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PHOTOTOXIC POLYACETYLENES FROM *VIGUIERA ANNUA* AND ADAPTATIONS OF A CHRYSOMELID BEETLE, *ZYGOGRAMMA CONTINUA*, FEEDING ON THIS PLANT

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Key Word Index—*Viguiera*; Compositae; *Zygogramma*; phototoxin; behavioural adaptation; antioxidant enzymes.

Abstract—Two major polyacetylenes, *trans*-1,3,5,11-tridecatetraen-7,9-diyne and 1,*cis*-3,*trans*-5,*trans*-11-tridecatetraen-7,9-diyne, possessing light-activated toxicity (phototoxicity) were isolated from the leaves of *Viguiera annua* (M. E. Jones) Blake (Asteraceae). Despite the insecticidal properties of these polyacetylenic derivatives, a field survey revealed that larvae of *Zygogramma continua* Le Conte (Coleoptera: Chrysomelidae) were able to feed and develop normally on the highly phototoxic leaves of *V. annua*. Results suggested that the light-avoidance behaviour, and the inducibility of some antioxidant enzymes, in *Z. continua* larvae were important features explaining the tolerance of this insect to the phototoxic polyacetylenes occurring in *V. annua*.

INTRODUCTION

More than 1000 species of Asteraceae are now reported to produce polyacetylene derivatives [1–3], many of which exert potent insecticidal effects when sensitized by near-UV light (phytotoxicity) [4] due to the production of activated oxygen species or other radicals that damage the lipid membranes [5]. However, some insects that are often exposed to phototoxic polyacetylenic derivatives in their host plants possess either some light avoidance behaviour [6] or enzymatic and non-enzymatic antioxidant adaptations that may protect them against phototoxicity [5, 7].

A new phototoxic plant, *Viguiera annua* (M. E. Jones) Blake (Asteraceae), as well as a chrysomelid beetle, *Zygogramma continua* Le Conte (Coleoptera: Chrysomelidae) feeding preferentially on this plant, were investigated. *Viguiera* is an important genus of the subtribe Helianthinae and is mostly found in Central America, South America and in the southwest United States [8, 9]. The genus *Viguiera* appears to be represented by opportunistic species that grow and develop quickly after the first rains of the wet season [10, 11], as was observed for the annual goldeneye, *V. annua*, in our field survey near Tucson, AZ (U.S.A.). The annual goldeneye has been reported as potentially poisonous to livestock due to its ability to sequester potassium nitrate in its tissues at up to 4.7% dry weight [12]. In rangelands of New Mexico, *V. annua* occupies up to 50% of the total foliar cover [13].

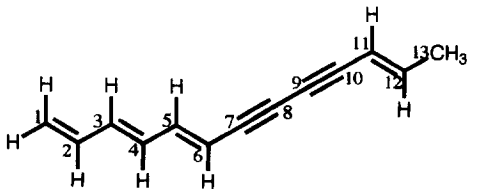
The insect genus *Zygogramma* has been reported to be distributed in Mexico, Costa Rica, Guatamala and Nicaragua [14]. *Zygogramma* contains several species that are known to feed restrictively on a particular hostplant. For examples, *Z. bicolorata* and *Z. suturalis* have sufficiently narrow host range that they have been introduced into Europe as biological control agents for their respective hostplant, *Parthenium hysterophorus* and *Ambrosia artemisifolia* [15, 16].

In the present study, we elucidated the structures of two phototoxic polyacetylenes, *trans*-1,3,5,11-tridecatetraen-7,9-diyne (**1**) and 1,*cis*-3,*trans*-5,*trans*-11-tridecatetraen-7,9-diyne (**2**), which occur in the leaves of *V. annua*. The behavioural and biochemical investigation of *Z. continua* larvae that were observed to selectively feed on the phototoxic leaves of *V. annua* suggested that both light-avoidance behaviour and inducibility of some antioxidant enzymes are useful to circumvent plant phototoxic defences.

RESULTS AND DISCUSSION

Structure elucidation of polyacetylenes

The UV spectra of **1** and **2**, displaying λ_{max}^{hexane} at 360, 334, 315, 280, 265 and 255 nm, suggested the presence of triene-diyne-ene chromophores [1]. Mass spectral analysis established a molecular ion of 168 for each compound. They were determined to be closely related structural isomers of one another, with molecular for-



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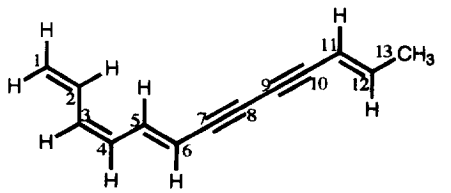

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mulae of $C_{13}H_{12}$, as verified from their ^{13}C NMR spectra, which revealed 13 carbon signals. ^1H-^1H coupling patterns, along with data from 2D COSY and HMQC NMR experiments, confirmed the arrangement of double and triple bonds. Compound **1**, the all-*trans* geometrical isomer, displayed typical ^1H-^1H coupling constants (J = 12–18 Hz) across each of its *trans* double bonds: 14.0 Hz (H-1_{trans}, H-2), 15.5 Hz (H-5, H-6), and 15.8 Hz (H-11, H-12) (H-3 and H-4 resonated at almost identical frequencies, thereby preventing ready identification of second-order coupling patterns). Compound **2** had a ^1H NMR spectrum very similar to that of compound **1**, only in this case, the C3-C4 double bond clearly showed *cis* geometry (J = 6–12 Hz). The *trans* and *cis* ^1H-^1H coupling constants observed for compound **2** were 16.7 Hz (H-1_{trans},H-2), 11.4 Hz (H-3, H-4), 15.4 Hz, (H-5, H-6), and 15.8 Hz (H-11, H-12). Compounds **1** and **2** thus differ only in the geometry ground the C3-C4 double bond. The concentrations of compounds **1** and **2** in the leaves of *V. annua* were *ca* 5 mg/g dry weight and 3.5 mg/g dry weight, respectively. 1,3,5,11-tridecatetraen-7,9-diyne has been reported to occur in 27 species of the Heliantheae tribe [3], but this is the first report to specify the occurrence of the 3-*cis*-isomer (**2**).

Insect tolerance to phototoxins: behavioural and biochemical adaptations

Compounds **1** and **2**, when tested in our standard yeast bioassay [17], were found to be highly phototoxic (with UV treatment, 22 mm of inhibition of 10 μg applied to a paper disk of 7 mm diameter, but no inhibition in dark). In previous experiments, we also demonstrated that polyacetylenes with similar chromophores to compounds **1** and **2** were highly phototoxic to mosquito larvae [18].

In a laboratory behavioural experiment (Fig. 1) beetle larvae assumed positions on the phototoxic leaves of *V. annua*, that were influenced by the light regime (Kruskall–Wallis test, $P < 0.001$). From 45 min until

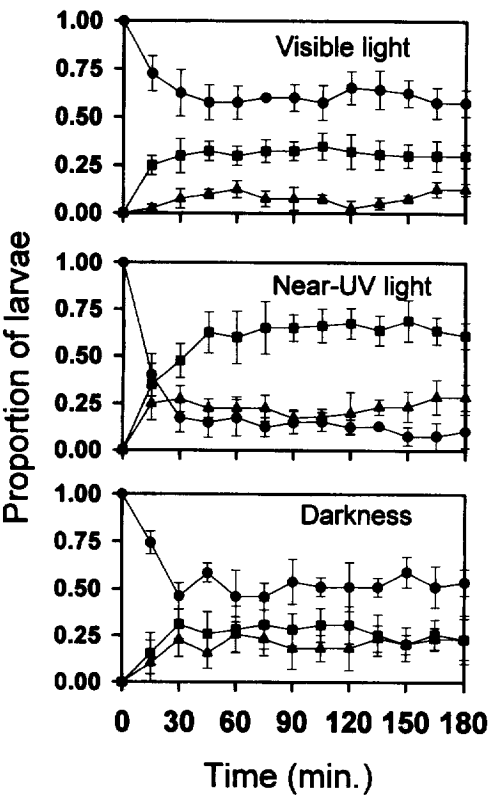

Fig. 1. Behavioural response of third-instar *Zygogramma continua* larvae feeding on phototoxic leaves of *Viguiera annua* under different light regimes. The symbols ●, ■, and ▲, respectively, represent the proportions of larvae above the leaves, under the leaves and those without any contact with the leaves. Each point represents the average of duplicate trials and vertical bars represent the standard deviation.

the end of the observation period, the number of larvae that had either escaped, that were no longer touching the leaves, or that had concealed their head under the leaf, was significantly higher for the near-UV treatment than in the visible light or dark treatments (Dunn's procedure, $P < 0.05$). For example, about 55% of the larvae remained above the leaves after 45 min in both visible light and darkness, while only 16% remained exposed to the presence of photosensitizing near-UV light (Fig. 1). Under field conditions, the first *Z. continua* larval instars were often observed hiding at the basal part of a leaf with their heads located between a node and the base of a petiole on *V. annua* plants. Such larval concealment in fields may constitute an analogous hiding behaviour to that detected in our laboratory experiment.

In the laboratory experiment, the feeding activity was also affected by the light regime since the area of tissues ingested was nearly two-fold higher in darkness than either under near-UV or visible light (Table 1).

We have previously reported the efficiency of behavioural adaptations alone to reduce the mortality of insects feeding on phototoxic plants in nature [6, 19]. However, we hypothesized that antioxidant capacity may also be induced in these insects after exposure to

Table 1. Feeding activity of *Zygogramma continua* third-instar larvae on phototoxic leaves of *Viguiera annua* under different light regimes

Treatment	Ingestion* (mm^2/larva/3 hr)
Near-UV light	1.77[a] (0.22)
Visible light	1.61[a] (0.26)
Darkness	3.27[b] (0.19)

* Values followed by the same letter are not significantly different (LSD, $P < 0.05$). Values provided in parentheses represent the standard error of the mean.

phototoxins, in order to enhance the protection of insects. Table 2 shows induction by the related phototoxin α-terthienyl of glutathione-*S*-transferase (GST) activity and glutathione reductase (GR) activity in both midgut and fat body ($P < 0.05$). Conversely, superoxide dismutase (SOD) activity was inhibited in the midgut and catalase (CAT) activity appeared unaffected when *Z. continua* larvae were topically applied with α-terthienyl. These results suggest a predominant role for GST and GR in the tissues of *Z. continua* to buffer the α-terthienyl-induced oxidative stress that leads to the peroxidation of lipids in sensitive insects [5] and, ultimately, to larval mortality. The previous demonstration of the importance of a high GSH:GSSG ratio in tobacco hornworm larvae to prevent the α-terthienyl-induced peroxidation of lipids [5] and our current results with the glutathione-dependent enzymes, GST and GR, indicate that glutathione is an important defence against photoxidants.

A plausible explanation for the fact that SOD and CAT activities were not induced by α-terthienyl may be that it is primarily a generator of singlet oxygen that can lead to lipid peroxidation without the involvement of either superoxide or hydrogen peroxide. Nivsarkar *et al.* [20] also demonstrated that SOD was inhibited by a α-terthienyl in the anal gills of mosquito larvae.

The activities of antioxidant enzymes in *Z. continua* larvae were also affected by the nature of the tissues (Table 2). For example, CAT was five-fold more active in the fat body than in the midgut while GST and GR had similar activity in both tissues (less than 22% variation). SOD showed a slight trend to higher activity in the midgut than in fat body either for α-terthienyl-treated larvae (39% higher) or control larvae (69% higher).

On the whole, the present results illustrate well how photochemistry and light environment may interact to alter the outcome of plant–insect relationships involving phototoxic polyacetylenic derivatives. While it is unknown if the integrated behavioural and biochemical strategies reported in *Z. continua* larvae have directly arisen due to the presence of phototoxins in its host-plant, these adaptive traits may represent major features that make this insect tolerant to the phototoxic polyacetylenes present in *V. annua*. In terms of an optimal strategy for the chrysomelid, it is hypothesized that the behavioural response of *Z. continua* larvae is the primary defence. The substitution of biochemical for behavioural adaptation when light avoidance is not feasible, is the best strategy to minimize the cost required to prevent phototoxicity.

EXPERIMENTAL

Extraction, isolation and structure elucidation of polyacetylenes. Plants of *V. annua*, as well as the chrysomelid larvae, were collected in Buenos Aires National Wildlife Refuge located at approx. 100 km south of Tucson, AZ (U.S.A.). The plants were identified at the herbarium of the University of Arizona in Tucson and a voucher specimen was deposited at the

Table 2. Effects of α-terthienyl topical applications on the activities of antioxidant enzymes in third-instar *Zygogramma continua* larvae†

	CAT‡			SOD‡			GST‡			GR‡		
Tissue	−αT	+αT	Ind (%)	−αT	+αT	Ind (%)	−αT	+αT	Ind (%)	−αT	+αT	Ind (%)
Midgut	222 (57)	189 (42)	−17	4.9 (0.4)	3.2* (0.5)	−35	1420 (85)	2005* (90)	+44	1.9 (0.3)	2.8* (0.3)	+45
Fat body	1174 (239)	1372 (150)	+17	2.9 (0.3)	2.3 (0.3)	−21	1270 (70)	1840* (50)	+45	1.6 (0.4)	2.5* (0.2)	+50

† Activity units for catalase (CAT), superoxide dismutase (SOD), glutathione-*S*-transferase (GST) and glutathione reductase (GR) are μmol H_2O_2 decomposed/mg protein/min, units/mg protein/min, nmol 1-chloro-2,4-dinitrobenzene conjugate/mg protein/min and nmol NADPH oxidation/mg protein/min, respectively.

‡ Values in parentheses represent the standard deviation. Treatments with and without α-terthienyl are referred to as +αT and −αT. Values from the +α-T treatments that are followed by an asterisk are significantly different than the corresponding −α-T treatments (LSD, $P < 0.05$). Ind represents the relative inducibility of enzymatic activity between +α-T and −α-T.

herbarium of the University of Ottawa. For photochemical isolations, seeds collected were sown in vermiculite and grown to maturity in the greenhouse. Foliar tissues, 650 g, were homogenized in 2.0 l EtOH (95%), filtered, and then combined with 2 l H_2O before being partitioned twice with a total volume of 1.5 l hexane. The hexane fr. was reduced to a vol. of 2 ml by rotary evapn, and the polyactylenes were sepd by prep. HPLC (model LC-908, Japan Analytical Industry Co. Ltd) using a reversed phase C-18 column with acetonitrile–H_2O (7:3) at a flow rate of 3 ml/min. The two major peaks determined by UV absorption at 254 nm and by refractive index, were separately collected, evapd to dryness, and then submitted for GC-MS and ^1H and ^{13}C NMR analysis.

Compound **1**. ^1H NMR (500 MHz, C_6D_6): δ 6.64 (1 H, *dd*, J = 15.5 Hz, 10.2 Hz, H-5), 6.24–6.14 (3 H, *m*, H-2,3,4), 6.11 (1 H, *dd*, J = 15.8 Hz, 6.9 Hz, H-12), 5.68 (1 H, *d*, J = 15.5 Hz, H-6), 5.49 (1 H, *dd*, J = 15.8 Hz, 1.4 Hz, H-11), 5.14 (1 H, *d*, J = 14.0 Hz, H-1_{trans}), 5.02 (1 H, *d*, J = 8.8 Hz, H-1_{cis}), 1.48 (3 H, *dd*, J = 6.9 Hz, 1.4 Hz, H_3-13); ^{13}C NMR (125 MHz, C_6D_6) δ 145.12 (C-5), 144.65 (C-12), 137.31 (C-3*), 136.84 (C-4*), 132.34 (C-2*), 120.51 (C-1), 110.27 (C-6), 109.89 (C-11), 82.91 (C-7*), 81.36 (C-8*), 78.00 (C-9*), 72.90 (C-10*), 18.77 (C-13); MS *m/z* 168 (M, 78), 167 (28), 166 (21), 165 (57), 153 (40), 152 (100), 151 (16), 141 (16), 139 (19), 128 (17), 115 (35), 77 (15), 63 (18), 39 916).

Compound **2**. ^1H NMR (500 MHz, C_6D_6): δ 7.00 (1 H, *ddd*, J = 15.4 Hz, 11.4 Hz, 1.0 Hz, H-5), 6.39 (1 H, *ddt*, J = 16.7 Hz, 11.4 Hz, 1.0 Hz, H-2), 6.00 (1 H, *dd*, J = 15.8 Hz, 6.9 Hz, H-12), 5.76 (1 H, *dt*, J = 11.4 Hz, 0.9 Hz, H-3), 5.61 (1 H, *t*, J = 11.4 Hz, H-4), 5.38 (1 H, *d*, J = 15.4 Hz, H-6), 5.31 (1 H, *ddd*, J = 15.8 Hz, 1.8 Hz, 1.0 Hz, H-11), 5.00 (1 H, *dt*, J = 16.7 Hz, 0.9 Hz, H-1_{trans}), 4.92 (1 H, *d*, J = 11.4 Hz, H-1_{cis}), 1.24 (3 H, *dd*, J = 6.9 Hz, 1.8 Hz, H_3-13); ^{13}C NMR (125 MHz, C_6D_6) δ 143.60 (C-12), 139.58 (C-5), 133.23 (C-3) 132.09 (C-2), 128.77 (C-4), 119.87 (C-1), 111.37 (C-6), 110.35 (C-11), 82.89 (C-7*), 81.22 (C-8*), 78.85 (C-9*), 73.72 (C-10*), 18.48 (C-13); MS *m/z* 168 (M, 77), 167 (32), 166 (29), 165 (66), 153 (42), 152 (100), 151 (15), 141 (17) 139 (20), 128 (17), 115 (35), 90 (15), 89 (21), 78 (15), 77 (15), 63 (20), 39 (17).

Light avoidance behaviour of Zygogramma continua *larvae*. The coleopteran larvae collected on the leaves of *V. annua* were identified by Dr Carl Olson from the Department of Entomology at the University of Arizona at *Zygogramma continua* Le Conte (Coleoptera: Chrysomelidae). Intense phototoxicity of the *V. annua* leaves was detected using a standard yeast inhibition assay [5]. For the behavioural experiment, oblong-elongated leaves of *V. annua*, *ca* 12 cm long and 0.8 cm wide, were cut in squares (0.9 × 0.9 cm) and individually placed in a compartment of a Falcon tray (1.6 cm in diameter). One third-instar larva was deposited onto each leaf section and the trays were recovered with a plastic wrap transparent to visible and near-UV wavelengths. Trays containing larvae and leaves were then placed under visible light (cool-white, 80 W/m^2), near-UV light (300–400 nm) (Westinghouse F20T12/BLB bulbs, 1.0 m W/cm^2) or kept in darkness at room temperature. Observations were made every 15 min over a 3 hr period on the relative position of the larvae compared to the foliar squares. Larvae without any contact with the leaf tissues were classified as 'escaped' while those still in contact with the leaf were divided into two groups depending on whether their heads were located above or under the leaves. At the end of the 180 min observation period, the feeding activity of each larva was determined by placing the leaf parts over a 1 mm graph paper and then evaluation the area removed using a dissecting microscope. Due to a limitation in the availability of third-instar larvae, the experiment was repeated twice using each time 20 larvae per light treatment so that 120 larvae were used in total (3 treatments × 20 larvae/treatment × 2 experiments). For statistical analysis, larvae having either escaped or moved under the leaf were considered as responding while those remaining above the leaf were counted as non-responding. The Kruskall–Wallis test for non-parametric data was used and then the Dunn procedure was performed to compare the insect response between the different light regimes [21].

Biochemical adaptations of Zygogramma continua *larvae: inducibility of antioxidant enzymes in presence of α-terthienyl*. The hypothesis that inducible antioxidant enzymes may buffer the oxidative stress generated by polyacetylenic derivatives in insects was tested using third-instar *Z. continua* larvae. Because oral administration with a feeding syringe would have been impractical due to the small size of *Z. continua* larvae, we performed topical applications. Five microlitres of EtOH containing 10 μg of α-terthienyl (control insects received only EtOH) were dorsally applied on each larva. Because the polyacetylenes contained in *V. annua* had not yet been purified at the time of our field survey, we used α-terthienyl as an alternate photo-oxidative polyacetylenic derivative [22]. Larvae were then kept in darkness for 8 hr to let the α-terthienyl diffuse through larval tissues. Our previous work has shown that over 50% of the topically applied α-terthienyl diffused in the internal tissues of lepidopteran insect larvae in this time frame [23]. The fact that the toxicokinetic parameters of α-terthienyl are comparable in both topical application or oral ingestion [23] were used to justify our experimental approach. *Z. continua* larvae were subsequently exposed for 4 hr under near-UV irradiation as above to stimulate the α-terthienyl photosensitization. Insects were then dissected on ice, rinsed in H_2O containing NaCl (0.9%), homogenized in PBS (0.1 M, pH 7.4) containing 1 mM EDTA and centrifuged at 5000 *g* for 5 min. Tissues of 10 insects were pooled for each analysis and 3–5 replicates were performed for

* Assignment ambiguous.

each treatment. Activities of superoxide dismutase (SOD) and catalase (CAT) were determined as described in ref. [24], while glutathione reductase (GR) and glutathione *S*-transferase (GST) were performed as in refs [25] and [26], respectively. Protein content was determined as in ref. [27].

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