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JATROPHA GOSSYPIIFOLIA (EUPHORBIACEAE), A SOURCE OF PROTEINS TOXIC TO *PHENACOCCUS HERRENI* (STERNORRYNCHA: PSEUDOCOCCIDAE)

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Abstract

The cassava mealybug *Phenacoccus herreni* (Sternorryncha: Pseudococcidae) is a pest of cassava, *Manihot esculenta* Crantz (Euphorbiaceae), in South America. Proteins, representing direct gene products, are prime candidates in genetic engineering manipulations for host plant resistance. *Jatropha gossypiifolia* L. (Euphorbiaceae), a plant species known to contain proteins toxic to insects, exhibited insecticidal properties to *P. herreni*. The toxic compounds consisting of proteins around 101.02 kDa appeared to be mostly located in the mature leaves. Further studies are needed to identify the proteins and to ensure that they are not toxic to mammals.

Key Words: Colombia, Euphorbiaceae, cassava, Sternorrhyncha, Pseudococcidae, artificial diet, host-plant resistance, toxic proteins

Resumen

El piojo harinoso de la yuca, *Phenacoccus herreni* (Sternorrhyncha: Pseudococcidae) es una plaga de la cassava (yuca), Manihot esculenta Crantz (Euphorbiaceae), en América del Sur. Las proteínas, que representan los productos directos de genes, son los principales candidatos en la ingeniería genética para la manipulación de la resistencia en las plantas. Jatropha gossypiifolia L. (Euphorbiaceae), una especie de planta conocida de contener proteínas que son tóxicas para los insectos, presenta propiedades insecticidas hacia P. herreni. Los compuestos tóxicos consisten de proteínas alrededor de 101,02 kDa que parecen estar ubicadas principalmente en las hojas maduras. Se necesitan más estudios para identificar las proteínas y para asegurarse de que no son tóxicos para los mamíferos.

In South America, and especially in Northeast Brazil, the cassava mealybug, *Phenacoccus herreni* Cox & Williams (Sternorryncha: Pseudococcidae), is a pest of cassava, *Manihot esculenta* Crantz (Euphorbiaceae) (Cox & Williams 1981; Bellotti et al. 1983; Noronha 1990).

Host plant resistance is a sustainable management option. However, cassava cultivars from either Africa or Latin America have not shown promise for obtaining good levels of resistance (Bellotti et al. 1999). Wild species of *Manihot* and other closely related genera may offer a potential source of resistance genes for the control of cassava pests (Asiedu et al. 1992). The identification of resistance sources of wild relatives of cassava and other closely related genera as a source of pest resistant genes for genetic engineering in order to obtain transgenic cassavas resistant to pest insects is currently pursued by the International Centre for Tropical Agriculture (CIAT) in Cali, Colombia.

Complex multi-gene characters are not easily engineered. Proteins, representing direct gene products, are in general first considered in genetic engineering manipulations for crop improvement (Meeusen & Warren 1989; Boulter at al. 1990). To date, several genes encoding plant entomotoxic proteins have been introduced into crop genomes, and some of these plants are being tested in the field and are awaiting commercialization (Pefer-

oen 1997; Jouanin et al. 1998; Schuler et al. 1998; Hilder & Boulder 1999). Protein inhibitors (proteinase and α-amylase inhibitors), lectins and δ-endotoxin proteins are different classes of proteins considered as chemical defensive factors against pest insects in transgenic plants (Meeusen & Warren 1989; Murdock et al. 1989; Czapla & Lang 1990; Gatehouse et al. 1992; Habibi et al. 1993; Powell et al. 1993; Rahbé & Febvay 1993; Rahbé et al. 1995; Davidson et al. 1996; Valencia-Jiménez et al. 2000). Transgenic strategies constitute a proven technology for control of crop pest insects and can contribute to integrated pest management systems with a strong biological control component (Schuler et al. 1998; Romeis et al. 2006).

Nevertheless, none of the wild *Manihot* species available at the CIAT germplasm collection showed insecticidal properties to *P. herreni* (P.-A. Calatayud, IRD c/o CNRS, LEGS, Gif-sur-Yvette, France, unpublished). The bellyache bush, (*Jatropha gossypiifolia* L. [Euphorbiaceae]), native from tropical America and now widespread in tropics, is used for medicinal purposes in Africa, Thailand and tropical America. In Florida, it is cultivated as an ornamental tree. However, seeds are toxic to humans (Morton 1981; Dev & Koul 1997). Few insects have been observed to be associated with this plant species apart from a single whitefly species (N. Sauvion, INRA, UMR-BGPI, Campus International de Baillarguet, Montpellier, France, personal communication) and occasional infestations by thrips and mealybugs in fields (P.-A. Calatayud, IRD c/o CNRS, LEGS, Gif-sur-Yvette, France, unpublished). In addition, leaf extracts of this plant were shown to be toxic to *Tribolium castaneum* (Coleoptera: Tenebrionidae), *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and *Ostrinia nubilalis* (Hubner) (Lepidoptera: Pyralidae) (Dev & Koul 1997; Valencia-Jiménez et al. 2006).

The purpose of this work was to test *J. gossypiifolia* for insecticidal properties to *P. herreni,* and to characterize the type of compound(s) involved in the toxicity of the most toxic plant to *P. herreni*.

MATERIALS AND METHODS

Insects

A culture of *P. herreni* was maintained at CIAT on cassava (var. 'CMC 40') in a greenhouse at 27-33°C and L12:D12 photoperiod. Egg masses were collected and incubated at 24°C until eclosion. Newly hatched larvae were used in all experiments.

Plants

Jatropha gossypiifolia plants were used. The plants were obtained directly from the field germplasm collection at CIAT and propagated by stem cutting.

The plants were grown individually in a growth chamber in 15 L plastic pots containing a 75:25 peat-sand mixture. The plants were watered 3 times weekly and received a complete nutrient solution (NPK, 15-15-15) each mo. The environmental conditions were 30/21°C (day/night), relative humidity of 70-80%, and L12:D12 photoperiod. Five-month old plants were used in the experiments.

Artificial Infestation

For each plant, the second and the third mature leaf below the apex were each infested with 100 neonates. Each treatment was replicated 6 times. The middle part of each petiole was covered with pure petroleum jelly to prevent larval migration between leaves, plants and pots. For each plant, the percentage of larvae remaining was assessed at 15 days after infestation. The percentage of larvae remaining on a plant will be referred to as survival though it was not determined how many larvae emigrated or immigrated.

Plant-Organ Collection and Extraction Method

The seeds, the second mature and the first immature leaf below the apex were collected. Mature leaves and seeds of the cassava variety 'CMC 40' were also used as a non-toxic control sample. Prior to extraction, seeds or leaves were collected and frozen in a portable ice chest, and extraction was initiated within 30 min after harvesting, based on the method described by Valencia-Jiménez et al. (2000). The seeds or leaves were flash frozen in liquid nitrogen and then ground. The ground plant materials were extracted at 4°C with 4 volumes of 0.1 M sodium chloride solution (1 g of crushed material in 4 mL of sodium chloride solution) for 6 h. The slurry was filtered and centrifuged at 10,000 × g at 4°C during 20 min. The supernatant was retained and centrifuged again until no suspended material was observed. Then the supernatant was dialyzed against water for 3 days at low temperature (2-4°C) with a 3.5 kDa molecular weight dialysis cellulose membrane, and freeze-dried.

The resulting powder was stored at -20°C and used for toxicity experiments. Long-term dialysis was appropriate as it is assumed that most of the metabolic compounds (primary and secondary metabolites) were removed and only molecules with molecular weights >3.5 kDa, such as proteins, were retained.

Phago-Deterrence and Toxicity Tests

Except for sucrose, which was adjusted to 200 g/L, the control diet (herewith referred to as Ap3)

developed by Calatayud et al. (1998) as a derivative of A0 of Febvay et al. (1988) was used as standard medium. The deficiency in tyrosine, caused by its low solubility, was compensated for by adding extra phenylalanine at 17.86 µmol/mL. Based on Ap3, 10 mg of leaf or seed powder was incorporated into 1 mL of the artificial diet. KCN at the same concentration was also tested as a toxic control. For all diets the pH was adjusted to 7.5 with potassium hydroxide, and the medium was then filter-sterilized (0.22 µm Millipore units). The diet was enclosed in sterile Parafilm® sachets and stretched on the top of a standard film box (black; height, 5 cm; diam., 3.2 cm), which constituted the rearing unit.

Phago-Deterrence Test

Because non-preference is often linked to toxicity, phago-deterrence tests were first done. For each aforementioned diet, ten groups of 30-40 neonate larvae each were deposited in each rearing units with a single Whatman N°1 paper disc, which covered the entire bottom of the box to collect honeydew, according to the method of Calatayud et al (2002). A droplet of liquid diet was placed at the middle of the top of each rearing unit. The plane of the Parafilm® surface through which the mealybugs feed was oriented horizontally. Larvae were left overnight under dark conditions (12 h) after which the number of mealybugs feeding on the diet was counted; the honeydew droplets on the Whatman paper discs were stained using ninhydrin reagent (0.2% in N-butanol). The number of honeydew droplets was counted and their diameter (in µm) measured with a micrometer. The volumes of droplets (in nL) were estimated using a calibration curve [volume = 0.076 × diameter] according to Calatayud et al. (2002). The volume of diet ingested per mealybug estimated by the volume of honeydew excreted was calculated and expressed as nL/mealybug.

Toxicity Test

For each aforementioned diet, another eight groups of 50-60 neonate larvae each were placed in each experimental rearing units. After 4 d (the minimum time necessary to show plant extract toxicity in an artificial diet), the percentage of larvae surviving on each diet was calculated.

In addition, to assess thermolability of the compounds, the plant extracts were boiled before addition to the artificial diet; toxicity was tested at the same concentration and experimental conditions as above.

All tests were conducted at 24°C, 80% R.H. and L12:D12 photoperiod.

Evidence of Protein(s) Involved in the Toxicity

To evidence the involvement of protein(s) in plant toxicity to *P. herreni* the following procedure was used. From the plant organs that caused the highest mortality in toxicity tests using artificial diets (see results of Table 3), an extraction procedure similar to the above procedure was used, except that the supernatant obtained before dialysis was directly used. The subsequent purification steps were carried out at 4°C. Sufficient solid ammonium sulfate was added slowly to the supernatant by continuous stirring to achieve 20% saturation. The suspension was kept at 4°C for 6 h, followed by centrifugation at 10,000 g at 4°C for 30 min, and the pellet was discarded. Using the same procedure, solid ammonium sulfate was added to the supernatant to obtain, sequentially, 20% increments of ammonium sulfate fractionation. The precipitated proteins obtained at 20-40, 40-60, 60-80 and 80-100% ammonium sulfate saturation were resuspended in 50 mL of 0.1 M sodium chloride solution and dialyzed for 3 days at low temperature (2-4°C) with molecular weight cut-off (MWCO) 3.5 kDa cellulose membrane against water and freeze-dried. Each fraction was tested for toxicity to *P. herreni* over 4 successive days using a similar toxicity test procedure.

Thereafter, from the most toxic fraction, the proteins were separated using native gel electrophoresis. Ten (10) mg of protein was separated on a slab gel (16 × 14 cm, 1 mm thick) containing 4-14% polyacrylamide. Electrophoresis was run for 12 h at 4°C, 7 mA, with molecular weight standards (20.42-194.24 kDa, BioRad). The gel was then stained in 0.01% (w/v) Coomassie blue stain R250 (BioRad), 0.5% (v/v) methanol, 0.1% (v/v) acetic acid solution for 2 h.

Statistical Analysis

Statistical tests were performed with Statview software (Abacus Concepts, USA). The volume of diet ingested and percent insect survival were log and arcsin transformed, respectively. Untransformed results are presented in the tables and in Fig. 1. All means were separated by Tukey's HSD test following one-way analysis of variance (ANOVA).

RESULTS

Jatropha gossypiifolia leaves yielded 5% and 0% of survival only after 24 and 48 hours, respectively.

The extracts tested in Table 1 influenced significantly the volume of diet ingested by *P. herreni* neonates within the first 12 h of feeding (ANOVA: $F_{5,54} = 18.9$, $P < 0.0001$). However, only mature leaf extracts reduced feeding of *P. herreni*, and the reduction was similar to that by KCN, which served as a
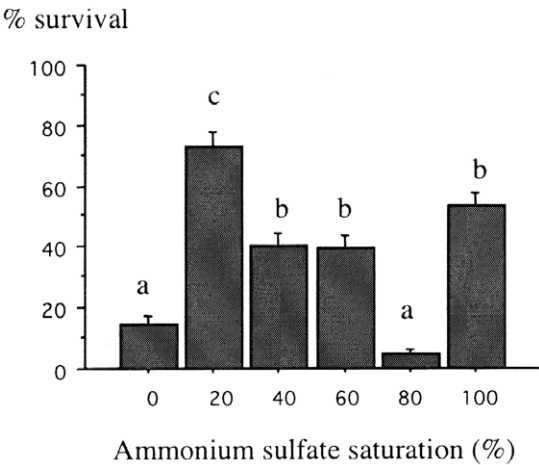
% survival

Fig. 1. Ammonium sulfate fractionation. The extract obtained from *Jatropha gossypiifolia* mature leaves was subjected to ammonium sulfate fractionation. Data represent percent survival (mean ± SE, n = 10) of *Phenacoccus herreni* obtained with each precipitated fraction after 4 days. Bars with different letters are significantly different at 5% level (Tukey's HSD test following ANOVA).

positive control for toxicity. The volume of diet ingested by *P. herreni* was significantly higher with Ap3 alone as compared to the diets containing plant extracts. No significant difference was observed between extracts of young leaves, seeds of *J. gossypiifolia*, and CMC40 leaf extracts.

The extracts significantly influenced the survival of *P. herreni* larvae after 4 days of feeding (Table 2) (ANOVA: $F_{7,56}$ = 59.7, P < 0.0001). Diets including either extracts of mature leaves or seeds in the Ap3 diet caused toxicity to *P. herreni*, which, however, was not as high as with KCN. By contrast, survival was significantly higher with Ap3 alone and with leaves of 'CMC 40' as well as young leaves of *J. gossypiifolia*. Boiling the extracts of mature leaves and seeds of *J. gossypiifolia* increased *P. herreni* survival significantly (Table 2), and survival was greater with boiled extracts of mature leaves than seeds.

Both phago-deterrence and toxicity tests showed that the active compound(s) involved in toxicity of *J. gossypiifolia* was mostly associated with mature leaves. Therefore subsequent preliminary purification was done with mature leaves.

Ammonium sulfate fractionation of the extract indicated the presence of toxicity toward *P. herreni*, mostly in the 80% fraction after 4 d of bioassay (Fig. 1) (ANOVA: $F_{5,54}$ = 40.2, P < 0.0001). The toxicity of this fraction was similar to the control (0% fraction = extract without precipitation treatment). The precipitated proteins of this fraction were separated using native gel electrophoresis (Fig. 2). The electrophoresis profile showed the presence of proteins from 21.36 to 188.05 kDa. Among them, the proteins around 101.02 kDa appeared to be the most abundant.

Discussion

Jatropha gossypiifolia was shown to be highly toxic to *P. herreni*. The findings indicate that the active compound(s) of *J. gossypiifolia* involved in the toxicity to *P. herreni* were mostly located in mature leaves. As generally observed with plant toxins (Schoonhoven et al. 1998), the toxic compound(s) were also phago-deterrent to *P. herreni*. The presence of several secondary compounds in *J. gossypiifolia* leaves, including flavonoids (e.g. apigenin, isovitexin, vitexin) and diterpenoids (e.g. jatrophone), and their implication in animal toxicity, were determined by Kupchan et al. (1970) and Subramanian et al. (1971). However, these compounds are generally not water soluble and are thus not identical to the compounds extracted from the leaves in our study. In addition, these molecules possess molecular weights lower than 3.5 kDa and would have been removed during the dialysis. Only molecules with molecular weight greater than 3.5 kDa could therefore be involved in the toxicity observed in the present study. Also, Euphorbiaceae plants are known to possess polyisoprenes in the form of latex with high molecular weights (Archer 1980). Such compounds are mostly soluble in organic solvents such as benzene and chloroform. Their presence in the leaf extract can also be ruled out. There-

TABLE 1. VOLUMES OF DIET INGESTED PER NEONATE LARVA OF *PHENACOCCUS HERRENI* (IN NL/MEALYBUG, MEAN* ± SE, N = 10) AFTER 12 HOURS ON DIFFERENT ARTIFICIAL DIETS.

Diet	Volume ingested
Ap3 alone (control)	109.0 ± 12.5 b
Ap3 with 'CMC 40' leaves extract	40.5 ± 7.4 b
Ap3 with KCN (toxic control)	6.4 ± 1.5 a
Ap3 with *J. gossypiifolia* young leaves extract	32.6 ± 5.6 b
Ap3 with *J. gossypiifolia* mature leaves extract	4.7 ± 1.6 a
Ap3 with *J. gossypiifolia* seeds extract	21.8 ± 11.9 b

*Means followed by different letters are significantly different at 5% level (Tukey's HSD test following ANOVA).

TABLE 2. SURVIVAL OF *PHENACOCCUS HERRENI* (MEAN* ± SE, N = 8) LARVAE REARED DURING 4 DAYS ON DIFFERENT ARTIFICIAL DIETS.

Diet	% survival
Ap3 alone (control)	93.0 ± 3.1 c
Ap3 with 'CMC 40' leaves extract	90.1 ± 3.1 c
Ap3 with KCN (toxic control)	0 a
Ap3 with *J. gossypiifolia* young leaves extract	88.4 ± 2.6 c
Ap3 with *J. gossypiifolia* mature leaves extract	17.2 ± 4.9 a
Ap3 with *J. gossypiifolia* seeds extract	18.7 ± 4.3 a
Ap3 with *J. gossypiifolia* mature leaves extract previously boiled	87.0 ± 2.3 c
Ap3 with *J. gossypiifolia* seeds extract previously boiled	58.9 ± 5.7 b

*Means followed by different letters are significantly different at 5% level (Tukey's HSD test following ANOVA).
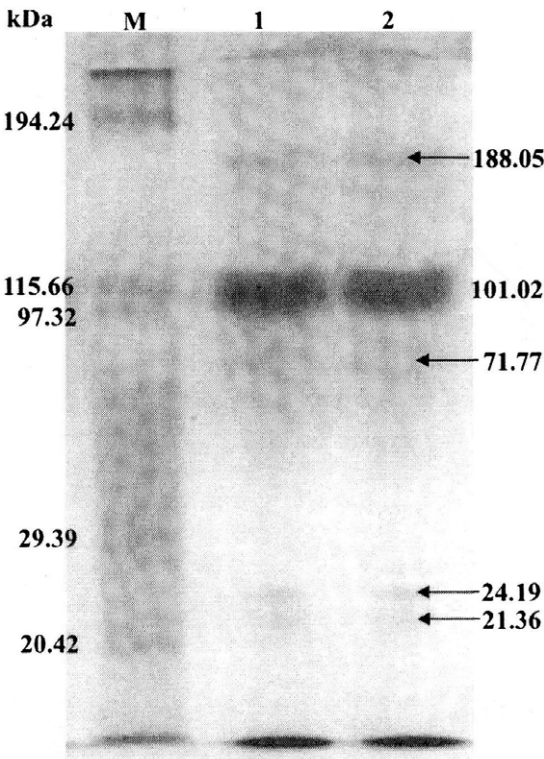

Fig. 2. Electrophoresis separation of the precipitated proteins obtained from the 80% ammonium sulfate saturation (lanes 1 and 2). Molecular weights (M) are indicated in kDa. Arrows on the right indicate approximate molecular weights of 21.36, 24.19, 71.77, 101.02 and 188.05 kDa from bottom to top.

fore, the compounds involved in the toxicity to *P. herreni* are most likely proteins or peptides since they were thermo-labile and were precipitated by ammonium sulfate fractionation, and had molecular weights greater than 3.5.

A toxic protein designated as "curcin" with hemagglutinating activity (Lin et al. 2010), was iso-lated from the seeds of *Jatropha curcas* L. (Euphorbiaceae) for the first time by Felke (1914). Curcin is known to be toxic and may be present in our leaf extract of *J. gossypiifolia*. However, this protein was reported by Barbieri et al. (1993) as a type I Ribosome-Inactivating Protein (RIP), consisting of a single polypeptide chain with molecular weight of 28-35 kDa. In this study, no bands were observed between 28 to 35 kDa in the precipitated proteins obtained from the 80% ammonium sulfate saturation and the most abundant proteins were approximately 101 kDa.

Although additional purification steps are necessary to identify the toxic protein(s), this study indicated that proteins in mature leaves of *J. gossypiifolia,* most probably around 101 kDa, are involved in the toxicity to *P. herreni*. Moreover, they are also toxic to another cassava mealybug species, *P. manihoti* Matile-Ferrero (Sternorrhyncha, Pseudococcidae), where the same precipitated proteins obtained from the 80% ammonium sulfate saturation induced around 90% mortality after 4 days (P.-A. Calatayud, IRD c/o CNRS, LEGS, Gif-sur-Yvette, France, unpublished). These proteins can therefore be considered as important factors in host plant resistance programs against cassava mealybugs and this plant can be therefore considered as a source of pest resistant genes for developing a GMO cassava. Nevertheless, additional work is required to identify the proteins involved in the toxicity, as well as to ensure that they are not toxic to mammals.

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