Gel Documentary System, Bio-Rad Laboratories, Hercules, CA) from the resis-

tant and susceptible populations were similar to those determined from the specific activity of the esterase (X.G.Z., unpublished data).

Biochemical-based assays, which diagnose both the qualitative and quantitative differences in detoxification enzymes, significantly improve the speed, accuracy, and sensitivity of resistance monitoring. The main advantage of biochemical resistance assessment methods is the ability to monitor subtle changes in the resistant gene frequency caused by chemical selection, unlike traditional bioassays that measure the average response of a heterogeneous population (Devonshire 1989). Because of the esterase polymorphism observed in *D. v. virgifera* and the distinctive gel pattern toward model substrates exhibited by the resistant individuals on the nondenaturing PAGE gels, group II esterase isozymes are a legitimate biochemical marker for identifying resistant individuals directly from the field. Based on the 1998 and 1999 assay results, the single beetle nondenaturing PAGE technique was highly comparable to the conventional diagnostic concentration bioassay, and further established itself as a biochemical diagnostic tool for monitoring the frequency of resistance-associated esterase genes in field populations.

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