Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 1869–1872, March 1991
Applied Biological Sciences

Evolution of diabroticite rootworm beetle (Chrysomelidae) receptors for *Cucurbita* blossom volatiles

(phenylpropanoids/kairomones/coevolution)

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Contributed by Robert L. Metcalf, November 30, 1990

ABSTRACT **The diabroticite rootworm beetles coevolved with plants of the family Cucurbitaceae as demonstrated by their feeding dependence on the tetracyclic triterpenoid cucurbitacins. These beetles also exhibit strong attraction to phenylpropanoid volatile components of *Cucurbita* blossoms. A mixture of 1,2,4-trimethoxybenzene, indole, and (*E*)-cinnamaldehyde, all blossom components, is highly attractive to the several species of diabroticite cucumber beetles and corn rootworms and is considered a simplified *Cucurbita* blossom kairomone odor. The evolutionary divergence in antennal receptor complementarity is best understood by comparing the species-specific responses of several *Diabrotica* to structural analogues of (*E*)-cinnamaldehyde, the major attractant for *Diabrotica undecimpunctata howardi*. Cinnamyl alcohol is a strong attractant for *Diabrotica barberi*, and 4-methoxycinnamaldehyde is an exceptional attractant for *Diabrotica virgifera*. The very closely related species *D. barberi* and *Diabrotica cristata* are most strongly attracted to 4-methoxyphenethanol, which is unattractive to the other species studied.**

The blossoms of Cucurbitaceae are well known to be highly attractive to many species of diabroticite rootworm beetles, including such major pests as the northern corn rootworm *Diabrotica barberi* Smith and Lawrence, the southern corn rootworm *Diabrotica undecimpunctata howardi* Barber, the western corn rootworm *Diabrotica virgifera virgifera* LeConte, the banded cucumber beetle *Diabrotica balteata* Leconte, and the striped cucumber beetle *Acalymma vittatum* (Fabricius) (1–4). The aggregations of diabroticite beetles in *Cucurbita* blossoms are the result of the concerted attraction from an array of blossom volatiles that increases the arrival rate, acting together with the singular arrestant and phagostimulant properties of the tetracyclic triterpenoid cucubitacins in the blossoms that delay departure (2, 4–7). The blossoms of *Cucurbita maxima* produce an array of >40 volatile chemicals (1, 2), and 22 of these chemicals have been identified by MS (8). Extensive field evaluations have shown that at least 10 of the volatile compounds are attractive to adults of both sexes of the diabroticite rootworms (4, 9).

Most of the highly attractive *Cucurbita* volatiles are phenylpropanoids, C_6C_3 compounds formed in the shikimic acid pathway through phenylalanine to cinnamic acid (10). Phenylpropanoids are produced from cinnamic acid in relatively large amounts during pollen maturation in blossoms (11, 12). Thus, the remarkable long-range attraction of many species of diabroticites to the blossoms of Cucurbitaceae implies that the original coevolutionary association between Cucurbitaceae and diabroticites (5) was that of pollen seeking and consequent fertilization. Coleoptera are considered to be the most primitive pollinators of angiosperms and were probably associated with open bowl-shaped flowers, where the beetles fed on floral secretions, nectar, and pollen (13).

All of the diabroticite species examined are attracted to chemically related phenylpropanoids; however, each species displays a distinctive pattern of response when exposed to an array of *Cucurbita* blossom volatiles (9, 14). Indole, a condensed phenylpropanoid, is a component of the rich heavy aroma of squash blossoms and is attractive to *D. virgifera virgifera* and *A. vittatum* adults (1, 7). Indole alone is not appreciably attractive to *D. barberi* or *D. undecimpunctata howardi*, but in admixture with the other squash blossom volatiles 1,2,4-trimethoxybenzene and (*E*)-cinnamaldehyde (8) shows synergistic attraction to *D. barberi, D. cristata, D. undecimpunctata howardi, D. virgifera virgifera*, and *A. vittatum* (7, 14, 15). This trimethoxybenzene/indole/cinnamaldehyde mixture is, therefore, a simplified mimic of the squash-blossom aroma.

Cinnamaldehyde is a strong attractant for *D. undecimpunctata howardi* and a weak attractant for *D. virgifera virgifera* (16). Cinnamyl alcohol, 2-phenylethanol (phenethanol), 3-phenylpropanol (phenpropanol), and eugenol are attractants for *D. barberi* (17) and for the closely related *D. cristata* (Harris) breeding in big bluestem *Andropogon gerardi* of relict prairie (15). Recently, we have found that two plant-produced phenylpropanoids, 4-methoxycinnamaldehyde for *D. virgifera* (16) and 4-methoxyphenethanol for *D. barberi* and *D. cristata* are extraordinarily effective attractants (Figs. 1 and 2). These kairomones are not attractive to *D. undecimpunctata howardi* or *A. vittatum*.

The spectrum of olfactory responses of the several diabroticite species to individual volatile components of *Cucurbita* blossoms indicates that antennal olfactory receptors of the rootworm beetles have evolved as speciation has occurred over the 30 million years (0.7 genetic distances) since *Acalymma* separated from *Diabrotica* and the latter diverged into the polyphagous *fucata* group of neotropical nondiapausing species—e.g., *D. undecimpunctata howardi* and *D. balteata*—and the *virgifera* group of nearctic, diapausing, univoltine species—e.g., *D. barberi, D. cristata*, and *D. virgifera virgifera* (18).

Here we examine the specific interactions of the array of kairomone attractants with the antennal sensory receptors of the several *Diabrotica* spp. and propose an evolutionary scenario for the chemical ecology of *Cucurbita*–diabroticite coevolution.

MATERIALS AND METHODS

Chemicals. The substituted cinnamaldehydes were prepared by condensing the appropriate benzaldehyde and acetaldehyde (19). The chemicals were vacuum distilled, and composition and purity were determined by GC/MS. The

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Abbreviations: LR, limit of response; WCR, western corn rootworm; NCR, northern corn rootworm.

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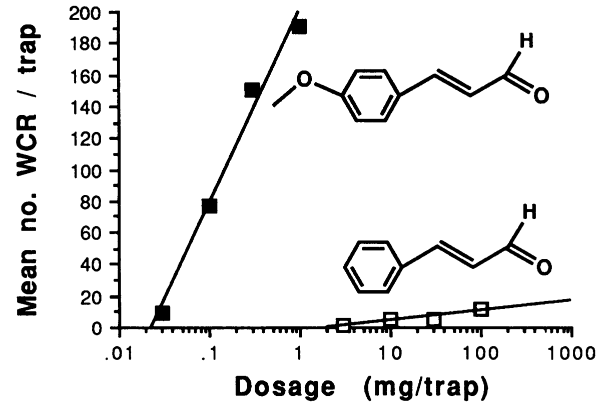

FIG. 1. Attractancy of *D. virgifera virgifera* (WCR) as function of trap dose ($n = 4$). □, Cinnamaldehyde ($y = 6.43 \log x - 2.16$, $R^2 = 0.838$); ■, 4-methoxycinnamaldehyde ($y = 12.87 \log x + 199.85$, $R^2 = 0.984$).

meta-CH_3O- and *para*-CH_3O-cinnamaldehydes were >99% pure, and the *para*-F-, *para*-CH_3, *para*-CH_3S- and pentafluorocinnamaldehydes were >90% pure. The F_3CO-cinnamaldehyde (20) was only 62% pure but was not purified further because of the very small quantity available. Furylacrylaldehyde, mp 49°C, semicarbazone, mp 217°C (21), was reduced with sodium borohydride to furylacryl alcohol, bp. 64–66°C per 0.4 mm, n^{23} sodium D line 1.5825. All other chemicals evaluated were Aldrich white-label (96–99% pure).

Evaluation of Attractancy. The attractancy of the plant kairomones and analogues was evaluated in the field against natural populations of rootworm adults in corn, cucurbits, and reconstituted prairie. The chemicals were applied by capillary micropipette to cotton dental wicks attached to the tops of 1.0-liter cylindrical paper cartons covered with insect adhesive (Tangle Trap, Grand Rapids, MI). Comparisons of attractant chemicals were made at a dose of 100 μl or 100 mg (in saturated acetone solution) using four replicates at each dose positioned randomly 10 m apart on 1-m-high stakes and compared with untreated controls (14). To determine the limits of response to the attractants, sticky traps were baited with decreasing doses of 100, 30, 10, 3, 1, 0.3, and 0.1 mg. Attraction of *Diabotica* spp. adults was measured as the mean number of beetles caught after 1-day (≈24 hr) exposure. Significance of the trap catches was determined by analysis of variance, and the individual means were separated by Duncan's multiple-range test (22). The data were logarithmically transformed for statistical analysis, and significance levels were set at $P < 0.05$.

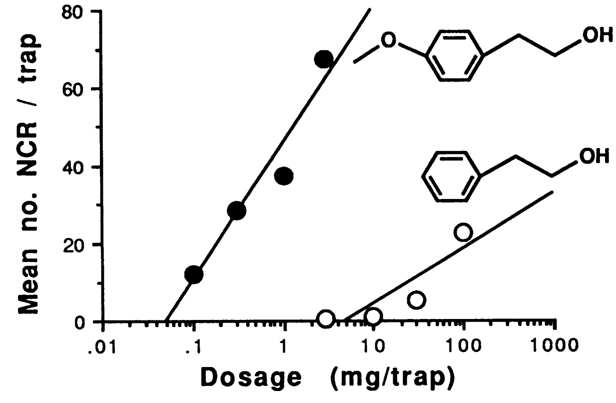

FIG. 2. Attractancy to *D. barberi* (NCR) as function of trap dose ($n = 4$). ○, Phenethanol ($y = 14.08 \log x - 10.16$, $R^2 = 0.780$); ●, 4-methoxyphenethanol ($y = 35.2 \log x + 45.2$, $R^2 = 0.937$).

The release rate of a number of the chemical lures was determined by weighing ≈20 mg of lure to 0.1 mg in 30-mm aluminium planchets. The test chemical was distributed as evenly as possible over the bottom of the planchet by adding 0.1–0.2 ml of acetone. After the acetone evaporated, the planchets were weighed and placed in a ventilated hood at a constant air velocity of 75 cm per sec (150 ft per min or 1.7 miles per hr). The hood temperature was relatively constant at 23–25°C. The planchets were weighed at appropriate intervals, and the loss in weight was determined as the average of four replicates for each compound. The rates of volatilization were calculated from the initial approximately straight-line portion of the curve of weight loss vs. time.

RESULTS AND DISCUSSION

Receptor Site Mapping for *D. virgifera virgifera*. 4-Methoxycinnamaldehyde is the most effective attractant yet found for western corn rootworm (WCR) adults (16). From the averages of four field evaluations with four replicates each made during 1987–1989, the ratios of numbers of WCR adults attracted as compared with 4-methoxycinnamaldehyde were as follows: (*E*)-cinnamaldehyde 0.08, (*E*)-cinnamyl alcohol 0.01, and 4-methoxycinnamonitrile 0.70. *Ortho*- and *meta*-methoxycinnamaldehyde were not statistically more attractive than untreated controls or were *para*-CH_3S-, *para*-F-, *para*-NO_2-, and *para*-CF_3O- and *para*-CH_3-substituted cinnamaldehydes (Table 1). Pentafluorocinnamaldehyde was also completely unattractive. These differences were highly significant ($P > 0.05$) by Duncan's multiple-range test.

These results indicate strong receptor binding at the *para*-CH_3O group and with the terminal aldehyde C(O)H. Hydrogen bonding with the planar phenyl ring is important. An interesting bioisosterism exists between 4-methoxycinnamaldehyde and 4-methoxycinnamonitrile, indicating the key role of C=O or C≡N dipoles. Saturation of the C=C bond of 4-methoxycinnamaldehyde, as in 4-methoxyphenylpropanal, reduced activity to ≈0.01. Replacement of aldehyde C(O)H by alcohol CH_2OH, as in cinnamyl alcohol, phenethanol, phenpropanol, and 4-methoxyphenethanol, produced compounds unattractive to *D. virgifera virgifera* (Table 2).

Receptor Site Mapping for *D. barberi*. Northern corn rootworms (NCRs) are strongly attracted to phenylpropanoids with alcoholic moieties—e.g., cinnamyl alcohol (17). From the averages of four field evaluations during 1988–1989, the ratios of numbers of *D. barberi* adults attracted compared with cinnamyl alcohol were as follows: phenpropanol, 0.84; phenethanol, 0.74; and (*E*)-cinnamaldehyde, 0.25. The presence of a *para*-CH_3O group substantially increased attraction, and 4-methoxyphenethanol was 3.9-fold more attractive than cinnamyl alcohol and 5.3-fold more attractive than phenethanol. 4-Methoxyphenpropanol was 1.13-fold more attractive than phenpropanol, but 4-methoxycinnamyl alco-

Table 1. *D. virgifera virgifera* adults per sticky trap after 1 day (July 24, 1989)

Trap treatment	Adults, mean no.
Control untreated	4.7 ± 3.3a
$4\text{-}CH_3OC_6H_4CH{=}CHC(O)H$	20.0 ± 8.4b
$3\text{-}CH_3OC_6H_4CH{=}CHC(O)H$	2.0 ± 1.7a
$2\text{-}CH_3OC_6H_4CH{=}CHC(O)H$	4.7 ± 1.3a
$4\text{-}CH_3SC_6H_4CH{=}CHC(O)H$	5.5 ± 3.1a
$4\text{-}ClC_6H_4CH{=}CHC(O)H$	5.5 ± 3.1a
$4\text{-}CH_3C_6H_4CH{=}CHC(O)H$	4.7 ± 1.5a
$4\text{-}CH_3OC_6H_4CH{=}CHC{\equiv}N$	15.5 ± 3.3b

Data are mean ± SEM for four replicate treatments, means followed by the same letter are not significantly different. ($P < 0.05$ by Duncan's multiple-range test). All attractants were applied at 100 mg per trap.

Table 2. *D. barberi* adults per sticky trap after 1 day (August 24, 25, 1989)

Trap treatment	Adults, mean no.	
	Aug. 24	Aug. 25
Control untreated	7.5 ± 3.3a	4.7 ± 3.9a
$C_6H_5CH_2CH_2OH$	50.0 ± 20.9b	12.2 ± 5.5a
$4\text{-}CH_3OC_6H_4CH_2CH_2OH$	184.7 ± 62.5d	60.3 ± 26.8b
$3\text{-}CH_3OC_6H_4CH_2CH_2OH$		7.2 ± 3.4a
$2\text{-}CH_3OC_6H_4CH_2CH_2OH$		13.5 ± 9.3a
$4\text{-}FC_6H_4CH_2CH_2OH$	38.0 ± 6.2b	
$4\text{-}CH_3C_6H_4CH_2CH_2OH$	89.2 ± 7.8c	
$4\text{-}O_2NC_6H_4CH_2CH_2OH$	7.7 ± 2.7a	
$4\text{-}NH_2C_6H_4CH_2CH_2OH$	10.5 ± 2.1a	
$C_6H_5CH{=}CHCH_2OH$		50.7 ± 50.9b
$4\text{-}CH_3OC_6H_4CH{=}CHCH_2OH$		5.2 ± 3.1a

Data are mean ± SEM for four replicate treatments, means followed by the same letter are not significantly different ($P < 0.05$ by Duncan's multiple-range test). All attractants were applied at 100 mg per trap.

hol was only ≈0.03 as attractive as cinnamyl alcohol because of its very low release rate.

The greatly increased attraction of 4-methoxyphenethanol is essentially a function of the *para*-CH_3O substitution, and attraction was greatly decreased by *ortho*- or *meta*-CH_3O substitution or by *para*-substitution with F, Cl, CH_3, NO_2 or NH_2 (Table 2). None of these phenethanol analogues was attractive to *D. virgifera virgifera*. Changes in the aromatic phenyl ring of cinnamyl alcohol decreased attractivity to 0.34 in furylacryl alcohol and to 0.05 in cyclohexylpropanol. Surprising findings were the marked field attractancy of phenethylamine and phenpropylamine, which were 1.37-fold and 0.88-fold as attractive as cinnamyl alcohol, respectively (Table 3). None of these phenalkylamines were attractive to *D. undecimpunctata howardi* or *D. virgifera virgifera*. We tentatively attribute the attractivity of the phenalkylamines to similarity in the interaction of the unshared electron pair of the N and O atoms with the receptor site for the OH group. Cinnamyl acetate (17) and the acetates of phenethanol and phenpropanol were significantly less attractive than the corresponding free alcohols (unpublished data).

Receptor Site Mapping for *D. undecimpunctata howardi*. (*E*)-cinnamaldehyde is the most effective lure yet found for southern corn rootworm adults (16). From the averages of four field evaluations during 1988–1989, the ratios of numbers of adults attracted compared to (*E*)-cinnamaldehyde were as follows: (*E*)-cinnamonitrile, 0.75; (*E*)-cinnamyl alcohol, 0.20; phenpropanal, 0.55; (*E*)-4-methoxycinnamaldehyde, 0.07; and 4-methoxycinnamonitrile, 0.05 (16, 17). Phenethanol was slightly attractive (9), but both 4-methoxyphenethanol and 4-methoxyphenpropanol were unattractive. Cinnamyl fluoride was approximately as attractive to *D. undecimpunctata howardi* adults as was cinnamaldehyde. Furylacrylaldehyde was 0.27 as attractive as cinnamaldehyde, but 2,3,4,5,6-pentafluorocinnamaldehyde was only 0.09 as attractive, and cyclohexylpropanaldehyde was unattractive. Substitution of the aryl ring with *para*-CH_3O eliminated attraction not only for cinnamaldehyde but also for cinnamonitrile and for allylbenzene (16). Thus, receptor interaction with *D. undecimpunctata howardi* strongly depends upon an aromatic ring and hydrogen bonding. The receptor interaction with aldehyde and nitrile moieties resembles that of *D. virgifera virgifera*.

Table 3. *D. barberi* adults per sticky trap after 1 day (August 16, 1989)

Trap treatment	Adults, mean no.
Control (untreated)	5.5 ± 4.7a
$C_6H_5CH{=}CHCH_2OH$	127.0 ± 24.2f
$C_6H_5CH_2NH_2$	57.5 ± 29.6e
$C_6H_5CH_2CH_2NH_2$	206.7 ± 32.3f
$C_6H_5CH_2CH_2CH_2NH_2$	131.5 ± 16.5f
$C_6H_5CH_2CH_2CH_2CH_2NH_2$	12.2 ± 6.1bc
$C_6H_5CH_2CH_2NHCH_3$	47.2 ± 16.3cd
$C_6H_4CH_2CH_2N^+(CH_3)_3$	9.2 ± 5.3ab
$4\text{-}CH_3OC_6H_4CH_2CH_2NH_2$	24.2 ± 15.4cd

Data are mean ± SEM for four replicate treatments; means followed by the same letter are not significantly different ($P \leq 0.05$ by Duncan's multiple-range test). All attractants were applied at 100 mg per trap.

Limit of Response (LR). This parameter represents the least amount of lure (mg per sticky trap; mean of four replicates) that resulted in a trap catch significantly greater (Duncan's multiple-range test) than that of an unbaited control. The LR value, therefore, is a most significant measure of lure efficiency. Diabroticite beetle responses to volatile attractants are directly proportionate to the logarithm of dose as predicted by the Weber–Fechner Law (7, 17). This relationship is shown in Fig. 1 for *D. virgifera virgifera* attracted to cinnamaldehyde and 4-methoxycinnamaldehyde and in Fig. 2 for *D. barberi* attracted to phenethanol and 4-methoxyphenethanol. The LR values under field trapping conditions can be estimated from these graphs, and they indicate that 4-methoxycinnamaldehyde (LR, 0.03 mg) is at least 33-fold more effective than cinnamaldehyde (LR, 1.0 mg) (Fig. 1), and that 4-methoxyphenethanol (LR, 0.1 mg) is at least 100-fold more attractive than phenethanol (LR, 10 mg) (Fig. 2). LR values for various kairomone attractants determined from our field studies are given in Table 3 (refs. 1, 7, 14, 16, and 17 and unpublished work).

Release Rates. Odor sources release volatiles at widely different rates, as determined by the vapor pressures of the individual chemicals. Therefore, to estimate the relative effectiveness of the diabroticite beetle kairomones, the LR values must be corrected by applying an additional factor for relative volatility (23, 24). Release rates of the effective lures determined under standard conditions are converted to relative rates, as shown in Table 4. Thus for *D. virgifera virgifera*, cinnamaldehyde has a release rate ≈83-fold greater than 4-methoxycinnamaldehyde, and the latter is 33-fold more effective in LR. The relative attractancy of 4-methoxycinnamaldehyde is, therefore, 83 × 33 = 2750 times greater than cinnamaldehyde. Similarly for *D. barberi*, phenethanol has a release rate 62-fold greater than 4-methoxyphenethanol, and the latter is 100-fold more effective in LR. Therefore, the relative attractancy of 4-methoxyphenethanol is 62 × 100 = 6200 times greater than for phenethanol. The relative attractancy values in Table 3 are based on values of 1.0 for cinnamaldehyde to *D. virgifera virgifera* and 1.0 for cinnamyl alcohol to *D. barberi*. From the data in Table 4, lures such as phenethanol and cinnamaldehyde apparently are effective attractants due to moderate affinity for diabioticite receptors and high release rates, whereas 4-methoxycinnamaldehyde, 4-methoxycinnamonitrile, and 4-methoxyphenethanol have low release rates and are effective because of their high affinity for the diabroticite receptors.

Phenyl Propanoids in Plant Biosynthesis. Most effective kairomone compounds for diabioticite beetles—e.g., indole, cinnamaldehyde, cinnamyl alcohol, and phenethanol—have been identified in *Cucurbita* blossom aroma (2, 8). However, neither 4-methoxycinnamaldehyde nor 4-methoxyphenethanol have yet been identified in the array of blossom volatiles. The probability of their existence there, however, is high, as 4-methoxybenzaldehyde and 4-methoxybenzyl alcohol are present in *Cucubita* aroma, and methoxylation to form 1,4-dimethoxybenzene and 1,2,4-trimethoxybenene is an efficient process in aroma metabolism of the *Cucurbita* (2, 8).

Table 4. LR, release rate, and relative chemosensory activity of kairomone lures to *D. barberi* and *D. virgifera virgifera*

	LR, mg		Relative release rate, molecules·sec^{-1}	Relative chemosensory activity
Lure	*D. barberi*	*D. virgifera virgifera*		
Cinnamyl alcohol	1.0		1.0	1.0
Eugenol	3.0		5.2	0.06
Phenethanol	10		30	0.003
Phenpropanol	3.0		4.4	0.2
4-Methoxyphenethanol	0.1		0.11	20
Phenethylamine	1.0		8.8	0.11
Phenpropylamine	3.0		6.0	0.05
Cinnamaldehyde		1.0	1.0	1.0
Indole		1.0	1.0	1.0
Estragole		3.0	8.0	0.04
β-Ionone		1.0	0.3	3.0
Cinnamonitrile		1.0	1.0	1.0
4-Methoxycinnamaldehyde		0.03	0.012	2750
4-Methoxycinnamonitrile		0.1	0.11	130

The *D. virgifera virgifera* attractant 4-methoxycinnamaldehyde has been identified in the essential oils of *Agastache rugosa, Orthodon methylchavicoliferum, Oscimum basilicum* (Lamiaceae), *Acorus gramineus* (Araceae), *Limnophila rugosa* (Scrophulariaceae), and *Spheranthus indicus* (Asteraceae) (16). The *D. barberi* attractant 4-methoxyphenethanol is also a natural product and has been isolated from the flowers of *Thalictrum rugosum* (Ranunculaceae) (25) and from *Aubrieta hybrida* (Cruciferae) (26).

In summary, it is evident that an ancestral diabroticite rootworm coevolved with primitive Cucurbitaceae and became a specific pollinator while receiving sustenance from pollen and sites for host selection. This coevolutionary relationship was cemented by cucurbitacin phagostimulants. Under evolutionary diversification, antennal sensory receptors, perhaps originally entirely complementary to cinnamaldehyde, evolved into receptors more specifically attuned to such modifications as *para*-methoxylation and aldehyde reduction to alcohols. As plant species and beetle species diversified, these specifically tuned receptors appear to have become key factors in controlling rootworm beetle behavior especially for host plant selection. Presently, even *Diabrotica* spp. whose larvae feed exclusively on the roots of grasses—e.g., *D. barberi, D. cristata,* and *D. virgifera virgifera*—are frequently found feeding on pollen and nectar in *Cucurbita* blossoms.

We thank Dr. Craig Reid, Lesley D. Dickson, and Jefferson Schott for excellent technical assistance. This work was supported, in part, by the U.S. Department of Agriculture Competitive Research Grants Office, AG 87-CRCR-1-2373, and by National Science Foundation Grant PCM-8312778.

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