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VOLATILE MONOTERPENES IN *POROPHYLLUM GRACILE* AND *P. RUDERALE* (ASTERACEAE): IDENTIFICATION, LOCALIZATION AND INSECTICIDAL SYNERGISM WITH α-TERTHIENYL

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Porophyllum gracile*; *Porophyllum ruderale*; asteraceae; monoterpene; α-terthienyl; synergism.

Abstract—Volatiles occurring in *Porophyllum gracile* and *P. ruderale* (Asteraceae) were identified in order to assess their role in integrated chemical defences against insects. Headspace and steam distillation techniques yielded a total of 12 mono- and sesquiterpenes and fatty acid derivatives. The glandular secretory cavities present on leaves of *P. ruderale* were large enough to permit direct sampling and were found to be particularly rich in volatile monoterpenes (73.6% of the integrated FID trace). Using reduction of relative growth rate of third instar *Ostrinia nubilalis* (Lepidoptera: Pyralidae) larvae as an index of insecticidal activity, the volatiles released from the secretory cavities located on the leaves of *P. ruderale* had no significant effect alone, but they synergized the effects of α-terthienyl, a toxic light-activated secondary compound also present in *P. gracile* and *P. ruderale*. This synergistic interaction was shown to be related to an enhanced accumulation of the α-terthienyl in *O. nubilalis* larvae when they were exposed to the volatiles emitted from the foliar secretory cavities of *P. ruderale*.

INTRODUCTION

The genus *Porophyllum* (Asteraceae), which occurs in an area extending from the southwest of the United States to South America [1, 2], is characterized by the presence of translucent glandular secretory cavities (GSCs) that are located along the leaf margin and, to a less extent, scattered throughout the lamina [3]. The lumen of these GSCs has a lysigenous origin and is delimited by a multilayered epithelium [4]. In a previous study we reported that the GSCs were also an important feature that confers resistance against insect herbivory in *Porophyllum gracile* (Jacq.) Cass. Var. *macrocephalum* (DC.) and *Porophyllum ruderale* Benth. (Asteraceae) [5]. Under field conditions, individuals of *P. gracile* having an average of 4.9 GSCs per leaf had only 4.3% of their leaves damaged by herbivores, mostly generalist grasshoppers, while plants with a mean value of 2.5 GSCs per leaf suffered a five-fold higher rate of herbivory. The same study also confirmed under laboratory conditions that the volatile compounds emitted from the foliar GSCs of both *P. ruderale* and *P. gracile* exert a repellent activity against adults of the red-legged grasshopper, *Melanoplus femurrubrum femurrubrum* (Orthoptera: Acrididae).

Different monoterpenes, sesquiterpenes and phototoxic thiophenes, have been identified in the genus *Porophyllum* [6–12]. It is, however, not known which insecticidal compounds typically occur in the foliar GSCs of *Porophyllum* spp. since the chemicals reported in the above studies were in most cases extracted from tissues of either entire aerial parts, roots or whole plants.

In this study we investigated by GC-MS the volatiles emitted from the aerial parts of *P. gracile* and *P. ruderale* and confirmed their identity. The hypothesis that these volatiles exert a synergistic effect on the insecticidal properties of α-terthienyl, a phototoxic polyacetylenic derivative also present in *P. gracile* and *P. ruderale*, was also tested against larvae of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae). Although *O. nubilalis* is not a

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Table 1. Chemical constituents in the leaves of Porophyllum ruderale according to different sampling techniques.

	Sampling technique		
Chemical constituent	Direct*† (% of the integrated FID trace)	Steam distillation* (% of the integrated FID trace)	Head-space* (% of the integrated FID trace)
Monoterpenes			
α-pinene	n.d.	n.d.	n.d.
sabinene	15	13	22
2,3-dihydro-1,8-cineole	n.d.	2	n.d.
myrcene	6	5	7
limonene	714	732	889
trans-β-ocimene	1	n.d.	n.d.
terpinolene	n.d.	n.d.	n.d.
Sesquiterpene			
β-cubebene	n.d.	n.d.	n.d.
Fatty acid derivatives			
7-tetradecene	n.d.	10	n.d.
cis-4-decenal	n.d.	7	n.d.
pentadecanal	16	20	n.d.
heptadecanal	230	2	n.d.

*The abbreviation n.d. refers to not detected.

†The extract was obtained from a direct sampling of the liquid secreted from the glandular secretory cavities present on the leaves.

pest of the genus *Porophyllum*, this insect has been reported to feed on over 200 herbaceous plants including many Asteraceae [13]. Given the frequent occurrence of monoterpene [14] and thiophene [15] derivatives in the Asteraceae family, *Ostrinia nubilalis* is therefore likely to encounter in its natural host plants these two types of phytochemicals.

RESULTS

Chemical Analysis

Volatiles that were collected from *P. gracile* and *P. ruderale* using the static head-space technique were mostly monoterpenes. Sabinene, myrcene and limonene constituted 91.8% of the integrated FID trace of chemicals emitted from the leaves of *P. ruderale* and α-pinene, sabinene and myrcene accounted for 83.9% of the volatile substances collected from the leaves of *P. gracile* (Tables 1 and 2).

The essential oil obtained by steam distillation of leaves provided a similar profile of monoterpenes to the results obtained with the head-space technique for *P. ruderale* except that a small amount of 2,3-dihydro-1,8-cineole, 0.2% of the integrated FID trace, was also detected (Table 1). As for *P. gracile*, the same monoterpenes were identified by both sampling techniques, but the relative proportions differed. This was especially evident for α-pinene and sabinene, which represented 17.7% and 3.5% respectively of the extract obtained with the head-space sampling and 2.3% and 20.5% of the extract obtained through steam distillation (Table 2). Different fatty acid derivatives, 7-tetradecene, cis-4-decenal, pentadecanal and heptadecanal, were also detected in the extracts obtained by the steam distillation of leaves, which contributed 3.9% and 5.3% of the total extracts in *P. ruderale* and *P. gracile* respectively (Tables 1 and 2). A sesquiterpene, β-cubebene, was also identified in the steam distillation extract of *P. gracile* (Table 2).

Chemical analysis of the liquid sampled directly from the GSCs in the leaves of *P. ruderale* revealed a similar profile of monoterpenes to the one obtained with the head-space technique except that a small amount of trans-β-ocimene (0.1%) was found (Table 1). The relative amount of the fatty acid derivatives in *P. ruderale* was 24.6% of the integrated FID trace in the direct sampling compared to 3.9% and undetectable levels in the steam distillation and head-space techniques respectively (Table 1).

One substance, 7-tetradecene, constituted 77.0% and 90.2% of the integrated FID trace obtained from the stems of *P. ruderale* by steam distillation and head-space samplings respectively (Table 3). Monoterpenes were not detected in the stems of *P. ruderale* according to head-space sampling, although limonene constituted 8.6% of the integrated FID trace of the extract collected by steam distillation (Table 3).

Insect Toxicity

The relative growth rate of *O. nubilalis* larvae exposed to volatiles emitted from the foliar GSCs of *P. ruderale* was not significantly different from the

Table 2. Chemical constituents in the leaves of Porophyllum gracile according to different sampling techniques.

	Sampling technique	
Chemical constituent	Steam distillation* (% of the integrated FID trace)	Head-space* (% of the integrated FID trace)
Monoterpenes		
α-pinene	2.3	17.7
sabinene	20.5	3.5
2,3-dihydro-1,8-cineole	n.d.	n.d.
myrcene	40.6	62.7
limonene	n.d.	n.d.
trans-β-ocimene	n.d.	n.d.
terpinolene	n.d.	n.d.
Sesquiterpene		
β-cubebene	9.1	n.d.
Fatty acid derivatives		
7-tetradecene	1.4	n.d.
cis-4-decenal	n.d.	n.d.
pentadecanal	2.9	n.d.
heptadecanal	1	n.d.

*The abbreviation n.d. refers to not detected.

Table 3. Chemical constituents in the stems of Porophyllum ruderale (Jacq.) Cass. var. macrocephalum (DC.) according to different sampling techniques.

	Sampling technique	
Chemical constituent	Steam distillation* (% of the integrated FID trace)	Head-space* (% of the integrated FID trace)
Monoterpenes		
α-pinene	n.d.	n.d.
sabinene	n.d.	n.d.
2,3-dihydro-1,8-cineole	n.d.	n.d.
myrcene	n.d.	n.d.
limonene	8.6	n.d.
trans-β-ocimene	n.d.	n.d.
terpinolene	n.d.	n.d.
Sesquiterpene		
β-cubebene	n.d.	n.d.
Fatty acid derivatives		
7-tetradecene	77	90.2
cis-4-decenal	n.d.	n.d.
pentadecanal	n.d.	n.d.
heptadecanal	n.d.	n.d.
β-cubebene	n.d.	n.d.

*The abbreviation n.d. refers to not detected.

control treatment (Fig. 1). The relative growth rate of *O. nubilalis* larvae was, however, reduced by 17% when α-terthienyl was added in a meridic diet at a concentration of 50 ppm. The reduction of the larval relative growth rate mediated by α-terthienyl was amplified 2.4-fold, *i.e.* 41% vs 17%, when insects were concurrently exposed to the volatiles released from the GSCs of the leaves of *P. ruderale* (Fig. 1). A nearly

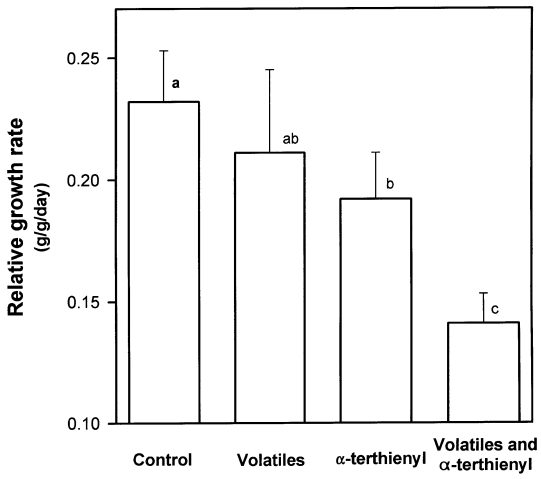

Fig. 1. Synergistic effects of the volatiles released from the glandular secretory cavities of the leaves of *Porophyllum ruderale* (Asteraceae) and α-terthienyl on the relative growth rate of third instar *Ostrinia nubilalis* larvae (the P value for the interaction between volatiles and α-terthienyl was 0.052). Error bars represent the standard deviation and treatments associated with distinct letters differ significantly (Tukey HSD multiple comparisons, $P < 0.05$).

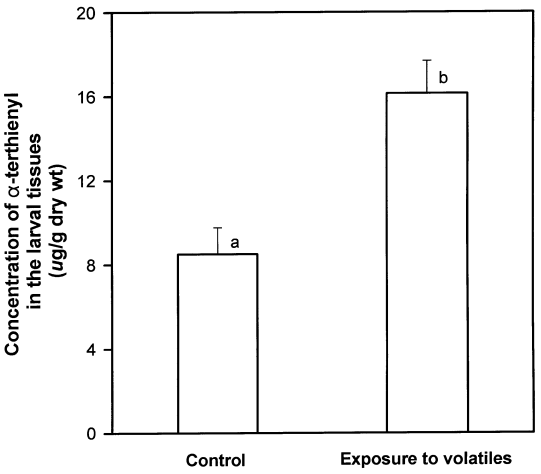

Fig. 2. Effects of the volatile monoterpenes released from the glandular secretory cavities of the leaves of *Porophyllum ruderale* (Asteraceae) on the retention of α-terthienyl in fifth instar *Ostrinia nubilalis* larvae fed with a meridic diet containing α-terthienyl. Error bars represent the standard deviation and treatments associated with distinct letters differ significantly (Tukey HSD multiple comparisons, $P < 0.05$).

two-fold increase in the retention of α-terthienyl in larval tissues of the European corn borer was also observed in presence of the volatiles released from the foliar GSCs of *P. ruderale* (Fig. 2).

DISCUSSION

The present study suggests that the volatile monoterpenes released from the leaves of *P. ruderale* are mainly sequestered in the GSCs. There was indeed no monoterpene detected in the stems of *P. ruderale* using static head-space sampling, although a small level of limonene was found with the steam distillation extraction. A similar profile of monoterpenes was furthermore observed in the extracts obtained by either the head-space or the steam distillation techniques using whole leaves, and the direct sampling of the liquid directly secreted from the GSCs (Table 1). It is expected that a sequestration of volatile monoterpenes in the GSCs also occurs for *P. gracile*—a typical pungent smell was released when the GSCs of both *P. gracile* and *P. ruderale* were squeezed—, although the content was not measured. The sequestration of monoterpenes into storage organs is well established [16]. For example, a mixture of monoterpenes including among others sabinene, camphor and α-pinene is contained in the trichomes of *Mentha piperita* and *Cleome spinosa* [17, 18]. Indole and monoterpenes are also sequestered in GSCs in leaves of *Tagetes erecta* [19].

It is likely that the monoterpenes, which constitute the major part of the chemicals emitted from the GSCs of *P. ruderale* (Tables 1 and 2), are responsible for the repellent activity previously reported for *P. gracile* and *P. ruderale* against red-legged grasshopper adults [5]. Although our results showed that the volatile monoterpenes released from the GSCs of the leaves of *P. ruderale* did not reduce the relative growth rate of European corn borer larvae, these monoterpenes synergized the effects of α-terthienyl against the same insect (Fig. 1). This synergism between volatile monoterpenes and α-terthienyl in the reduction of the relative growth rate of *O. nubilalis* was apparently due to a nearly two-fold increase in the concentration of α-terthienyl in larvae when they were exposed to the volatiles released from the foliar GSCs of *P. ruderale* (Fig. 2).

Other monoterpenes have been demonstrated to enhance the uptake of both hydrophilic and lipophilic chemicals into organic structures. For example, Yamane *et al.* [20] showed that limonene and cineole enhance by 3.6- and 95-fold respectively the partitionning of a hydrophilic drug, 5-fluorouracil, from aqueous solutions into human stratum corneum. Ogiso *et al.* [21] observed that cineole had a similar effect on the transdermal penetration of a lipophilic drug, indomethacin, in rat. Monti *et al.* [22] also used different terpenes as penetration enhancers of a chemotherapeutic drug, dapiprazole, through hairless mouse skin.

The increase of the chemical uptake into organic tissues mediated by some monoterpenes appears to be related to an alteration of the lipid organization in cellular membranes or in intercellular lamellar structures [20]. In sophisticated studies involving comp-

lementary results obtained from differential scanning calorimetry, small-angle X-ray diffraction and enhancer uptake in human stratum corneum, Cornwell *et al*. [23] and Yamane *et al*. [24] have shown that some monoterpenes, including cineole and limonene, partially disrupt the lipid bilayer organisation of cellular membranes.

An alternative explanation for the higher retention of α-T in *O. nubilalis* larvae exposed to volatiles (Fig. 2) is an inhibitory effect that these volatiles may exert on the metabolic detoxification of α-T which relies on the cytochrome P-450 enzymatic complex in this insect [25]. In support of this hypothesis, it has been established that β-myrcene and d-limonene inhibit the *in vitro* metabolic activity of a cytochrome P-450 monooxygenase (CYP2B1) in rat liver microsomes [26].

Overall, results of the present study indicated that the co-occurrence of monoterpenes with α-terthienyl may represent an integrated defensive strategy used by *Porophyllum* spp. to improve, by synergism, their chemical protection against herbivorous insects. A 'solvent hypothesis' has already been proposed that suggests monoterpenes may serve as agents for storage and delivery of lipophilic secondary compounds to an attacked site of host plants [27] However, the present study suggests that monoterpenes may also enhance the retention of toxic secondary compounds in insect tissues. Given the frequent co-occurrence in plants of monoterpenes and toxic higher molecular weight compounds and assuming that a similar synergism to the one observed in the present study also occurs between monoterpenes and other classes of secondary compounds, it is expected that monoterpenes may represent a widespread component of plant chemical defences.

EXPERIMENTAL

Plants. Seeds of *P. ruderale* were provided by Xavier Lozoya from Xochitepec (Mexico) while those of *P. gracile* were collected by Guillet in the area of Tucson (Arizona, USA) in August 1994. Seeds of both species were germinated in water and then transplanted in vermiculite. Plants were fertilized weekly with a Hoagland's solution [28] and grown under greenhouse conditions at 20°/8 hr night and 25°/16 hr day. Natural lighting supplemented with high-pressure sodium lamps provided a daytime irradiance of 300 ± 50 $\mu mol\, m^{-2}\, s^{-1}$ photosynthetic photon flux density.

Chemical sampling. The techniques used to extract the volatile compounds from plant tissues, steam distillation and static head-space techniques have been described previously [37, 38]. The steam distillation technique provides the full spectrum of volatiles in the tissue, although some rearrangements may occur, while the static head space technique yield the more readily volatalized materials. Direct sampling by micropipette of the liquid contained in the GSCs of the leaves of *P. ruderale* was also undertaken to compare constituents with those obtained with volatilization techniques as above. Direct sampling was not attempted with the foliar glands of *P. gracile* given their smaller dimensions.

Chemical analysis. GC analysis was performed with a FID and the columns used were a fused silica Durabond DB-5 (0.25 μm film thickness, 30 m × 0.25 mm i.d.), a fused silica Durabond DB-1 (1.0 μm film thickness, 30 m × 0.25 mm i.d), and a fused silica Durabond DB-Wax (0.25 μm film thickness, 30 m × 0.25 mm i.d.) (J & W Scientific, Folsom, CA). For all analyses, the temperature program was as follows: 40° initial temperature, 2°/min to 250° with helium as the carrier gas. The injector and detector temperatures were 230° and 250°, respectively. Injections of 1 μl were made in the splitless mode and changed to the split mode after 0.6 min. Retention indices were calculated relative to *n*-alkane standards. Electron impact MS (*ca* 100 ng sample) were obtained using an ionizing potential of 70 eV.

Insect toxicity. To verify if α-terthienyl and volatile monoterpenes, which co-occur in *P. ruderale* and *P. gracile*, exert additive toxic (insecticidal) effects against the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae), a simple factorial experiment was designed. The following treatments were performed: non-exposed larvae (control), larvae exposed to either volatile monoterpenes or α-terthienyl, and larvae simultaneously exposed to both chemicals.

Newly molted third instar *O. nubilalis* larvae obtained from a laboratory colony were maintained and fed a meridic diet as described in [29]. A meridic diet was warmed to 35° before adding α-terthienyl at a concentration of 50 ppm via a Me_2CO soln added at a rate of 5 μl/g. This concentration of 50 ppm of α-terthienyl is representative of the amount of thiophene derivatives occurring in tissues of *Porophyllum* spp [7]. and other Asteraceae [7, 30–32]. An equivalent amount of Me_2CO was added in the diet for treatments without α-terthienyl. Diets were then incubated in darkness at room temperature for two hr to let agar solidify and Me_2CO evaporate. Exposure of insects to volatile monoterpenes was controlled by inserting a leaf of *P. ruderale* harboring 5–7 GSCs of 2–3 mm of length inside a glass petri dish in which larvae were fed. Leaves were individually inserted under a fine metallic mesh of 25 mm of diameter that was glued on the inside of petri lids. This protocol made the leaves inaccessible to *O. nubilalis* larvae, although it did not prevent the volatiles released from GSCs from reaching the larvae. For treatments with exposure to volatiles, the GSCs were physically crushed at the beginning of the experiment whereas the GSCs were gently dissected out of leaves with a razor blade and discarded in treatments with no exposure to volatiles. Leaves from each dish were replaced every day. At the beginning of the experiment, 15 third instar larvae were weighed together then transferred in a glass petri dish containing five cubes of diet, *ca* 0.9 cm^3 each, with

or without α-terthienyl according to the respective treatment. For each of the four treatments, ten replicates were performed and insects were exposed to near-UV light as described in [33] to stimulate the photosensitization mediated by α-terthienyl [34]. The experiment lasted 48 hr and the final wt of insects was measured to determine the relative growth rate (weight gain/initial weight/day). The potential insecticidal activity was assumed to be higher in treatments with lower growth rate [35].

In a second experiment, the effects of the volatiles released from the foliar GSCs of *P. ruderale* on the retention of α-terthienyl in *O. nubilalis* larvae were investigated. Actively feeding mid-fifth instar larvae were exposed to the volatiles released from the GSCs of a leaf of *P. ruderale* as above while insects were fed for 24 hr in darkness on a meridic diet containing α-terthienyl at a concentration of 50 ppm. The peritrophic membrane of larvae was then removed before determining their α-terthienyl content to reduce variations related to diet remains possibly occurring in gut lumen of insects. This was done by cutting the head and the two or three last abdominal segments of each larva so that the peritrophic membrane and its content could be pulled out using fine dissection tweezers. Insect tissues without the peritrophic membrane were dipped in 10 ml of hexane and agitated for 48 hr in darkness to solubilize α-terthienyl. An aliquot of 1.5 ml of the hexane in which larvae were placed was then centrifuged (16 000 g, 5 min) and the supernatant was kept for HPLC analysis. The yield for the extraction of α-terthienyl according to this procedure was shown to be higher than 90% in a preliminary experiment. Insect tissues were then dried and weighed to determine the concentration of α-terthienyl in a dry wt basis. Ten *O. nubilalis* larvae were used per replicate and six replicates were performed for each treatment.

α-terthienyl analysis. Concentration of α-terthienyl was determined by HPLC using a Beckman Gold System equipped with a solvent module (model 126), a UV- detector (model 168) set to 350 nm and an autosampler (model 502). A C-18 reverse phase column (Beckman, 5 ODS, 25 cm* 4.6 mm) was used with acetonitrile:water (3:1) at a flow rate of 1.0 ml/min. Under these conditions, α-terthienyl had a retention time of 16.3 min and its concentration was determined by comparing the peak areas of samples to those of a standard curve made with pure α-terthienyl synthesized as described in [36].

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