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Bioactivity of Anacardic Acid against Colorado Potato Beetle (*Leptinotarsa decemlineata*) Larvae

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Anacardic acid (2-hydroxy-6-alkylbenzoic acid) produced and secreted from glandular trichomes of zonal geranium (*Pelargonium* × *hortorum*; Geraniaceae family) provides resistance to small pests (aphids and spider mites). To assess the potential bioactivity of anacardic acid against larger insect pests and to determine if an alternate mode of application (ingestion rather than the topical application) could impart resistance to pests, the effects of anacardic acid consumption on the development of Colorado potato beetle larvae were tested. Analysis of dose−response curves indicated a significant effect on weight gain and mortality. Assessment of food preference (treated versus untreated) indicated larvae avoid food containing anacardic acid and have a lower feeding rate on food containing anacardic acid. On the basis of these results, it is suggested that anacardic acid, applied as a chemical spray or through bioengineering production in crop plants, may provide a new tool in the arsenal to minimize damage to plants caused by pests.

KEYWORDS: Colorado potato beetle; zonal geranium; anacardic acid; pest resistance

INTRODUCTION

Anacardic acid (AnAc; 2-hydroxy-6-alkylbenzoic acid) is produced in a relatively small number of plants, mostly in the Anacardiaceae family that includes cashew (*Anacardium occidentale*) (*1*) and pistachio (*Pistacia vera*) (*2*). AnAc is also found in other families and includes, as examples, ginkgo (*Ginkgo biloba*; Ginkgoaceae family) (*3*), zonal geranium (*Pelargonium* × *hortorum*; Geraniaceae family) (*4*), *Knema elegans* (Myristicaceae family) (*5*), *Philodendron scandens* (Araceae family) (*6*), and *Schoepfia californica* (Olacaceae family) (*7*). Although AnAc has been found in numerous plant species, zonal geranium is the only plant in which a physiological function of this phytochemical is known.

The pest resistance mediated by glandular trichomes of zonal geranium has been studied extensively using two highly inbred lines (one pest-resistant and the other pest-susceptible) (reviewed in ref *8*) and has been reported to be effective against small pests such as spider mites (*4*, *9*, *10*) and aphid (*11*, *12*). The tall glandular trichomes found on all aerial tissues of zonal geranium (with the exception of flower petals) have been shown to produce AnAc (**Figure 1**) (*4*, *12*−*16*). Zonal geranium lines differ in the accumulation of either high amounts of saturated AnAc (AnAc 22:0 and AnAc 24:0) in the pest-susceptible phenotype or high amounts of unsaturated AnAc (AnAc 22:$1^{\omega5}$ and AnAc 24:$1^{\omega5}$) in the pest-resistant phenotype (*10*, *17*). The secretion of AnAc from glandular trichomes of zonal geranium acts as a physical barrier or "sticky trap" to impede pest movement, thereby reducing feeding and oviposition

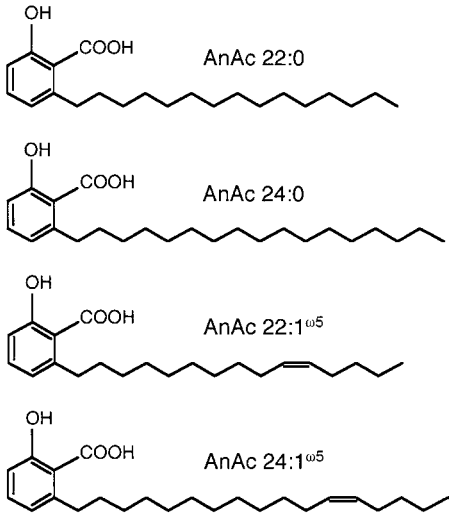

Figure 1. Structures of the dominant AnAc types found in glandular trichomes of *Pelargonium* × *hortorum.* AnAc 22:0 and AnAc 24:0 are most abundant in the pest-susceptible phenotypes, whereas AnAc 22:$1^{\omega5}$ and 24:$1^{\omega5}$ are most abundant in the pest-resistant phenotypes.

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behavior (*18*). In addition, AnAc functions as a toxin toward arthropods (*9*). The production of the AnAc 22:1$^{\omega 5}$ and AnAc 24:1$^{\omega 5}$ has been found to be conditioned by a single dominant Mendelian trait (*10*) that encodes the Δ9 14:0-ACP fatty acid desaturase gene (*19*). The effectiveness of pest resistance in this system is therefore dependent on the fluidity of the fatty acids used as precursors to AnAc; that is, saturated AnAc derived from saturated fatty acids will be more viscous than unsaturated AnAc derived from unsaturated fatty acids. The major effect of fluidity is on the effectiveness of the sticky trap. However, because AnAc also acts as a toxin, application to pests, regardless of saturation, is correlated with increased mortality. Therefore, in addition to influencing the effectiveness of the sticky trap, fluidity also influences the effectiveness of application of a potential toxin. Trichome-secreted AnAc that is saturated (and therefore less fluid) does not impede pest movement and will not be applied effectively to the pest.

AnAc produced in, and secreted from, zonal geranium trichomes forms an effective pest resistance system against small pests. On the basis of demonstrated antimicrobial activity (*20*−*29*), AnAc may also be expected to be involved in pathogen resistance in other plants or may also have a role in pest resistance in the non-zonal geranium species, although such roles have not been experimentally determined. If in fact AnAc does impart pest resistance to a broader range of plants, the mode of action would likely be distinct from the sticky trap of zonal geranium as this is the only species known to secrete AnAc from glandular trichomes. In all other plant species, AnAc is found within tissues (e.g., leaf, fruit, nut shell). This brings to light an interesting question: can AnAc function in pest resistance only when applied topically to pests (as in zonal geranium), or does AnAc function when ingested by pests (as might be the case in non-zonal geranium species)? If AnAc is toxic to pests when ingested, production of this phytochemical could be bioengineered into otherwise susceptible plants, thereby imparting an endogenous pest resistance system, or application of AnAc as a spray may prove to be effective at controlling pest populations.

Defining the effectiveness of AnAc as it relates to mode of application is therefore increasingly important. We report here that in a dose-dependent manner, AnAc consumed as part of a diet negatively affects numerous facets of Colorado potato beetle (CPB) larval growth and development. We also show that when given a food choice, the larvae avoid food that is treated with AnAc and show reduced feeding rates on food treated with AnAc.

MATERIALS AND METHODS

Isolation and Purification of AnAc from Zonal Geranium. *Pelargonium* × *hortorum* (accession 88-51-10, Penn State Geranium Breeding Program) were clonally propagated and maintained year-round in greenhouses with no supplemental light. Complete flowers were removed from the plant, flowers or buds were cut off, and then the 1−3 cm long pedicles were cut from the receptacle and collected into glass bottles. AnAc was purified as described (*16*) with the following modifications. The dissected pedicle material was briefly washed, three times, with a minimum volume of chloroform. The extracts were pooled and concentrated to dryness in a rotary evaporator, and the resulting residue was dissolved in a minimum volume of hexane. The hexane-soluble extract was then applied to a silica gel G column (50-mL bed volume in a 2 cm × 16 cm glass column with porous glass fret). Samples were eluted sequentially with 50 mL of hexane, 100 mL of 1% diethyl ether/1% acetic acid in hexane (v/v/v), 100 mL of 10% diethyl ether/1% acetic acid in hexane (v/v/v), 100 mL of 50% diethyl ether/1% acetic acid in hexane (v/v/v), and 50 mL of 25% methanol in diethyl ether (v/v) from the column and collected in 8-mL fractions. Fractions containing AnAc were detected using a small aliquot (~50 μL) spotted onto TLC plates (10 × 20 cm silica gel G, Whatman Co.) that were developed to the full length of the plate in petroleum ether/diethyl ether/acetic acid (60/40/1, v/v/v). AnAc was visualized as fluorescent spots under short-wave UV light (λ_{254nm}). Fractions containing AnAc were pooled and concentrated under a light steady stream of N_2(g). Concentrated crude AnAc extracts were dissolved in hexane at a concentration of 200 mg/mL, and the two dominant AnAc (22:1$^{\omega 5}$ and 24:1$^{\omega 5}$) were separated by HPLC with modifications to the procedures described (*16*, *30*, *31*). HPLC purification was carried out using a Premier C18 reversed phase column (25 cm × 4.6 mm; 5 μm particle, Shimadzu) at a flow rate of 2.5 mL/min of acetonitrile/dH_2O/acetic acid (100/20/1, v/v/v) with UV detection at 254 nm (Shimadzu VP series HPLC system). HPLC fractions (1.5 mL) were collected using a Gilson fraction collector (FC203B). Fractions correlating to the two dominant peaks (8−9- and 15−17-min retention times) were pooled separately. An equal volume of water was added to each sample followed by acidification through addition of acetic acid to 1%. Each sample was extracted three times with an equal volume of hexane. Hexane extracts were concentrated to dryness under a steady stream of N_2(g), weighed, and then dissolved in hexane at a final concentration of 10 mg/mL.

The purity of the HPLC peaks was further confirmed by running samples with an alternate HPLC solvent system (3 mL/min of acetonitrile/methanol/acetic acid; 82/16/1, v/v/v) and by analysis on TLC plates (as described above) with extended I_2 vapor staining to ensure no contaminating products were present. On the basis of TLC and HPLC analyses, peak purity was >95% with the remaining additional minor AnAc peaks that were not separated in our primary HPLC system. Initial experiments did not indicate a significant difference between AnAc 22:1$^{\omega 5}$ and AnAc 24:1$^{\omega 5}$ in our assays. Therefore, only AnAc 24:1^{5} was used for more detailed studies.

The identities of the two main peaks isolated by reversed phase HPLC were further verified by 1D and 2D NMR methods. High-resolution ^{13}C NMR was first employed to determine the length of the hydrocarbon chain (i.e., 22:1 vs 24:1 AnAc). Typically, the entire peak fraction (after hexane extraction and evaporation to dryness) was dissolved in 0.5 mL of $CDCl_3$ (Cambridge Isotopes) and subjected to overnight ^{13}C acquisition (17500−25000 scans) on a 5 mm BB probe operating at 125.7 MHz and employing continuous waltz 1H decoupling (Varian INOVA 500 NMR system). All backbone carbons (**Table 1**) could be fully resolved by extending the fid to 128K data points prior to 0.5 Hz exponential multiplication and Fourier transformation.

The position of the double bond was verified by 2D heteronuclear correlation spectroscopy (^{1}H−^{13}C HMQC, ^{1}H−^{13}C HMBC) on a 5-mm triple-resonance probe operating at a proton frequency of 499.7 MHz. Standard Varian gradient enhanced pulse sequences were employed typically using either 4 scans (HMQC) or 8 scans (HMBC) and 256 t_1 increments. A high-resolution HMBC experiment (32 scans, 512 t_1 increments, reduced spectral widths in both ^{1}H and ^{13}C) was also performed to further clarify the aliphatic region.

CPB Colony. To establish a colony of *Leptinotarsa decemlineata* (CPB), adults, larvae, and egg masses were collected from gardens and small farms in the Louisville metropolitan area. CPB were maintained in large plastic containers (with small air holes drilled) with a mature (approximately 1 m) eggplant (*Solanum melongena*; Solanaceae family). All eggplants (Imperial Black Beauty) were grown from seed and maintained in growth chambers with a 16-h light cycle (approximate photon flux of 200 μmol/m^2/s). Plants were replaced as needed to maintain the CPB population. Population size was maintained so that a continuous production of newly hatched larvae was readily available in sufficient numbers for all assays. In all assays, newly hatched larvae (first instar) were selected (within a day of hatching) and randomly assigned to treatments.

Larval Growth and Development Assays. To determine the influence of AnAc 24:1$^{\omega 5}$ on growth and development, newly emerged larvae (described above) were selected and provided eggplant leaf disks (30-mm diameter) for food. Larval weight was recorded daily for the duration of each experiment. Eggplant leaf disks were prepared by cutting leaf disks from developing young leaves with a 30 mm bore template. Each experiment replicate consisted of five treatments. AnAc

Table 1. ^{1}H and ^{13}C Chemical Shifts of HPLC-Purified Anacardic Acid in $CDCl_3$

	$^{13}C\ \delta$		$^{1}H\ \delta$	
fragment	22:1$^{\omega5}$	24:1$^{\omega1}$	HMQC[a]	HMBC[b]
–**C**H$_3$	14.23	14.24	0.90 (3H, t, $J = 7$ Hz)	1.32
–**C**H$_2$CH$_3$	22.57	22.57	1.32	0.90, 1.32, 2.03
=CH**C**H$_2$–	27.14, 27.42	27.13, 27.42	2.03 (4H, m)	5.36
–CH$_2$(**C**H$_2$)$_n$CH$_2$	29.53, 29.70, 29.76, 29.81, 29.98, 30.05	29.54, 29.71, 29.78, 29.85, 29.87, 29.89, 30.00, 30.05	1.25–1.40[c]	1.25–1.40,[c] 1.60
–**C**H$_2$CH$_2$CH$_3$	32.19	32.19	1.32	0.98, 1.32, 2.03
–**C**H$_2$CH$_2$Ar	32.30	32.30	1.60 (2H, quintet)	2.98, 1.30
–CH$_2$**C**H$_2$Ar	36.76	36.75	2.98 (2H, dd, $J = 7.7$ Hz)	6.78, 1.60
Ar**C**–CO$_2$H	110.40	110.44		6.78, 2.98, 6.87
Ar**C**H	116.04	116.06	6.87 (1H, d, $J = 8.3$ Hz)	6.78
Ar**C**H	122.90	122.94	6.78 (1H, d, $J = 7.6$ Hz)	6.87, 2.98
=**C**H	130.09, 130.11	130.06, 130.13	5.36 (2H, t, $J = 5$ Hz)	2.03, 1.32
Ar**C**H	135.57	135.62	7.36 (1H, t, $J = 7.9$ Hz)	6.78, 6.87
Ar**C**-alkyl	147.77	147.88		2.98, 7.36, 1.60, 6.78
Ar**C**–OH	163.90	163.90		7.36, 6.87
CO$_2$H	174.65	175.23		6.87

[a] Values in parentheses (integration, multiplicity, coupling) were obtained from 1D ^{1}H NMR and are shown for 22:1$^{\omega5}$ AnAc (24:1$^{\omega5}$ ^{1}H data were essentially identical). [b] More intense correlations are given first. [c] Specific proton–carbon correlations could not be determined due to spectral overlap.

24:1$^{\omega5}$ was applied to leaf disks at four concentrations (0.1, 0.5, 1.0, and 2.5 mg/mL) and were compared with a buffer control treatment. Each treatment concentration was prepared by transferring a known concentration of AnAc 24:1$^{\omega5}$ to a glass tube, and the hexane was fully evaporated under N_2(g). The samples were then dissolved in 50 mM CHES buffer (pH 9.0). To facilitate solubilization of AnAc, all samples were heated to 50 °C prior to leaf disk treatment. Leaf disks were incubated in the sample or control solutions for 5 min. Each leaf disk was drip-dried briefly and then placed on a dH_2O moistened 3 MM Whatman paper disk (30 mm) in a 35-mm plastic Petri dish. Each Petri dish was sealed using Tenderskin tape (Kendall). Leaf disks, treated as just described, were replaced daily. Four newly emerged larvae (each <1 day old) were placed on each of the five leaf disk treatments, and each experiment was replicated five times. Larval weight was recorded as a cohort of four larvae in each treatment until day 5 (phase I) due to the low individual weight of the larva during this initial developmental time period. On day 6, the four larvae in each treatment were transferred to individual leaf disks, and weight was recorded on an individual basis for the remainder of the experiment (phase II). At day 10, each Petri dish was transferred to a small glass baby food jar containing ~4 cm of soilless medium (Metro mix 360), and the jar was capped. Upon completion of development through the fourth instar, CPB larvae buried themselves in the soil to pupate. The date that larval development was completed was recorded when the larva was no longer visible and the leaf disk showed no signs of herbivory. After pupation began, each jar was observed daily until beetle emergence so that duration of pupation could be recorded.

Food Preference Assay. To assess the ability of larvae to detect and potentially avoid food containing AnAc 24:1$^{\omega5}$, food preference assays were conducted. Cohorts of newly emerged larvae were randomly selected and reared on untreated leaf disks (prepared as previously described) for 7 days. On day 7, four larvae were randomly selected from individual cohorts for the food preference assays. Four leaf disks (two controls and two treated with 2.5 mg/mL) were distributed equally in an alternating pattern on dH_2O moistened 3 MM Whatman filter paper (85-mm disks) in 90-mm Petri dishes. The four larvae were placed at the center of the Whatman paper, equidistant from all leaf disks. At time points 1, 2, 4, and 8 h, each dish was observed and the number of larvae feeding on either control or treated leaf disks was recorded. Each experimental trial was replicated 10 times.

After the final observation at 8 h, remaining leaf disk surface area was measured using a Fluor-S MultiImager (Bio-Rad) to scan the surface area with Quantity One software (version 4.0.3) analysis to calculate surface area. Surface area consumed was equal to the average initial surface area minus the remaining surface area at the end of the experiment, multiplied by 100. Average initial surface area value was an average of six independent leaf disk surface area measurements.

Statistical Analysis. *Larval Growth Rate.* Growth during the first 5 days (phase I) was analyzed separately from the remaining days of growth (phase II) because during this period all four larvae in a given replication were weighed together. After day 5, each larva was individually weighed and, thus, variability among larvae could be included in the analysis. A similar analysis was done on both data sets. The growth rate data were modeled using a mixed general linear model with a repeated measures analysis in which larva identification number was the subject effect and day was the repeated effect. When (phase I) data were analyzed, the subject effect was replication number because all four larvae were weighed together. AnAc dose was a between-subjects effect and in some analyses treated as a discrete valued variable. Thus, contrasts to test for differences between the control and specific doses of AnAc could be conducted. In some analyses, AnAc dose was treated as a continuous variable. Because the entire experiment was replicated five times, replication number was included as a block variable that had a random effect interpretation. A number of covariance structures were tested, and the ante-dependence structure was found to give the best fit to the data by comparison of the values of the log likelihood ratio and Aikikos information criterion. For all models, residuals were examined for fit to the normal distribution and homogeneity of variance. The log transform of the original data was used to achieve normality and homogeneity of variance of the model residuals. All analyses were performed with the MIXED procedure of SAS/STAT version 9.1 (*32*).

Analysis of Survivorship. The effect of AnAc dose on survivorship was examined using the Kaplan−Meier (product limit) method that allowed for the inclusion of right-censored data. All analyses were performed with the LIFETEST procedure of SAS/STAT version 9.1 (*32*).

Analysis of Eclosion Rates. All individuals reaching the pupal stage were observed for eclosion. The effect of AnAc dose on proportion of eclosion was tested for by the Cochran−Armitage trend test and also by an exact test of independence for a two-way contingency table. All analyses were performed with the FREQ procedure of SAS/STAT version 9.1 (*32*).

Food Preference Test. Homogeneity of replicate trials for the proportion of larvae on the two types of leaf disks was tested at each time (1, 2, 4, and 8 h) by the Mehta−Patel exact test for a two-way contingency table. All were found to be homogeneous and thus were pooled. The pooled data were used in a Cochran−Armitage test to test for a trend in proportion on each type of food, over time. No significant trend over time was found, and a test for homogeneity of proportions over time was not significant, so the data were summed over the time periods. The final pooled data were tested for goodness of fit to the model of equal frequency, on control disks and on treated disks, by a

chi square goodness of fit test. All analyses were performed with the FREQ procedure of SAS/STAT version 9.1 (*32*).

Leaf Surface Area Consumption. Leaf surface area was analyzed with a mixed general linear model. Replicate trials were found to have unequal variances, and thus unequal variances were included in the final model. The replicate trials were specified to have a random effect interpretation as were their interactions with treatment. Residuals of the models were examined for goodness of fit to the normal distribution and equality of variance and found to be acceptable. All analyses were performed with the MIXED procedure of SAS/STAT version 9.1 (*32*).

Larval Feeding Rate. The larval feeding rate on control and treated leaf disks was estimated separately for each of the ten trials. The number of larvae hours on each disk treatment (control versus AnAc) was estimated from the observed data, and the feeding rate per larvae per hour was estimated by dividing the change in leaf surface area by the number of larvae hours for each type of disk for each trial. The difference in feeding rate between control and treated disks (control − treated) for each trial was computed, and its distribution was found to be highly skewed. The null hypothesis of a median difference of zero was tested using the sign test.

RESULTS AND DISCUSSION

Isolation and Purification of AnAc. Previous GC and HPLC analyses of glandular trichome exudates from pest-resistant zonal geranium lines indicated that AnAc 22:1$^{\omega5}$ and AnAc 24:1$^{\omega5}$ represent 80−90% of the total AnAc profile (*10*, *16*, *17*, *33*). HPLC analysis of isolated AnAc indicated two dominant peaks (8−9- and 15−17-min fractions) that represent ∼85% of all AnAc. Each peak was tentatively identified as AnAc 22:1$^{\omega5}$ (8−9-min peak) and AnAc 24:1$^{\omega5}$ (15−17-min peak) and subsequently purified. Purity was assessed using an alternative HPLC system and TLC analysis with extended staining (data not shown). A single TLC spot was detected (by UV fluorescence and iodine staining), indicating each sample contained only AnAc. HPLC analysis indicated each AnAc was >95% pure (with the remaining 5% unresolved minor AnAc that appeared as a shoulder). High-resolution ^{13}C NMR analysis (**Table 1**) was initially used to confirm the hydrocarbon identity of each AnAc peak (22:1 at 8−9 min and 24:1 at 15−17 min), consistent with predicted elution behavior. Finally, analysis of the proton−carbon connectivities elucidated from 2D heteronuclear NMR spectroscopy (HMQC and HMBC, **Table 1**) confirmed the placement of the double bond at the ω5 position.

Dose−Response of CPB Larval Growth to Increasing Concentrations of AnAc. Larval growth was analyzed by measuring larval weight gain over a 15-day duration. Weight gain was recorded until larvae pupated or died. CPB larval development generally takes between 14 and 21 days. In almost all experimental replications, larvae completed development or died by day 17. One larva in the 0.1 mg/mL AnAc 24:1$^{\omega5}$ treatment lived to day 24 before dying and one larva in the 1.0 mg/mL AnAc 24:1$^{\omega5}$ treatment died on day 24. Average larval weight gain over time was plotted for each treatment for which at least three larvae were alive (among five replications) and had not yet pupated (**Figure 2**). To assess the significance of increasing concentrations of AnAc 24:1$^{\omega5}$ on larval weight, growth curves were analyzed using a repeated measures design. Weight gain was analyzed in two phases (phase I includes days 0−5 and phase II includes days 6−15). In phase I, due to small individual size the larvae were weighed as a cohort to increase measurement accuracy. No significant differences could be detected between treatments during phase I. During phase II growth, larvae were weighed as individuals, and each dose−response curve was found to be linear but differed in slope. The trend for age (days development) by treatment dose interactions was found to be highly significant ($p = 0.0007$).

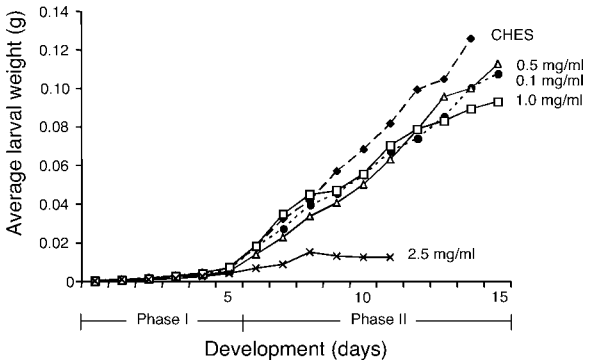

Figure 2. Influence of AnAc 24:1$^{\omega5}$ on CPB larval weight gain during development. CPB larvae were weighed daily for the duration of this experiment. Larvae were weighed as a cohort (phase I) until day 6, at which time larvae were weighed separately (phase II). Each replication consisted of four AnAc 24:1$^{\omega5}$ treatment concentrations (0.1, 0.5, 1.0, and 2.5 mg/mL) and one control (CHES buffer only). Each treatment in an experiment contained four newly hatched larvae. Data are presented as the average larval weight for which at least three larvae (sum of all replicates) were alive and had not yet pupated.

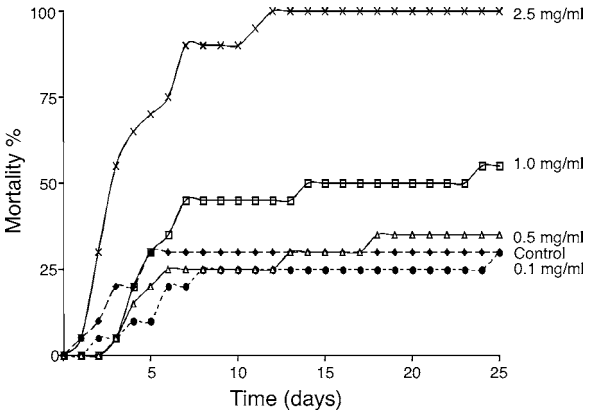

Figure 3. Influence of AnAc 24:1$^{\omega5}$ on CPB larval mortality. CPB larvae were monitored daily, and all deaths were recorded. Percent mortality was calculated for each treatment replication, and the average mortality of the five replications for each treatment was plotted over time (larvae development). Each replication consisted of four AnAc 24:1$^{\omega5}$ treatment concentrations (0.1, 0.5, 1.0, and 2.5 mg/mL) and one control (CHES buffer only). Each treatment in a replication contained four newly hatched larvae.

Thus, as time increased, the influence of treatment concentration had a proportionally higher effect on weight gain. Of significance, all larvae in the highest treatment (2.5 mg/mL AnAc 24:1$^{\omega5}$) perished before day 12. Therefore, an additional important aspect of larval development to consider is survivorship.

Larval death (average of percent mortality in each treatment) was plotted over time (**Figure 3**). Larval development ended at one of two points with larvae either dying prior to pupation or with the larvae pupating. During the daily weight measurements, larval death or pupation was also recorded. By day 25, all larvae (in all replications and treatments) had either died or pupated. Using the Kaplan−Meier estimator method to analyze a trend model of survivorship, a highly significant relationship ($P < 0.0001$) between treatment and mortality was detected such that increasing concentrations of AnAc 24:1$^{\omega5}$ were correlated with increased larval mortality. Taken together, analysis of larval development with respect to weight gain and mortality indicates that ingested AnAc negatively affects insect larval development in a concentration-dependent manner.

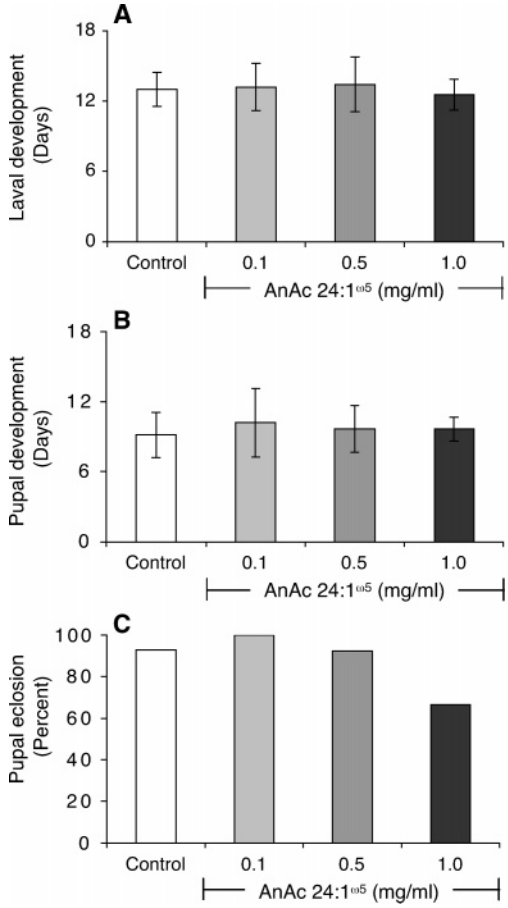

Figure 4. Influence of AnAc 24:1$^{\omega 5}$ on duration of CPB larval and pupal development and pupal eclosion. Total time (days) to complete larval (**A**) or pupal (**B**) development was averaged for all treatments. Error bars represent standard deviation. The number of individuals that pupated in each treatment and successfully eclosed and emerged as adult beetles was recorded and used to calculate a total percentage for each treatment (**C**).

Influence of AnAc on Duration of Larval Development and Pupation. Because AnAc affects larval development with respect to weight gain, we sought to determine if the duration of developmental phases (larval and pupal) for individuals that did not perish during larval development was also affected (**Figure 4**). The end point of larval development was recorded as either death of the larva or the beginning of pupation (noted when the larvae entered the soil and displayed no further feeding). Larvae in the 2.5 mg/mL AnAc 24:1$^{\omega 5}$ treatment remained small and did not appear to progress through normal development including progression through four distinct instars, and thus we speculate that AnAc may act to impede larval development when consumed in a diet. However, all larvae in treatment groups with 2.5 mg/mL AnAc 24:1$^{\omega 5}$ perished prior to pupation and thus could not be used to directly assess larval or pupal developmental time. In all other treatments, a proportion of the larvae completed development (**Figure 3**). To determine the impact of AnAc treatment on the duration of larval development to pupation, treatments were compared to controls. Treatment and control larvae averaged ~13 days for larval development but were not found to be statistically different (**Figure 4A**).

The mode of action by which this toxin affects pests has not been determined. However, studies have been conducted that indicate possible mechanisms of action. AnAc inhibits tyrosinase (*34*), an enzyme suggested to have a role in the molting process (*35*). However, tyrosinase has also been proposed to have a more limited role in cuticle development (restricted to wounding), whereas laccase occupies a more central role in cuticle development (reviewed in ref *36*). Recently, an RNAi-based approach confirmed laccase and not tyrosinase was the major enzyme involved in sclerotization (*37*), yet a minor role for tyrosinase during cuticle formation cannot be conclusively ruled out. We clearly observed a delay in instar development at our highest treatment concentration (2.5 mg/mL); however, these larvae died before development was complete. Additionally, we cannot exclude explanations unrelated to the molting process. For example, AnAc at high concentrations may disrupt the digestive system of the larvae and thereby delay development. Thus, further study will be required to determine the biochemical and physiological consequences to insect larvae that ingest AnAc at high concentrations. Analysis of lower treatment concentrations failed to show a significant relationship with larval development. In fact, the average larval developmental time in all treatments (13 days) is rapid compared to a typical range for CPB development (14−21 days). This is most likely due to the controlled environments used in these studies. Thus, it appears that AnAc may not directly affect larval development by inhibiting molting but more significantly may act to limit larval development by effecting processes that lead to decreased larval weight gain (**Figure 2**) and increased larval mortality (**Figure 3**).

A weakened larva would potentially have lower success in completing pupal development, marked by eclosion of the adult beetle. Because our treatments showed a significant effect of dose on larval growth rate, we hypothesized larval consumption of AnAc would affect pupal development duration or success noted by eclosion of the adult beetle. In all replications, we recorded the duration of time from the point at which the larvae burrowed into the soil to the time when an adult beetle emerged as an indirect measure of pupation (**Figure 4B**). Again, no results were recorded for the 2.5 mg/mL treatment because all larvae perished prior to pupation. Of all larvae that appeared to pupate (noted by burrowing into the soil), only 5 of a total of 50 did not eclose and emerge as adults (control, 13/14; 0.1 mg/mL, 14/14; 0.5 mg/mL, 12/13; and 1.0 mg/mL, 6/9; numbers denote eclosion and emergence of adults/larvae that began pupation). No significant difference was observed for the duration of successful pupation (average of ~10 days in all treatments, **Figure 4B**). However, the proportion of total beetles in each treatment that successfully eclosed and emerged from the soil as adults appeared to show a trend with only 67% success in the 1.0 mg/mL treatment compared with >92% in all other treatments (**Figure 4C**). However, statistical analysis (exact test) indicated no significance. Therefore, on the basis of our analysis of duration of larval and pupal development and successful completion of pupation, we cannot conclude any significant effect of AnAc treatment at the lower concentrations tested and, thus, the effectiveness of AnAc appears to be through increased larval mortality. Because the number of pupae studied here is not large and none of the larvae treated with the highest dose of AnAc reached the pupal stage, an effect of intermediate concentrations of AnAc on the pupal stage cannot be ruled out.

Food Preference Analysis. To further explore the potential mechanisms by which AnAc may affect pest populations, we utilized a food preference test to determine if AnAc could act as a feeding deterrent and to assess the impact of AnAc on relative leaf consumption (**Figure 5**). On the basis of our results from the larval development assay, it was apparent that the 2.5 mg/mL AnAc 24:1$^{\omega 5}$ treatment affected larval growth and

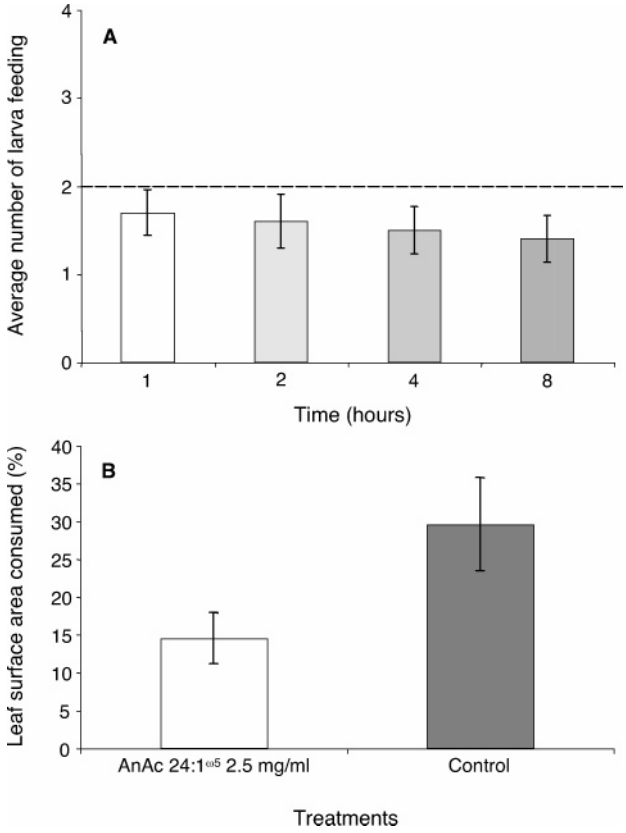

Figure 5. Food preference behavior and consumption rates of CPB larvae. CPB larvae were given a choice between two leaf disks treated with 2.5 mg/mL AnAc 24:1$^{\omega 5}$ and two leaf disks that were treated with a buffer control. Feeding behavior was recorded over an 8-h period and indicated for leaf disks treated with 2.5 mg/mL AnAc 24:1$^{\omega 5}$ (**A**). The number of larvae feeding on control or treatment was averaged for 10 experimental replications (error bars represent standard error). The dotted line indicates the expected distribution of larvae displaying random feeding behavior. At the end of each replication, the remaining leaf surface area was measured and used to calculate average leaf area consumed (**B**) for the 10 replications (error bars represent standard error).

development, and thus this treatment level was chosen for further study. In each of 10 replicates, four larvae of the same developmental age (7 days) were provided with two control leaf disks and two leaf disks treated with 2.5 mg/mL AnAc 24:1$^{\omega 5}$. Feeding behavior was recorded at 1, 2, 4, and 8 h (**Figure 5A**). In all cases larvae were feeding on treated or untreated leaf disks (i.e., at no time did we observe larvae not feeding on a leaf disk). Random feeding behavior (i.e., no food preference or avoidance) would lead to an equal distribution of larvae on the treated and untreated leaf disks. If larvae were able to detect and/or avoid the AnAc 24:1$^{\omega 5}$ treated leaf disks, either upon initial selection (at 0 h) or after sampling at a later time, a higher proportion of larvae feeding on untreated leaf disks would be expected. We found no statistically significant trend between treatment feeding behavior and time using the Cochran−Armitage test. A chi square test showed the proportions of larvae feeding on control and treated disks to be homogeneous with respect to time, so the data were summed over time. A chi square test of the model for no preference between control and treated disks by the larvae proved to be significant ($p = 0.0044$). On the basis of the chi square analysis of these results, it appears that larvae are able to avoid the treated samples and/or preferentially feed on the control leaf.

After the final observation times, the surface area of the remaining leaf disks was measured and used to calculate leaf consumption (**Figure 5B**). The average control leaf area consumed was 30%, whereas the average treatment leaf area consumed was 15%, and this difference was found to be highly significant ($p = 0.0022$). In addition to the larvae preferentially locating themselves on the control leaf disks, it is also possible they consume control and treated leaf tissue at different rates. To test this possibility, the rate of leaf area consumption per larvae per hour was computed for both control and treated leaf tissue for each replicate experiment. The median difference (control − treated) was 3.57 and is significantly different from a median of zero by a sign test ($p = 0.0215$). This result indicates larvae on control leaf disks feed at a higher rate than larvae located on treated leaf disks.

Ultimately, food preference of larvae is dependent on access to alternative, untreated food. In a plant producing AnAc in all leaf tissues or sprayed with AnAc, larvae would have no alternative food choice due to limited mobility. On the basis of our food preference test, larvae appear to avoid tissue treated with AnAc and display reduced consumption rates of tissue treated with AnAc. Thus, we speculate that plants producing AnAc or treated with AnAc would be more resistant to pests or display reduced damage relative to comparable plants lacking AnAc. Another important aspect to consider is that adult beetles could easily move between plants producing AnAc and those that do not. This point is particularly important when it is considered AnAc has been shown to inhibit lipoxygenase (*38*−*40*) and cyclooxygenase (*39*, *41*, *42*), the eicosanoid products of which can influence insect reproductive ability (reviewed in refs *43*−*45*). Thus, additional research is required to test if AnAc acts as a deterrent to adult beetle feeding and egg-laying activity.

In this study we show a dose−response between AnAc concentration and larval growth and development. Our results indicate that when ingested, AnAc is effective at inhibiting growth and development of CPB larvae, most likely through a toxic effect that lowers growth rate and increases mortality. We noted that larval development was stunted at our highest treatment concentration (2.5 mg/mL AnAc 24:1$^{\omega 5}$) with no larvae progressing past the second- or third-instar stage, although more thorough studies will be needed to assess the specific role of AnAc in inhibiting larval development by a direct (e.g., molting) or indirect (e.g., toxicity) mechanism. We further assessed the effectiveness of AnAc as a feeding deterrent by utilizing a food preference test. We found a significant preference by larvae for untreated leaf disks over those treated with AnAc, and on the basis of leaf surface area consumption rates, it was apparent that the larvae have lower consumption rates on treated versus control tissue.

Approaches to genetically engineer pest resistance have proven to be effective for the Bt-endotoxin (*46*−*48*). However, there is growing concern that an increasing population of target insects will become resistant to Bt (*49*). Specifically in potato, work has been conducted to evaluate additional mechanisms of resistance to CPB (*50*−*52*); whether they are factors that already exist in the germplasm or traits introduced through genetic engineering, it is clear that the trend is toward a combinatory approach to resistance. On the basis of the results of our study, we suggest that AnAc, applied as a chemical spray or through bioengineering production in crop plants, alone or in combination with other pest resistance approaches, may provide a new weapon in the arsenal to minimize damage caused

by pests. Future studies will be needed to determine LD_{50} values for AnAc as well as to assess any differences in bioactivity of AnAc homologues.

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ABBREVIATIONS USED

AnAc, anacardic acid; CPB, Colorado potato beetle.

NOMENCLATURE

Anacardic acid (AnAc) is used as a generic term to describe any 2-hydroxy-6-alkylbenzoic acid, regardless of alkyl chain length or saturation state. To designate a specific AnAc, the following nomenclature was adapted on the basis of fatty acid nomenclature: AnAc $X{:}Y^{\omega Z}$ indicates an anacardic acid (AnAc) with X total carbon atoms and Y double bonds in the alkyl group. Double-bond position (Z) is designated from the methyl (ω) end of the alkyl group.

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