RESISTANCE OF CITRUS ROOTSTOCKS AND *GLYCOSMIS PENTAPHYLLA* AGAINST LARVAL *DIAPREPES ABBREVIATUS* (COLEOPTERA: CURCULIONIDAE) IN LIVE ROOT OR DIET-INCORPORATION ASSAYS

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ABSTRACTS

The growth of larval *Diaprepes abbreviatus* L. was measured after rearing them on roots of rutaceous seedlings for 35 or 42 days. Larvae were fed on seedlings of two common citrus rootstocks, two new hybrids that are under development as rootstocks, and one citrus relative. Live weights of larvae reared on Carrizo or Swingle rootstocks for 42 days increased an average of 10.3- and 10.2-fold, respectively; weight increases on the citrus hybrids HRS-802 and HRS-896 for 35 days averaged 7.6- and 6.1-fold, respectively; and weight increase on *Glycosmis pentaphylla* Retzius for 42 days averaged 2.5-fold. A bioassay to test for potential phytochemical sources of resistance against the larvae was developed by incorporating finely milled roots into larval diet. Milled root samples were incorporated into a standard semi-defined diet at 5% concentrations (w/v), and growth of larval weevils was recorded following a 32-day feeding period. Roots collected from uninfested control seedlings in the previous experiment were used. On diet containing no roots, mean larval weight increased 16.8-fold, while weights increased 13.9-fold on diet containing roots of Carrizo, 12.0-fold on Swingle, 15.1-fold on HRS-802, 12.3-fold on HRS-896, and only 5.5-fold on *G. pentaphylla*. Both tests indicate that *G. pentaphylla* may represent a source of root resistance to *D. abbreviatus*, and the diet-incorporation tests indicate potential phytochemical or microbial sources of resistance.

Key Words: *Diaprepes abbreviatus*, citrus root weevil, rootstock resistance, larval growth, diet-incorporation assay

RESUMEN

Fue medido el crecimiento de larvas de *Diaprepes abbreviatus* L. criadas en raíces de plántulas de rutáceas durante 35 o 42 días. Las larvas fueron alimentadas en dos patrones comunes de cítricos, dos nuevos híbridos que están bajo desarrollo como patrones, y un pariente de los cítricos. El crecimiento en los patrones Carrizo o Swingle durante 42 días promedió 10.3 y 10.2 veces, respectivamente; el crecimiento sobre los híbridos HRS-802 y HRS-896 durante 35 días promedió 7.6 y 6.1 veces, respectimamete, y el crecimiento en *Glycosmis pentaphylla* durante 42 días promedió 2.5 veces. Se desarrolló un bioensayo para probar fuentes potenciales fitoquímicas de resistencia contra la larva mediante la incorporación de raíces finamente molidas a la dieta larval. Las muestras de raíces molidas fueron incorporadas a una dieta estándar semidefinida a concentraciones del 5% (peso/volumen), y el peso de las larvas fue registrado siguiendo un período de 32 días. Fueron usasas las raíces colectadas de las posturas control en el experimento previo. En una dieta sin raíces, el peso medio larval aumentó 16.8 veces, mientras que los pesos aumentaron 13.9, 12.0, 12.3 y 5.5 veces sobre la dieta que contenía raíces de Carrizo, Swingle, HRS-802, HRS-896 y *G. pentaphylla*, respectivamente. Ambos ensayos indicaron que *G. pentaphylla* puede ser una fuente de resistencia radicular hacia *D. abbreviatus*, y que los ensayos de incorporación a las dietas sirven para indicar fuentes fitoquímicas o microbianas de resistencia.

Cultivated varieties of crop plants are routinely evaluated for resistance of foliage or fruit to insect feeding and damage (Smith 1989, Panda & Khush 1995). Evaluating insect resistance in roots is less common, however, especially for long-lived horticultural crops such as citrus. In Florida, the citrus root weevil *Diaprepes abbreviatus* L. (Coleoptera: Curculionidae) causes most of its damage to citrus trees during the larval stage while feeding on the root system of the rootstock. There, larvae strip bark from the roots of the tree, weakening and eventually killing the root once its circumference is fully girdled. We recently reported results from a whole-plant test designed to compare root damage, larval survival, and larval growth among citrus rootstock cultivars, hybrids, and other species of citrus relatives (Shapiro & Gottwald 1995).

The phytochemical composition of citrus roots has been well studied (Shapiro 1991; Nordby & Nagy 1981, Gray & Waterman 1978). Structural characterizations of numerous coumarins, alkaloids, flavonoids, and limonoids from citrus roots have been reported, but with few references to biological activities. These classes of phytochemicals include many examples of defensive compounds. To discover whether such compounds impart any resistance against *Diaprepes*, roots must first be screened for biological activity, then the chemical source of an activity must be identified. A successful bioassay should enable rapid tests of small quantities of root material from a large number of samples. A whole-plant assay has already been developed (Shapiro & Gottwald 1995). Of eight commercial rootstocks and new hybrids that were tested in that assay, only one - Swingle - showed some resistance, in contrast to an earlier study (Beavers & Hutchison 1985). This observation was based on three parameters measured in the bioassay: larval weight gain, larval mortality, and root damage relative to uninfested control plants. The last parameter was measured by root volume, root weight, and by digitally integrating the visible areas of roots from photographs, all of which correlated well.

To enable the identification of roots that deter larval growth and of the active phytochemicals extracted from them, we have designed a simple diet-incorporation assay, and here compare it with our routine whole-plant assay for resistance to *Diaprepes* larvae (Shapiro & Gottwald 1995).

Materials and Methods

Insects

Larvae were obtained from a weevil colony maintained in isolation on a semi-defined diet for over 6 yr, with only occasional infusion of adult weevils from citrus groves located near Lake Jem in central Florida and in Homestead, Florida. Larvae from field-collected adults were added once or twice each year and comprised no more than 20% of the larvae in the colony. Larvae at one month of age were taken from the colony and individually weighed. Groups of ten were selected at average weights of 25-30 mg/group for placement on seedlings with one group of ten per seedling.

Plants

Seeds for rootstocks or hybrids were obtained from germplasm grown at the US Horticultural Research Laboratory (USHRL) Foundation Farm in Leesburg, FL, and for *G. pentaphylla* from the Florida Division of Plant Industry Arboretum in Winter Haven, FL. Test seedling plants were grown from seed at the USHRL Foundation Farm and transferred for weevil challenges to the USHRL greenhouses in Orlando, FL.

Seedling Challenges

Challenges were conducted as described by Shapiro and Gottwald (1995), except that the duration of those tests was 44 days. The starting weight of each *Diaprepes* larva was taken prior to placing ten larvae on each plant. Larvae were placed at 8 cm depth in the soil, evenly distributed midway between the trunk of a seedling and the circumference of the 1-gal pot. Each test consisted of seven replicates of each cultivar, one plant per replicate. Larvae were allowed to feed in the greenhouse on the roots of 5 selections including two widely used commercial rootstocks (Carrizo and Swingle; 42 days, from June 11 to July 23, 1996), one citrus relative, *G. pentaphylla* (42 days, from June 13 to July 25, 1996), and two citrus hybrids that are in final stages of testing as rootstocks (HRS-802 and HRS-896; 35 days, from May 22 to June 26, 1996). Seedlings were removed from pots, larvae were recovered, and survival rates and the live weight of each surviving larva were recorded.

Diet-Incorporation Tests

Roots for diet-incorporation assays were obtained from uninfested seedlings collected from seedling challenge experiments. Following storage at -80°C, roots were milled in a centrifugal mill (Retsch ZM-1000, Brinkmann, Westbury, NY) at 10,000 or 15,000 rpm to < 0.5-mm particle size. Diet for incorporation was prepared by first adding 14 g agar to approximately 800 ml water, and heating to approximately 100°C while mixing with a Braun (Lynnfield, MA) type 4169 hand-held homogenizer. As the agar cooled, 184 g of citrus root weevil diet premix #1675F (Bio-Serve, Frenchtown, NJ), which is used for routine rearing, was added. The mixture was thoroughly mixed and diluted to 1 L with water. Diet was distributed to 100-ml beakers, and 5 g of roots were blended with 100 ml of diet when diet had cooled to a temperature of approximately 50°C, the melting point of the agar used in the diet. Approximately 15 ml of diet were rapidly poured into each plastic 30-ml shot cup (Jet Plastica, Hatfield, PA), allowed to cool, and dried for approximately 6 h under a laminar flow hood. Controls consisted of diet only with no roots added. One larva was added to each of 30 cups of diet per treatment, cups were covered, and larvae were allowed to feed for 32 days in the insectary at an approximate temperature of 29°C under a light regime of 10:14 (L:D). Larvae were then separated from the diet and individually weighed for final weights.

Statistics

One-way ANOVA and post-hoc comparison of means (Tukey's HSD) tests were performed using the Statistica (StatSoft 1995) version 5.0 Basic Statistics module.

Results and Discussion

Larvae that fed for 35 days on roots of the two hybrid selections, HRS-802 and HRS-896, increased 7.6- and 6.1-fold over their initial live weight, to 222 and 184 mg, respectively (Table 1). Those that were fed for 42 days on the two commercial rootstocks, Carrizo and Swingle, increased 10.3- and 10.2-fold over their initial live weight, to 229 and 236 mg, respectively. In contrast, larvae that were fed on roots of *G. pentaphylla* for 42 days increased only 2.5-fold over their initial live weight, to a mean final weight of only 64 mg. There were no significant differences in mean final weights among larvae grown on Carrizo, Swingle, and HRS-802. Larvae on HRS-896 weighed significantly less than those on Swingle and Carrizo, although seven more

TABLE 1. CHANGE IN LARVAL WEIGHT AND SURVIVAL OF LARVAE AFTER FEEDING 35 DAYS (HRS-802 AND HRS-896) OR 42 DAYS (CARIZZO, SWINGLE, OR *G. PENTAPHYLLA*) ON ROOTS OF SEEDLING ROOTSTOCKS OR *G. PENTAPHYLLA*.

	Mean Weight (mg ± SD)			Survival[3]	
	Starting[1]	Final[2]	Weight Change (mg)	N	%
Swingle	23.0 ± 1.8b	235.5 ± 54.5a	212.0 ± 53.5a	60	86 ± 13a
Carrizo	22.3 ± 1.2b	228.8 ± 28.6ab	206.5 ± 28.7a	58	83 ± 14a
HRS-802	25.7 ± 1.0a	221.9 ± 28.2ab	196.2 ± 28.0ab	67	96 ± 5a
HRS-896	25.9 ± 1.2a	184.2 ± 14.0b	158.3 ± 13.7b	61	87 ± 10a
G. pentaphylla	26.1 ± 2.0a	64.3 ± 14.1c	38.2 ± 13.3c	40	57 ± 14b

[1]Mean ± SD (N = 7) of the starting weights of larvae placed 10 per plant on each of 7 plants per cultivar.
[2]Mean ± SD (N = 7) of the final weights of larvae surviving on each of 7 plants per cultivar.
[3]Number and mean percentage ± SD (N = 7) of the larvae that survived on each of 7 plants per cultivar.
Figures that are followed by the same letter within a column are not significantly different ($P < 0.05$; ANOVA followed by Tukey's HSD test).

days of feeding (42 days total) would have reduced this difference. However, larval weight gain on all four cultivars was significantly different from the mean final weight of the larvae fed on *G. pentaphylla* roots for 42 days. This was despite the fact that starting weights of the larvae on HRS-802, HRS-896, and *G. pentaphylla* were significantly, though only slightly, greater than the larvae placed on Swingle or Carrizo.

In diet-incorporation assays, larvae were fed for 32 days on the standard rearing diet with or without milled roots incorporated into it (Table 2). Changes in mean weights of larvae fed on diet alone were greater than those fed on live roots. Larval weights increased 16.8-fold on diet only, 13.9-fold on Carrizo, 12.0-fold on Swingle, 15.1-fold on HRS-802, 12.2-fold on HRS-896, and 5.5-fold on *G. pentaphylla*. Mean final weights of all larvae except those fed on HRS-802 were significantly lower than those fed on diet alone. Final weights of larvae fed on *G. pentaphylla* were significantly lower than those of larvae fed either on diet alone or on diet with any of the other root selections incorporated. In two additional diet-incorporation assays, larvae fed on *G. pentaphylla* also gained significantly less weight than larvae on Swingle or Carrizo (unreported results).

The results are significant from two perspectives. First, *G. pentaphylla* roots supported only very low growth rates, roughly one-third to one-fourth of those supported by the other root systems. Previous results (Shapiro & Gottwald 1995) showed growth rates on Swingle, Carrizo, and six other cultivars that were 2.8- to 5-fold greater than the growth rate on *G. pentaphylla* in this study. On diet that incorporated milled roots, larval growth rates on *G. pentaphylla* were approximately one-half to one-third as high as on the other selections. Secondly, results from the diet-incorporation assay mirrored those with live root tests. The comparison between larval growth rates on live roots and growth on roots incorporated into diet is striking and repeatable.

These tests highlight the usefulness of the diet-incorporation assay as a possible substitute for tests on live plants. Not only does the diet-incorporation assay require only a fraction of a plant's total root system, but roots can be stored indefinitely at -80°C for repeated tests whenever desired. Roots from any size of tree can also be readily collected from the field and tested without destroying the tree. This will allow comparable and parallel tests to be run on seedling, juvenile, and mature trees together. Effects of resistance discovered in seedlings can thereby be compared to the potential

TABLE 2. CHANGE IN LARVAL WEIGHT AFTER FEEDING 32 DAYS ON 5 G MILLED ROOTS ADDED TO 100 ML DIET.

	Weight (mg ± SD)[1]		
	Starting	Final	Change
Diet only[2]	23.2 ± 4.7a	390.0 ± 99.4a	366.8 ± 98.4a
Swingle	22.2 ± 4.5a	266.3 ± 116.8b	244.1 ± 115.3b
Carrizo	22.6 ± 4.4a	315.2 ± 103.2b	292.6 ± 102.2b
HRS-802	21.5 ± 4.6a	325.0 ± 86.8ab	303.5 ± 87.3ab
HRS-896	23.1 ± 4.6a	281.4 ± 112.3b	258.3 ± 112.0b
G. pentaphylla	20.6 ± 4.0a	113.9 ± 41.8c	93.3 ± 40.6c

[1]Mean ± SD (N=30) of the weights of 30 larvae, all of which survived. Figures that are followed by the same letter within a column are not significantly different ($P < 0.05$; ANOVA followed by Tukey's HSD test).

[2]Cups with 'diet only' contained no ground roots.

for resistance in other stages of tree grown from similar germplasm. Roots from plant species entirely unrelated to citrus can also be tested for their effect on growth and survival of *Diaprepes* larvae.

The diet-incorporation assay will also be very useful for further studies on chemical or biological constituents of roots that may be responsible for inhibited larval growth. Present results indicate a molecular or microbial source of larval growth inhibition. In the diet-incorporation test, finely milled roots of *G. pentaphylla* at only 5% concentration in diet produced the same relative growth inhibition seen in whole live roots. This low requisite concentration of roots will reduce the time and sample size required to identify active molecules or microbes. Although caveats in the use of diet assays for identification of active compounds have been examined and discussed (Shapiro, 1992), this assay affords a powerful tool for identification of active root constituents.

In the current search for active biochemical factors in citrus for defense of rootstocks against *Diaprepes*, we have focused primarily on natural products that are either small chemical constituents (Shapiro et al., 1988; Shapiro, 1991) or macromolecules such as defense-related proteins (Mayer et al., 1995; McCollum et al., 1995). Our discovery of growth-inhibiting activity in *G. pentaphylla* and the development of a bioassay to examine that activity should contribute to finding or developing a rootstock with resistance to the weevil.

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