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Response of *Tribolium castaneum* (Coleoptera, Tenebrionidae) to *Salpichroa origanifolia* Withanolides

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Biological effects on *Tribolium castaneum* larvae were evaluated for three withanolides isolated from *Salpichroa origanifolia* (Solanaceae), (20*S*,22*R*,24*S*,25*S*,26*R*)-5α,6α:22,26:24,25-triepoxy-26-hydroxy-17(13→18)-*abeo*-ergosta-2,13,15,17-tetraen-1-one (salpichrolide A, **1**), (20*S*,22*R*,24*S*,25*S*,26*R*)-22,26:24,25-diepoxy-5α,6*β*,26-trihydroxy-17(13→18)-*abeo*-ergosta-2,13,15,17-tetraen-1-one (salpichrolide C, **2**), and (20*S*,22*R*,24*S*,25*S*,26*R*)-5α,6α:22,26:24,25-triepoxy-15,26-dihydroxy-17(13→18)-*abeo*-ergosta-2,13,15,17-tetraen-1-one (salpichrolide G, **3**), and for several chemically modified analogues. The compounds were incorporated into the larval diet at concentrations of 500 and 2000 ppm. Salpichrolide C (**2**) produced a significant delay in the development of neonate larvae to adults at the highest concentration (2000 ppm); development delays and lethal effects were produced by salpichrolides A (**1**) and G (**3**) at both concentrations assayed. The size of surviving adults was used as a criterion for assessing feedant deterrent effects; the results suggest that these compounds act as feeding inhibitors. Influence of chemical modifications in development delay was analyzed.

KEYWORDS: ***Salpichroa origanifolia*; withanolides; natural insecticides; *Tribolium castaneum*; feeding deterrents**

INTRODUCTION

During coevolution, plants have evolved different kinds of special metabolites that act as chemical defenses against phytophagous insects. Many of these compounds do not have a direct lethal effect but act by deterring herbivores (*1*). This has been observed for some Solanaceae species, from which a group of specialized metabolites, the withanolides, has been isolated (*2*). Some of them exhibit activity as feeding deterrents (*3*−*5*) or ecdysteroid antagonists (*6*), and they have been related to chemical defense mechanisms (*7*). *Salpichroa origanifolia* is a member of the Solanoideae characterized by the presence of withanolides with a six-membered aromatic ring D, salpichrolides A (**1**), C (**2**), and G (**3**) being the major components (*8*−*11*). In a previous publication we demonstrated the feeding inhibition of compounds **1**−**3** and a few synthetic analogues against *Musca domestica* larvae (Diptera, Muscidae) (*12*). In this work we studied the biological effects of these compounds against a stored grain pest, *Tribolium castaneum*, to determine development delays and reduction in surviving adult size.

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MATERIALS AND METHODS

Insects. Bioassays were performed on *T. castaneum*, CIPEIN strain, obtained from a colony reared in the laboratory since 1983. Larvae were maintained at 25 ± 1 °C, 70 ± 5% relative humidity, and a 12:12 h photoperiod on a wheat flour, beer yeast, and cornstarch diet (10/1.5/10) (*13*).

Test Compounds. Aerial parts of *S. origanifolia* were collected in the surroundings of the university campus in Buenos Aires, Argentina. A voucher specimen has been deposited at the Museo Botánico, Universidad Nacional de Córdoba, Argentina, no. CORD 89. Salpichrolides A (**1**), C (**2**), and G (**3**) were isolated from fresh leaves and stems of *S. origanifolia* (*11*). Compounds **4**−**7** were obtained as previously described (*12*). Prior to biological testing, all compounds were tested by TLC on Silica Gel 60 F254 (Merck) plates using hexanes/EtOAc (4:1, 1:1, or 2:3 v/v) as mobile phase. Spots were visualized by spraying 10% H_2SO_4 in EtOH and heating. A purity >95%, as verified by ^{1}H NMR spectroscopy, was considered to be acceptable.

Bioassays. Groups of 10 newly hatched larvae (1−2 days old) were reared on an artificial diet into which the test compounds had been incorporated. This diet was prepared with wheat flour of commercial use that had been mixed with solutions of the test compounds in acetone to obtain final concentrations of 500 and 2000 ppm. Control larvae were exposed to flour treated only with acetone. All treatments were assayed in triplicate. Larvae were kept as described above. The numbers of larvae, pupae, and adults were recorded every 20 days, the percentage of individuals (as larvae, pupae, and adults) was calculated in relation

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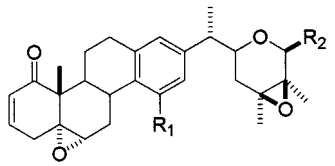

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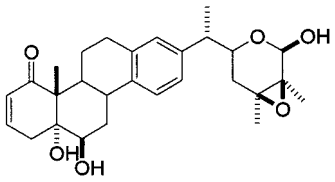
3 R_1 = OH, R_2 = OH
4 R_1 = H, R_2 = OAc



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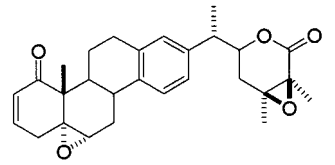

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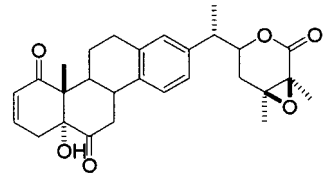

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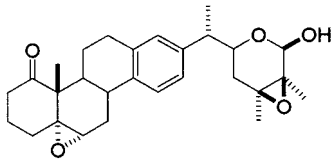

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to the total number of individuals found in each treatment. The length of individuals was determined in comparison to the control treated only with acetone. The size of individuals that reached the adult stage was recorded using a Nikkon SM 22 stereoscopic microscope. Significant reduction in the adult size of individuals emerged from exposed larvae and was considered as a criterion of feeding inhibition.

Statistical Analysis. Data of larvae, pupae, and adult numbers registered at 40, 60, and 80 days and data of adult size were subjected to analysis of variance (block design ANOVA) followed by a Tukey multiple-range test. Adult numbers at 40, 60, 80, 100, 120, and 140 days, expressed as a percentage in relation to the surviving individual numbers, were used to calculate by Probit analysis (*14*) the parameter DT_{50} (development time 50 or the necessary time to reach the adult stage for 50% of the exposed larvae). Significant development delay was assessed by no superposition of fiducial limits between DT_{50} of treated larvae and controls.

RESULTS AND DISCUSSION

Figure 1 shows the average percentages of larvae, pupae, and adults at 40, 60, and 80 days, respectively, after continuous exposure to salpichrolides A (**1**), C (**2**), and G (**3**). At 40 days (Figure 1a), the major percentage of control insects reached the fifth larval instar (50%), whereas 26.7% reached the sixth larval instar, 6.7% the fourth instar, and 6.7% the pupal stage. No pupae were observed in any of the treatments. Sixth-instar larvae were not found in treatments with compound **1** (500 ppm), and very low percentages appeared in treatments with **2** (500 ppm) and **3** (500 ppm). At the highest concentration (2000 ppm) neither pupae nor sixth-stage larvae were found for the treatments with compounds **1**−**3**. Evaluation after 60 days (Figure 1b) showed a development delay in all treatments, except for compound **2** at 500 ppm. At 80 days (Figure 1c) the adult percentage was significantly lower in all of the treatments, with the lowest percentage corresponding to treatments with **1** (2000 ppm) and **3** (2000 ppm).

Table 1. Development Time for *T. castaneum* Larvae Exposed to Natural Salpichrolides **1**–**3**

treatment[a]	concn (ppm)	DT_{50}[b] (95% FL) days
control		57.3 (52.5–60.8)
salpichrolide A (**1**)	500	70.7 (68.3–73.0)
	2000	85.9 (81.5–90.4)
salpichrolide C (**2**)	500	54.6 (49.1–58.1)
	2000	87.0 (82.0–92.2)
salpichrolide G (**3**)	500	69.1 (61.9–74.6)
	2000	106.6 (101.8–112.6)

[a] Groups of newly hatched larvae ($n = 30$) were reared on wheat flour treated with the different compounds under study. [b] DT_{50} indicates the necessary time to reach adult stage of 50% of the exposed larvae. Development delay was established as no superposition of fiducial limits.

Table 2. Development Time for *T. castaneum* Larvae Exposed to Modified Salpichrolides **4**–**7**

treatment[a]	DT_{50}[b] (95% FL) days
control	51.0 (43.1–55.7)
salpichrolide A (**1**)	63.6 (58.1–67.8)
4	54.4 (48.9–57.8)
5	54.0 (48.3–57.5)
6	57.9 (54.3–60.5)
7	64.2 (62.0–66.3)

[a] Groups of newly hatched larvae ($n = 30$) were reared on wheat flour treated with the different compounds evaluated to a final concentration of 500 ppm. Salpichrolide A (**1**) was included as a positive control. [b] DT_{50} indicates the necessary time to reach adult stage of 50% of the exposed larvae. Development delay was established as no superposition of fiducial limits.

Adult number, expressed as a percentage in relation to the surviving individual number, at the six different observation times (40−140 days), was used to calculate DT_{50} values according to Probit analysis (Table 1). Significant development delays from larva to adult were observed in treatments with salpichrolide C (**2**) at 2000 ppm and with salpichrolides A (**1**) and G (**3**) at 500 ppm and higher concentrations (Table 1). The latter two compounds also produced higher mortality and higher failures in adult emergence. These results are parallel to those previously obtained on *Musca domestica* larvae (*12*), in which salpichrolide A (**1**) showed the greatest development delay. However, we did not observe a development delay with compound **2** at 500 ppm. The different responses may be explained by species-specific detoxification mechanisms.

It is interesting to relate the concentrations at which these compounds are active with those actually present in the plant. Thus, the total withanolide content of *S. origanifolia* on a fresh weight basis ranges from ∼500 ppm (in winter) to >3000 ppm (in summer) in both leaves and stems (salpichrolide A accounts for ∼85% of the total) and 450 ppm in the fruits (*12*).

We also assayed derivatives **4** and **5**, with modifications on the side chain of salpichrolide A, the oxidized derivative of salpichrolide C (**6**), and the ring A-reduced analogue **7**. The number of adults in treatments with modified salpichrolides

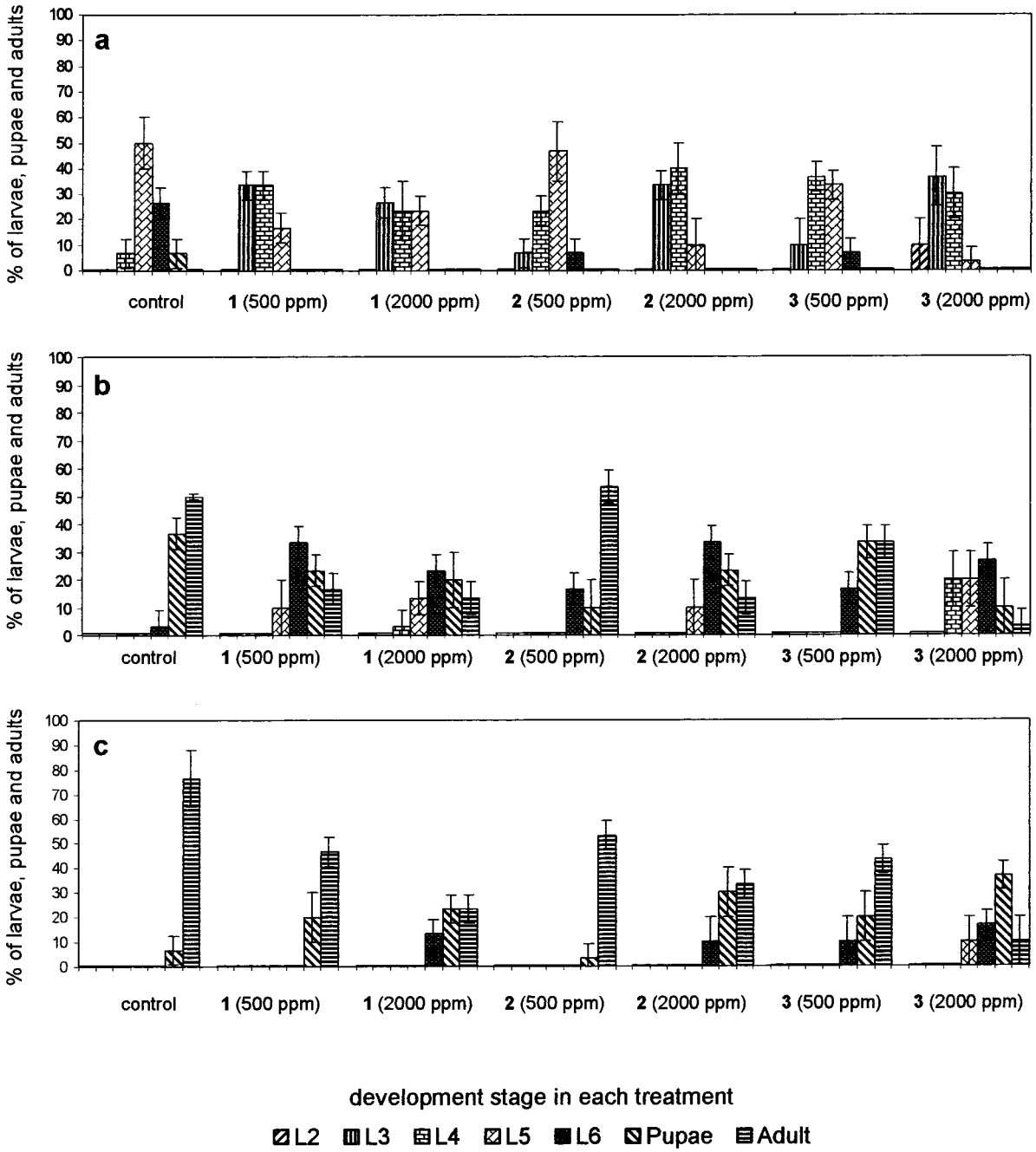

Figure 1. Development of *T. castaneum* larvae exposed to wheat flour treated with natural salpichrolides **1**–**3** at 500 and 2000 ppm: (a) 40, (b) 60, and (c) 80 days. Standard deviation is indicated at the top of each bar, $p \leq 0.05$. L, larval instar.

at 500 ppm, expressed as a percentage in relation to the surviving individual number at the six different observation times (40−140 days), was used to calculate DT_{50} values according to Probit analysis (Table 2). Treated replicates were compared with the control and also with salpichrolide A (**1**) as positive control, which in the previous bioassay (Table 1) was the most effective at 500 ppm. Results (Table 2) showed that compound **7** produced a significant development delay. On the other hand, acetylation of the hemiketal on the side chain (as in compound **4**) or oxidation to the lactone (compounds **5** and **6**) drastically reduced the observed effect, not being significantly different from the control. The presence of the hemiketal moiety was also shown to be necessary for exerting an effect on *M. domestica* larvae (*12*), although in that case acetylation did not block the effect, presumably owing to hydrolysis in vivo.

Ascher et al. have observed that four withanolides (nicalbine A, nicalbine B, and a mixture of withanicandrine and daturalactone A) acted as antifeedants on neonatae larvae of *T. castaneum* (*5*). In that paper, Ascher et al. used the weight of 14-day-old larvae as a criterion to evaluate the antifeedant effect. In the present work, we used the size of adults as the criterion for antifeedant activity, which proved to be a more reliable measure because false weights due to flour powder contamination of the external surface of the cuticle were avoided. Comparison of adult size data in treatments that produced developmental delays showed that control adults were significantly bigger (3.60 ± 0.10 mm) than individuals treated with

compound **1** at 500 ppm (3.22 ± 0.10 mm) and with compound **3** at 500 ppm (3.27 ± 0.15 mm), suggesting feeding inhibition by these compounds. However, the size of adults raised on derivative **7** at 500 ppm was not significantly different (3.42 ± 0.15 mm) from those of adults of the positive control treatment (compound **1**) or the control, suggesting that compound **7** has only a slight feeding inhibition effect.

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