NUTRITIONAL INDICES: DO THEY EXPLAIN TOXICITY OF CALLIANDRA AMINO ACIDS?

CHRISTOPHER S. SHEA AND JOHN T. ROMEO
Department of Biology,
University of South Florida
Tampa, FL 33620

ABSTRACT

Calliandra leaves and the nonprotein imino acids they contain possess insecticidal activity. In order to determine the role these compounds play in Calliandra resistance to herbivory, feeding experiments were performed using larvae of the polyphagous herbivore Spodoptera frugiperda Smith, and nutritional indices were calculated. Statistically significant growth inhibition occurred with the total amino acid fraction and trans-5-hydroxypropionic acid, and there was a trend toward similar inhibition with several other imino acids. The efficiency with which larvae converted assimilated food into biomass (ECO) was reduced by the total amino acid fraction as well as by two imino compounds. The consumption index (CI) and the diet digestibility (AD) were unaffected by amino acids. Leaf material caused significant growth inhibition, increased mortality, and a dramatic decrease in the growth and survival of progeny. Leaf material lowered AD, raised relative consumption rates (RCR) and lowered the efficiency of conversion of the ingested food (ECI). The data suggest that nonprotein amino acids acting as toxins via negative effects on insect nutritional physiology are part of Calliandra resistance to herbivory.

RESUMEN

Las hojas de Calliandra y los iminoácidos no-proteínicos que ellas contienen, poseen actividad insecticida. Para determinar el papel que estos compuestos juegan en la resistencia de Calliandra a la herbivoría, se desarrollaron experimentos de alimentación usando larvas del herbívoro polífago Spodoptera frugiperda Smith, y fueron calculados índices nutricionales. Se encontró una inhibición del crecimiento estadísticamente significativa entre la fracción total de aminoácidos y el ácido trans-5-hidroxipropionílico, así como una tendencia similar de inhibición en muchos otros iminoácidos. La eficiencia con la cual las larvas convirtieron alimento asimilado en biomasa (ECO) fue reducida por la fracción total de aminoácidos así como dos compuestos imino. El índice de consumo (CI) y la digestibilidad de la dieta (AD) no fueron afectadas por los aminoácidos. Las hojas causaron una significativa inhibición del crecimiento, un incremento en la mortalidad, y una reducción dramática en el crecimiento y la sobrevivencia de la progene. Las hojas disminuyeron el AD, incrementaron la tasa relativa de consumo (RCR) y disminuyeron la eficiencia de conversión del alimento ingerido (ECI). Los datos sugieren que aminoácidos no-proteínicos actuando como toxinas a través de efectos negativos sobre la fisiología nutricional de insectos, son parte de la resistencia de Calliandra a la herbivoría.

Many nonprotein amino acids play ecological roles in protecting plants from insect herbivores. The majority of studies, however, report insecticidal activity without addressing the mechanism, although several modes of action for these compounds are known. Nonprotein amino acids: 1. can function as antimetabolites of their protein amino acid analogues. Canavanine does this when it substitutes for arginine on an insect's arginyl-tRNA synthetase and produces anomalous insect proteins (Dahman & Roenthal 1976). Another nonprotein amino acid, abizzine, depresses asparagine synthetase activity (Lea & Fowler 1975). 2. can function as feeding deterrents affecting sensory perception as do Acacia amino acids to acridids (Evans & Bell 1979). 3. may
negatively affect nutritional physiology resulting in suboptimal foraging and growth. L-Dopa reduces the digestibility of the artificial diet of the black cutworm, *Agrotis ipsilon* Hufnagel, and also the efficiency of conversion of assimilated food into biomass (Reese & Beck 1976).

The tropical mimosoid legume *Calliandra* consists of some 150 + species of woody shrubs to large trees largely confined to the New World. Although not yet exploited on a large scale, at least one species is cultivated for firewood. Foliage is rich in protein, and cattle and goats consume it freely. Bees use its nectar for producing honey (National Academy Press 1983). The genus has been designated as a potentially exploitable tropical plant group needing further scientific investigation (Uribe et. al. 1984). *Calliandra haematocephala*, used in this study, is native to Bolivia and widely cultivated in south Florida. In the field and in cultivation, *Calliandra* plants are relatively free from insect predation.

The amino and imino acids (amino acids with a heterocyclic nitrogen ring) of *Calliandra* are individually moderately toxic to aphids and larvae of lepidopteran pests belonging to *Spodoptera* and *Heliotis* genera (Romeo 1984, Romeo & Simmonds 1989). It is common for a plant to produce a series of structurally related defensive compounds that may act additively or synergistically (Beresnaum 1985). Piperolic acid and its various hydroxylated derivatives (Fig. 1) found in the leaves, sap, and seeds of *Calliandra* species act synergistically in causing feeding deterrence and toxicity in aphids (Simmonds et al. 1988). The presence of a nonprotein sulphur-containing amino acid further enhances the activity of the piperolic acids.

Some modes of action previously have been eliminated as explanations of the biological activity of piperolic acids: 1. As higher homologues of proline they are too big to substitute for this imino acid and produce anomalous insect proteins as the lower homologue, azetidine-2-carboxylic acid, does (Fowden et al. 1967). 2. Although the mono and di-hydroxy piperolic acid derivatives are similar in structure to known alcaloidal glycosidase inhibitors, they lack enough hydroxyls to act as competitive sugar mimics (Fellows and Nash, personal communication). In contrast, *trans-trans-trans*-trihydroxypiperolic acid with its single extra hydroxyl is a specific inhibitor of human beta-D-glucuronidase and alpha-L-iduronidase (Ceci di Bello et al. 1964). 3. They do not appear to alter taste preception in lepidopterans. In electrophysiological recordings of taste sensilla of *Spodoptera littoralis*, responses did not correlate significantly with feeding behavior (Romeo & Simmonds 1989).

With the above explanations of toxicity excluded, we decided to use Waldbauer’s (1968) nutritional indices as a way of getting at the possible mechanism of action. A series of feeding studies was performed using the polyphagous herbivore *Spodoptera frugiperda*, and five nutritional indices were computed. All compounds and extracts were assayed using concentrations at or below their naturally occurring levels in *Calliandra*.

**Materials and Methods**

**Source of Chemicals.** *Trans*-5-hydroxy-piperolic acid was purchased from Sigma Chemical Co., St. Louis, MO. The other imino acids were from stock supplies previously isolated in our laboratory.

**Source of Insects.** Eggs of *Spodoptera frugiperda* were obtained from the USDA Basic Biology and Insect Attractants Laboratory in Gainesville, FL.

**Preparation of Diets and Extracts.** The control diet was the pinto bean based artificial diet of Burton (1969). Leaf powder and imino acid containing diets were prepared according to the method of Romeo (1984); crystalline chemicals and finely ground leaf powder were added to the dry ingredients of the pinto bean diet. Leaf powder was prepared from locally obtained *Calliandra haematocephala* Hasak leaves dried in an oven (24 hr, 100° C) and ground (40 mesh) in a Wiley mill. The aqueous fractions of *C.*
Fig. 1. Structure of Piceolic Acid and Derivatives.

Piceolic Acid \( R_1 = R_2 = R_3 = R_4 = H \)

T-5-OH PIP \( R_4 = OH, R_1 = R_2 = R_3 = H \)

C-5-OH PIP \( R_3 = OH, R_1 = R_2 = R_4 = H \)

TT-4,5-OH PIP \( R_2 = R_3 = OH, R_1 = R_4 = H \)

TT-4,5-OH PIP \( R_2 = R_4 = OH, R_1 = R_4 = H \)

Haematocephala were prepared by extracting from ground leaves with 50% ethanol. The extract was filtered, concentrated, redissolved in methanol-chloroform-water (12:5:1), and extracted again with chloroform and water. The aqueous portion was evaporated to dryness (in vacuo) and redissolved in a small amount of deionized water. The amino acid fraction was obtained by applying the aqueous extract to a series of ion-exchange columns (CG 120 cation exchange resin, elution with ammonia followed by CG 400 anion exchange resin, elution with acetic acid). For 100% plant equivalency experiments, an amount of leaf powder equal to the amount of diet to be made (on a dry weight basis) was extracted and the extract added to the dry ingredients of the artificial diet.

Bioassay. Treatment groups consisted of 20 neonate (less than 24 hours old) larvae which were kept in individual 2 oz. cups with lids and maintained in a growth chamber (25°C, 70-90% relative humidity, 12 hr light/12 hr dark). In the leaf powder experiment, larvae were fed with an excess of fresh weighed diet each day. Larvae and un Consumed diet were weighed daily after day 10. Frass was collected daily. In the imino acid and leaf extract experiments, neonate larvae were placed on diet cubes that were sufficient for the entire experimental period. In order to minimize error in computation of nutritional indices, size of diet cubes was adjusted so they would be largely consumed during the feeding program (Schmidt & Reese 1986). Larvae and un Consumed diet were weighed and frass collected at the end of these experiments. Frass dry weight was determined directly after drying in an oven at 100°C for 24 hr. Larval and diet dry weights were determined by aliquot. Five randomly chosen larvae from each of the last three instars were frozen, dried and weighed. Instars averaged 16% dry weight (range = 14-20). Similarly, diet cubes were dried to determine initial and final dry weights. Humidity in the cups containing larvae was high; diet desiccation ranged from 0-2%.
Nutritional Indices. These were calculated according to the method of Waldbauer (1968). Measurements were made of: Relative Consumption Rate (RCR), the consumption rate corrected for mean body weight (used in the leaf powder experiment); Consumption Index (CI), the consumption rate corrected for final body weight (used in the imino acids experiment due to the lack of daily weighing); Approximate Digestibility (AD), the proportion of ingested food actually assimilated; Efficiency of Conversion of Digested food (ECD), a measure of the animal's ability to convert assimilated food into biomass; Efficiency of Conversion of Ingested food (ECI), a measure of the ability to convert ingested food into biomass. ECI varies with AD and ECD and is dependent upon them.

\[
\text{RCR} = \frac{\text{food eaten (mg)}}{\text{mean larval wt (mg)/day}}
\]

\[
\text{CI} = \frac{\text{food eaten (mg)}}{\text{final larval wt (mg)/day}}
\]

\[
\text{AD} = \frac{\text{amount ingested (mg) - frass produced (mg)}}{\text{amount ingested (mg)}}
\]

\[
\text{ECD} = \frac{\text{weight gain (mg)}}{\text{amount ingested (mg) - frass produced (mg)}}
\]

\[
\text{ECI} = \frac{\text{weight gain (mg)}}{\text{amount ingested (mg)}}
\]

Significant differences in larval weights and in nutritional indices were determined by ANOVA and Tukey's test for unequal sample size. Significant differences in mortality were determined by Chi Square Analysis.

**RESULTS**

The effects of incorporation of leaf powder into artificial diet of the fall armyworm are illustrated in Table 1. Only the group fed 5.0% *C. haematocephala* leaf powder showed a significant increase in mortality, but all leaf powder diets caused growth inhibition in the early instars as reflected in the larval weights on day 10. Higher concentration leaf powder diets reduced the efficiency with which larvae converted ingested food into biomass (ECD). A dramatic 88% reduction was seen. This was accompanied by an increase in the consumption rate (RCR), so larvae were not deterred from feeding. Reduction or a trend towards reduction in AD was seen in all animals fed leaf powder. Only the 2.5% leaf powder diet significantly lowered assimilation conversion efficiency (ECD).

None of the individual imino compounds tested caused a significant increase in mortality or abnormal larvae, but all imino compounds incorporated into artificial diet slowed larval growth relative to controls (Table 2). The greatest effect observed on day 8, a 48% growth inhibition, resulted from the 0.5% *trans*-5-hydroxyipepecolic acid incorporation. The 0.1% concentration of this same compound still produced a 34% growth
TABLE 1. Effect of *Calliantra haematocephala* leaf material on *Spodoptera frugiperda* growth, survival and nutritional physiology.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mortality (%)</th>
<th>10 Day Wt (mg)</th>
<th>RCR</th>
<th>ECI</th>
<th>AD</th>
<th>ECD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>268</td>
<td>1.27</td>
<td>0.23</td>
<td>0.48</td>
<td>0.54</td>
</tr>
<tr>
<td>1.0% C h</td>
<td>25</td>
<td>162*</td>
<td>1.10</td>
<td>0.22</td>
<td>0.35*</td>
<td>0.65</td>
</tr>
<tr>
<td>2.5% C h</td>
<td>10</td>
<td>181*</td>
<td>1.99*</td>
<td>0.14*</td>
<td>0.40</td>
<td>0.36*</td>
</tr>
<tr>
<td>5.0% C h</td>
<td>35*</td>
<td>214*</td>
<td>1.75*</td>
<td>0.14*</td>
<td>0.34*</td>
<td>0.48*</td>
</tr>
</tbody>
</table>

*p < 0.05

Inhibition. The other imino acids show an apparent trend toward reduced growth. Nutritional indices were affected in only 3 instances. ECD's were lower in larvae fed trans-5-hydroxyippecolic acid and trans-trans-4,5-dihydroxyippecolic acid. ECI was also lowered by the latter compound. None of the compounds affected the consumption rate, as evidenced by the CI values, nor the AD of the food.

When the aqueous and amino acid extracts of *C. haematocephala* leaves were incorporated into artificial diet at 100% plant equivalency, dramatic effects on growth were seen (Table 3). Larvae fed the total aqueous fraction weighed less than 10% of control larvae on day 8 and seemed not to have grown at all. Nutritional indices could not be calculated for this group due to the small amount of diet consumed and frass produced. A 20% reduction in growth occurred with the amino acid fraction. As with individual amino acids there were no affects on CI or food digestibility (AD). ECD was significantly depressed, but no more than with individual amino acids.

A limited experiment in which second generation larvae were raised on the same diet as their parents produced striking results (Table 4). Eighth day treatment larval weights were less than 10% of control. Mortality was significant. Egg viability of second generation was also severely affected (8% for 2.5% leaf powder moths vs 54% for controls).

TABLE 2. Effect of artificial diets containing imino acids on *S. frugiperda* growth, survival and nutritional physiology.

(T5 = trans-5-hydroxyippecolic acid; C5 = cis-5-hydroxyippecolic acid; TC = trans-cis-4,5-dihydroxyippecolic acid; TT = trans-trans-4,5-dihydroxyippecolic acid)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mortality (%)</th>
<th>8 Day wt (mg)</th>
<th>CI</th>
<th>ECI</th>
<th>AD</th>
<th>ECD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>256</td>
<td>0.46</td>
<td>0.27</td>
<td>0.43</td>
<td>0.66</td>
</tr>
<tr>
<td>0.5% T5</td>
<td>5</td>
<td>134*</td>
<td>0.47</td>
<td>0.25</td>
<td>0.49</td>
<td>0.53*</td>
</tr>
<tr>
<td>0.1% T5</td>
<td>0</td>
<td>169*</td>
<td>0.48</td>
<td>0.30</td>
<td>0.43</td>
<td>0.71</td>
</tr>
<tr>
<td>0.1% TC</td>
<td>0</td>
<td>230</td>
<td>0.45</td>
<td>0.28</td>
<td>0.38</td>
<td>0.68</td>
</tr>
<tr>
<td>0.1% C5</td>
<td>10</td>
<td>195</td>
<td>0.47</td>
<td>0.26</td>
<td>0.45</td>
<td>0.59</td>
</tr>
<tr>
<td>0.1% TT</td>
<td>5</td>
<td>222</td>
<td>0.48</td>
<td>0.28*</td>
<td>0.49</td>
<td>0.52*</td>
</tr>
</tbody>
</table>

*p < 0.05
TABLE 3. EFFECT OF AQUEOUS AND AMINO ACID FRACTIONS OF C. HAEMATOCEPHALA LEAF EXTRACT (100% PLANT EQUIVALENCY) ON S. FRUGIPERDA GROWTH, SURVIVAL AND NUTRITIONAL PHYSIOLOGY.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mortality (%)</th>
<th>8 Day wt (mg)</th>
<th>CI</th>
<th>ECI</th>
<th>AD</th>
<th>ECD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>246</td>
<td>0.52</td>
<td>0.21</td>
<td>0.45</td>
<td>0.66</td>
</tr>
<tr>
<td>Amino acids</td>
<td>15</td>
<td>194*</td>
<td>0.52</td>
<td>0.27</td>
<td>0.49</td>
<td>0.57*</td>
</tr>
<tr>
<td>Aqueous fract.</td>
<td>15</td>
<td>2*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05

DISCUSSION

Most of the mortality and growth inhibition among larvae fed C. haematocephala leaf powder occurred during the first week after larval eclosion (i.e., in the first three instars). Greater sensitivity of young larvae to plant defenses is not unusual (Shaver & Parrot 1970). Feeding on a toxin free artificial diet for the first 48 hours after hatching can dramatically improve growth (Reese 1983). Less growth inhibition was observed in this study than in an earlier one (Romeo 1984). We attribute the discrepancy to the added stress of daily weighings in the earlier study, a major difference in experimental protocol.

Nutritional indices are useful in suggesting possible mechanisms of antibiosis (Scriber & Slansky 1981, Reese 1983, Manuwoto & Scriber 1986). The decreased diet conversion efficiency (ECI) seen in larvae fed the higher concentrations of leaf powder is reflective of both lower diet digestibility (AD) and lower conversion efficiency of digested food (ECD). Low ECD values usually result from the presence of a toxin or from a nutrient imbalance (House 1974, Scriber & Slansky 1981). Fiber content will lower AD, but the magnitude of the reduction in the treatment groups appears too great to be explained by fiber alone. Digestibility reducing compounds such as tannins and protease inhibitors may also contribute to this low AD. The increased RCR’s caused by the higher concentrations of leaf powder diets are probably a response to lower diet digestibility. Herbivorous insects often increase consumption to compensate for low digestibility (Scriber & Slansky 1981; Brown 1975). Diet digestibility and ECD are not necessarily independent of one another. Factors that caused lower ECD values may have caused an even greater depression of ECD if they had been incorporated into a more digestible diet. The often observed inverse relationship between AD and ECD can be the result of homeostatic mechanisms (Reese & Beck 1976).

Imino compounds did not deter larvae from feeding, as evidenced by the CI values obtained. This is consistent with previous antifeedant assays conducted on other lepidopteran species (Romeo & Simmonds 1989). As expected, imino compounds had no

TABLE 4. GROWTH AND SURVIVAL OF SECOND GENERATION S. FRUGIPERDA LARVAE ON C. HAEMATOCEPHALA LEAF POWDER DIETS. EACH TREATMENT INITIALLY CONSISTED OF 20 LARVAE.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mortality</th>
<th>8 Day Wts (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>333</td>
</tr>
<tr>
<td>2.5 % leaf</td>
<td>20*</td>
<td>13*</td>
</tr>
<tr>
<td>5.0 % leaf</td>
<td>25*</td>
<td>17*</td>
</tr>
</tbody>
</table>
effect on diet digestibility, but the reduction of conversion efficiency of assimilated food (ECD) shown by two of them (trans-5-hydroxyphenolic acid and trans-trans-4,5-dihydroxyphenolic acid) is in accord with other studies on the nutritional effects of nonprotein amino acids (Reese & Beck 1976; Dahlan 1977). These compounds behave like physiological toxins and are apparently taken up from the gut into the hemolymph where they adversely affect metabolism. The lowered ECD could be due to the energetic cost of detoxification or to interference with normal amino acid metabolism. Scriber & Slansky (1981) point out the effects of amino acid imbalances on increased catabolism, slowed growth and reduced conversion efficiency. Since our individual imino compounds were fed at such low levels, there appears to be more than a mere amino acid imbalance involved.

The effects of individual imino compounds on growth, survival and diet usage were assayed at or below the levels at which they are found in plants (Rome 1984). The effects of individual amino acids on growth and nutritional indices were limited to a few cases. If the synergistic effects on both toxicity and deterrence, previously observed in aphids (Simmonds et al. 1988) also occur in Spodoptera, we would expect a greater growth depression and also a depression of the consumption index from the total amino acid fraction. These were not observed (Table 3). This appears then to be another example of varying effects of the same compounds on different test organisms, and re-emphasizes the importance of using caution in forming generalizations. An additional point for consideration is that combinations of toxic/deterrent amino acids may be less so when combined with the normal protein amino acids present. This is usually overlooked in designing bioassays.

The second generation effects suggest a fruitful area for further study. The observance of such pronounced negative effects on fecundity and growth of progeny, in organisms which survive the initial round of selection, indicates that the toxicity of allelochemicals is even more complex than usually envisioned. How many times have potentially important bioactive compounds been overlooked because they produced no results in initial screenings?

In answer to the question raised by the title of the paper, this study suggests that nonprotein imino acids are partially responsible for some of the observed antibiotic effects of Calliandra leaves. Specifically, the reduction in assimilation conversion efficiency (ECD) appears to have a nonprotein imino acid component. The energetic costs of processing Calliandra leaves are probably increased by the presence of these compounds. It is difficult, of course, to compare bioassay results of leaf powder experiments with those of individual compounds, since the biological activity of an allelochemical depends upon the environment in which it is encountered (Scriber & Slansky 1981). In this regard it is noteworthy that the aqueous fraction (which includes the amino acids) essentially stopped all larval growth. This provides evidence for the existence of other potent water soluble allelochemicals in Calliandra.

ACKNOWLEDGMENT

This material is based upon work supported by the National Science Foundation under grant no. BSR 8400277.

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