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**TOXIC PHENOLIC GLYCOSIDES FROM *POPULUS*:
PHYSIOLOGICAL ADAPTATIONS OF THE WESTERN NORTH
AMERICAN TIGER SWALLOWTAIL BUTTERFLY, *PAPILIO
RUTULUS* (LEPIDOPTERA: PAPILIONIDAE)**

J. Mark Scriber¹, Richard L. Lindroth², and James K. Nitao¹

ABSTRACT

The phenolic glycosides tremulacin and salicortin found in quaking aspen, *Populus tremuloides*, and other members of the Salicaceae, are known to be toxic to larvae of the Eastern tiger swallowtail butterfly, *Papilio glaucus*, but not to the Canadian tiger swallowtail, *P. canadensis*. Larvae of the western tiger swallowtail, *P. rutulus*, were not killed nor were their growth rates suppressed when fed a mixture of tremulacin and salicortin on black cherry leaves. When the Salicaceae-adapted *P. rutulus* penultimate instar larvae were fed a combination of the two phenolic glycosides and the esterase inhibitor (DEF = S,S,S-tributylphosphorothioate), growth was reduced more than 50% compared to controls, and half of the larvae died before completing the instar. Our results indicate that esterase detoxification mechanisms are involved in the western tiger swallowtail, *P. rutulus*, as is also known to be the case for the northern tiger swallowtail, *P. canadensis*. It is not known whether the same esterase isozyme is involved in both species. From an evolutionary perspective such information could help resolve whether the Salicaceae-adapted swallowtails species are a monophyletic group (perhaps due to isolation in the Beringial Pleistocene glacial refuge of Alaska).

The family Papilionidae (swallowtail butterflies) has figured centrally in discussions of chemical ecology and coevolution (Dethier 1954, Ehrlich and Raven 1964, Wiklund 1975, Berenbaum 1983, Miller 1987), yet the chemical bases underlying interactions of this group with their host plants remain relatively unexplored. Much work has been done on the oviposition phase of host acceptance and suitability for a variety of papilionids (e.g., Feeny et al. 1983, 1989; Honda 1986, Nishida et al. 1987). In contrast, research on the biochemical adaptations of *Papilio* larvae to specific phytochemicals has focused on only a few species (Bull et al. 1984, Cohen et al. 1989, Lindroth 1989a). Determining whether related species use homologous enzymatic systems (Fig. 1A) or whether detoxification mechanisms have evolved independently a number of times (Fig. 1B & C) is crucial to understanding the evolution of papilionid host use (Fig. 1, Scriber et al. 1991).

The glaucus group in the genus *Papilio* exhibits some of the most intriguing and well documented patterns of host use in the Papilionidae (Scriber 1988). Although the species in this group are all oligophagous or polyphagous, the larvae exhibit striking differences in their abilities to use hosts. In particular, the ability to feed

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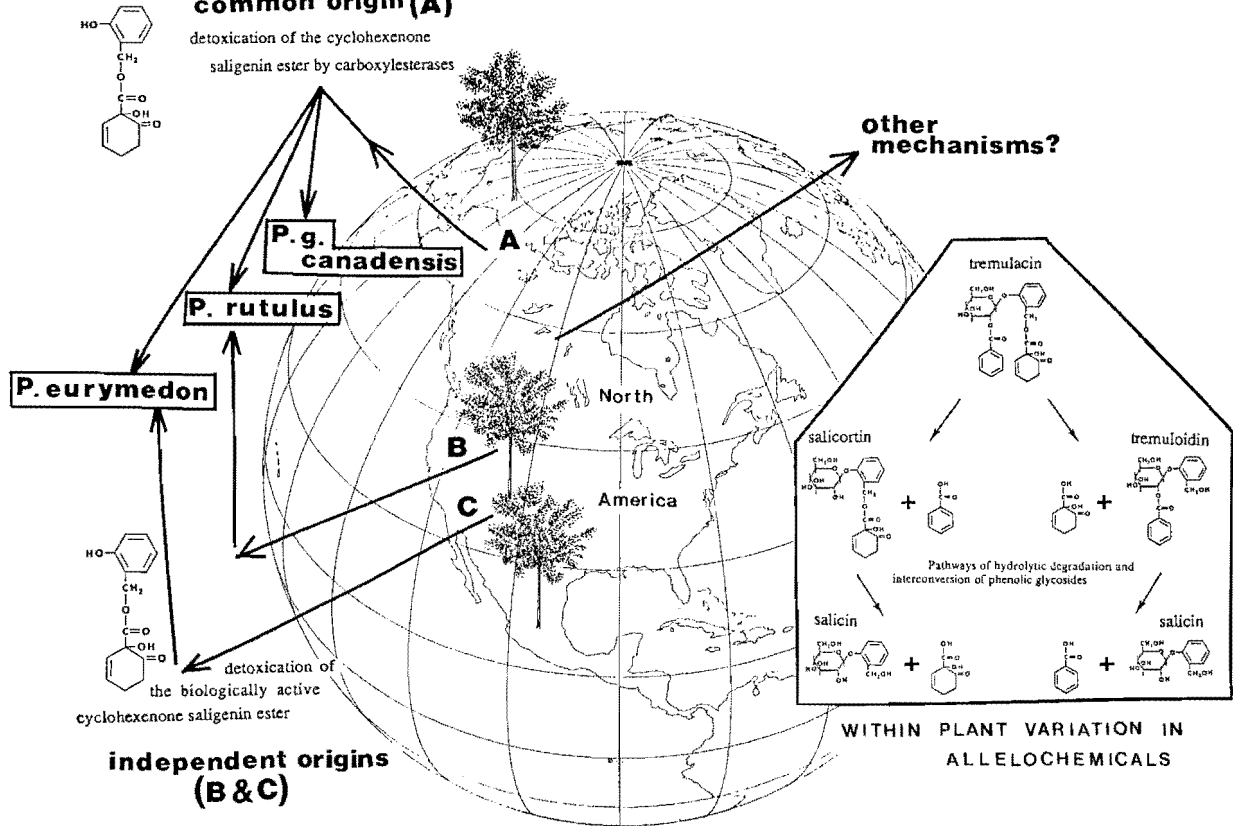


Figure 1. Phenolic glycosides from quaking aspen (Salicaceae) that have the cyclohexanone saligenin esters are toxic to *Papilio glaucus* but not to *P. canadensis*, *P. rutulus*, or *P. eurymedon*. It remains to be determined whether or not the same carboxylesterase enzyme is used in detoxification for all three western species, and if so whether a single or multiple origin is most likely (modified from Scriber et al. 1991).

upon members of the Salicaceae appears to be a significant trait in the evolution of this group. *Papilio glaucus* L., including its Florida populations, (*P. g. australis* R. & J.), and *P. alexiarses garcia* R. & J. are unable to survive on *Populus tremuloides* (quaking aspen); in contrast, larvae of *Papilio canadensis* R. & J., *P. eurymedon* Lucas, and *P. rutulus* Lucas can develop successfully on this plant (Scriber et al. 1989a, b, 1991; Dowell et al. 1990).

Phenolic glycosides, especially salicortin and tremulacin, are responsible for differential performances of *P. canadensis* (a recent elevation from subspecies; see Hagen et al. 1991) and *P. glaucus* on aspen. The compounds exhibit little toxicity against the adapted *canadensis*, but dramatically reduce survival and growth in *glaucus* (Lindroth et al. 1988). Phenolic glycosides are metabolized in *Papilio* by several enzyme systems, including esterase, b-glucosidase and possibly glutathione transferase (Lindroth 1989a, Scriber et al. 1989b). Esterases, however, are primarily responsible for detoxification of the compounds. Esterase activity is 3-fold higher in *canadensis* than in *glaucus*, and is inducible by prior consumption of phenolic glycosides in *canadensis* but not in *glaucus* (Lindroth 1989a, b). Moreover, when *canadensis* larvae consume diets containing phenolic glycosides and an esterase inhibitor, their survival rates decline significantly.

Scriber et al. (1989b) in a series of backcross studies with *canadensis* and *glaucus*, showed that esterase activities generally increased with the proportion of *Papilio canadensis* genes in the genotype, and activity paralleled overall trends in larval survival and feeding performance. They concluded that phenolic glycosides, such as tremulacin, are responsible for differential performance of *Papilio glaucus* subspecies, hybrids and backcrosses fed plants in the Salicaceae, and that detoxification of phenolic glycosides by midgut esterase explains why some *Papilio glaucus* genotypes can effectively utilize these plants.

The western tiger swallowtail, *P. rutulus*, performs very well on quaking aspen and utilizes that host where it co-occurs with *P. glaucus* in western North America (Scriber 1988, Dowell et al. 1991). The studies described in the following report were conducted to assess potential effects of phenolic glycosides on *P. rutulus*, and to determine whether esterases may be involved in their detoxification.

METHODS AND MATERIALS

Collecting and rearing of insects. Larvae for the feeding studies were obtained from a female *P. rutulus* (#7102) collected in California by Dr. Robert Dowell. Because larvae in the *glaucus* group perform poorly on artificial diets, and because all subspecies of *P. glaucus* effectively utilize black cherry (*Prunus serotina*) leaves, we used these as our basal diet. A crude phenolic glycoside mixture (38% tremulacin, 14% salicortin) was isolated from aspen and applied to cherry leaves using the methods of Lindroth et al. (1988). The four experimental treatments were methanol treated control leaves, the phenolic glycoside mixture applied with methanol, DEF (S,S,S-tributylphosphorotriothioate, an esterase inhibitor) applied with methanol, and a combination of glycosides and DEF (top and bottom sides of leaves, respectively). All larvae were reared on mature black cherry leaves prior to experiments.

For the feeding trials, each replicate consisted of a single, freshly molted fourth instar caterpillar placed in a petri dish (15 x 2.5 cm) containing a treated black cherry leaf. The phenolic glycoside mixture and DEF were applied at 4.0% and 0.01% fresh leaf weight, respectively to reflect natural occurrence. New leaves were provided as needed (3-4 day intervals) until completion of the fourth stadium. Upon completion of each trial we froze a subsample of larvae, then dried (3d, 60°C) and weighed the larvae, frass, and uneaten food. To estimate the proportion dry weight of larvae at the onset of each trial, a subset of newly molted fourth instars from the same cohort were dried and weighed. The proportion dry weight of leaves used in

feeding trials was determined for each batch of leaves provided ($36.6\% \pm 0.5\%$; $35.5\% \pm 1.09$; $39.9\% \pm 0.4\%$) by drying and weighing a sample of each batch of experimental leaves and nutritional indices were calculated on the basis of dry weights, using standard formulae (Waldbauer 1968, Scriber 1977). Growth and consumption rates were calculated on the basis of the duration of the fourth stadium, or until death (if larvae survived more than 5 days). Larvae were housed in a Percival® growth chamber maintained at 25°C with an 18:6 photo:scotophase cycle.

Enzyme assays. Because of the small number of larvae available, we used larvae reared on cherry leaves as well as some larvae from the feeding trials (all diets except phenolic glycosides + DEF) for enzyme preparations. Midguts were removed from fifth instars (3–6 days old, 3–5 larvae per replicate), washed and homogenized as described by Lindroth (1989a). The homogenate was centrifuged at 10,000 g (10 min), and the supernatant was then centrifuged at 100,000 g (60 min). We flash-froze the enzyme solutions in liquid nitrogen and stored them at -70°C until assayed. The final supernatant (containing soluble esterases) was used as the enzyme source in this study. Esterase activity was determined with the 1-naphthyl acetate assay (Lindroth 1989c). A modified Folin-phenol procedure (Schacterle and Pollack 1973) was used to measure protein concentration of the enzyme solutions.

Statistics. For the feeding trials we assessed differences among treatment effects by one-way analysis of variance (ANOVA). When the ANOVA F statistic was significant ($P = 0.05$), we compared treatment means via Tukey's test for unequal sample sizes (Winer 1962, Snedecor and Cochran 1967).

RESULTS

The mean growth rate for larvae fed the glycoside/enzyme inhibitor treatment was half that of those fed the methanol control. While not statistically significant, the leaves treated with glycoside alone produced the fastest growth, the greatest consumption rates, and the highest overall efficiency of all treatments (Table 1).

Larvae of *P. rutulus* grew significantly slower on the treatment with a combination of phenolic glycosides (tremulacin and salicortin) and the esterase enzyme inhibitor (DEF) than did larvae fed either the methanol control or the glycosides alone (Table 1). This poor growth was due to a combination of suppressed consumption rates and lowered biomass conversion efficiency (ECI).

It is noteworthy that half of 6 larvae in the glycoside plus enzyme inhibitor treatment died before completing the stadium, whereas all larvae in the methanol control and the glycoside treatments successfully molted. One larva in the DEF enzyme treatment died before completing the stadium.

Results from the enzyme assays with *P. rutulus* showed relatively low esterase activity. The mean activity for three enzyme preparations from final instar larvae was $1,409 \pm 154$ nmol/min/mg protein.

DISCUSSION

The phenolic glycosides tremulacin and salicortin from quaking aspen are known to be toxic to *P. glaucus* and its Florida populations (putative subspecies *australis*), but not to *P. canadensis* (Lindroth et al. 1988, Scriber et al. 1989). In this study, a combination of salicortin and tremulacin applied to black cherry leaves did not kill or reduce larval growth performance of the western tiger swallowtail, *Papilio rutulus*. This is consistent with *P. rutulus* feeding on quaking aspen and other members of the Salicaceae plant family (Brower 1958, Scriber 1988, Dowell et al. 1991).

As was observed with the Salicaceae-adapted *P. canadensis* (Lindroth 1989a), *P. rutulus* larvae fed a combination of phenolic glycosides and the esterase inhibitor

Table 1. — Growth performance of penultimate instar *Papilio rutulus* fed black cherry (*Prunus serotina*) leaves treated with methanol, a phenolic glycoside mixture (tremulacin and salicortin), an esterase inhibitor (DEF), and a mixture of glycosides and the esterase inhibitor. Data are presented as a mean \pm SE.*

Treatment	(n)	Survival through instar (%)	Instar Duration (T) (days)	Growth Rate (RGR) $\text{mg}\cdot\text{mg}^{-1}\cdot\text{day}^{-1}$	Consumption Rate (RGR) $\text{mg}\cdot\text{mg}^{-1}\cdot\text{day}^{-1}$	Digestibility (AD) (%)	Efficiency (ECD) (%)**	Efficiency (ECI) (%)**
control (methanol)	(6)	100%	8.00 a	.209 a	1.197 a	36.8 a	37.6 a	16.9 ab
			2.52	.031	.141	2.9	5.2	1.1
phenolic glycosides	(6)	100%	5.29 a	.220 a	1.238 a	34.4 a	56.9 a	17.7 a
			.31	.009	.034	5.5	7.8	0.4
inhibitor (DEF)	(6)	83%	6.96 a	.181 ab	1.195 a	37.8 a	37.8 a	14.7 ab
			1.23	.034	.147	1.9	4.9	2.2
glycosides and inhibitor	(6)	50%	7.63 a	.096 b	.916 a	29.6 a	41.8 a	9.3 b
			1.88	.036	.198	10.7	12.4	3.2
LSD			(n.s.)	.110	(n.s.)	(n.s.)	(n.s.)	7.9

*Significant differences (L.S.D.) among means are indicated at $p < 0.05$ level (Tukey's test; Snedecor and Cochran 1967; Winer 1962).

**E.C.D. = efficiency of conversion of digested food to larval mass, E.C.I. = efficiency of conversion of ingested food to larval mass.

DEF were drastically affected. Growth rates were reduced more than 50% compared to larvae on the control and glycoside treatments. Also, half of the larvae died before completing the penultimate instar when fed the glycoside and inhibitor combination, whereas none of them died on the control or glycoside treatments (Table 1). These data, although limited, suggest that functional esterase detoxification enzymes play an important role in the ability of *P. rutulus* to feed on members of the Salicaceae that contain tremulacin and salicortin.

DEF is generally believed to act as a noncompetitive inhibitor of insect esterases, most likely due to phosphorylation of the enzymes (Jao and Casida 1974). Our treatment with DEF applied alone showed that the compound may negatively affect larval performance. Growth rates tended to decline (although not significantly) and one larva died in the treatment. Marginally detrimental effects are not surprising, given that esterases are required for the metabolism of endogenous compounds (e.g., juvenile hormone).

The general esterase activity exhibited by *P. rutulus* was surprisingly low. The value of 1409 nmol/min/mg protein is only slightly higher than that of the unadapted *P. glaucus*, and less than half that of the aspen-adapted *P. canadensis* (Lindroth 1989a, Scriber et al. 1989b). This result illustrates one of the problems that can be encountered in use of model substrates—they do not necessarily indicate activity against the compounds of interest (e.g., phenolic glycosides). Activity of isozymes particularly effective against phenolic glycosides may be masked in determinations of general esterase activity. Unfortunately, the lability of phenolic glycosides in aqueous solutions has thus far precluded development of esterase assays with phenolic glycoside substrates (Lindroth, unpubl. data).

Given that our studies were conducted with offspring from a single butterfly, we caution that the results should be considered preliminary. Nevertheless, our results do indicate that phenolic glycosides, at naturally occurring concentrations, are not toxic to *P. rutulus*. Moreover, they suggest that esterases are the metabolic basis for resistance to the compounds. We hope in our future research to define more clearly the genetic basis and geographic extent of the esterase resistance mechanism in *P. rutulus*, and to determine if this detoxification system is the same as in *P. canadensis*. Such knowledge will be useful from an evolutionary viewpoint because it may contribute to our understanding of whether *P. canadensis*, *P. rutulus*, and *P. eury-medon* are of a monophyletic origin (e.g., in a Beringial refuge, Scriber 1988, Hagen and Scriber 1991, Hagen et al. 1991), or if this Salicaceae detoxification system arose several times independently (Fig. 1).

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