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Autotoxic effects of essential oils on photosynthesis in parsley, parsnip, and rough lemon

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Summary. Many plant species contain essential oils with allelochemical properties, yet the extent to which these same chemicals can be autotoxic is unclear. In this study, we tested the toxicity of several essential oil components to three species that produce them—*Pastinaca sativa* and *Petroselinum crispum* (Apiaceae), and *Citrus jambhiri* (Rutaceae). The effects of exogenous application of small amounts of essential oil components to the surface of foliage, followed by a pinprick to allow entry into the leaf, were monitored by chlorophyll fluorescence imaging. Rapid and spatially extensive declines in photosynthetic capacity were detected within 200 s. The most toxic constituents were monoterpenes. Two sesquiterpenes, caryophyllene and farnesene, and the phenylpropanoid myristicin, by comparison, were not toxic. Autotoxicity of endogenous essential oil was investigated by slicing through containment structures (glands or tubes); significant toxicity, manifested by reduced photosynthetic activity, was observed in all three species but was most pronounced in *P. sativa* and *P. crispum*, both of which possess oil tubes.

Key words. Autotoxicity – essential oils – Apiaceae – wild parsnip – parsley – *Citrus*

Introduction

According to optimal defense theory, the synthesis and containment of defensive compounds in a plant exact costs in energy and raw materials that could otherwise be directed to other functions, such as growth and reproduction (Zangerl & Bazzaz 1992). This cost is thought to increase with the autotoxicity of the defense, as the plant must allocate more resources to insure the safe separation of its toxins from sensitive tissues and processes (McKey 1979).

Although the potential for autotoxicity in plants has been hypothesized to exist (e.g. McKey 1974; Fowden & Lea 1979), evidence of autotoxicity *in vivo* under ecologically relevant circumstances is scarce. In one case, Baldwin and Callahan (1993) investigated whether species-specific nicotine tolerance in several solanaceous species is associated with ability of the plant to produce nicotine. Two plant species that typically produce large amounts of nicotine, *Nicotiana sylvestris* and *N. glauca,* were no more resistant to hydroponically fed nicotine than two species, *Datura stramonium* and *Lycopersicon esculentum*, that produce no nicotine (Baldwin & Callahan 1993).

Foliar essential oil constituents possess a wide variety of biological activities consistent with defense; not only do they have antimicrobial, fungicidal, and insect antifeedant properties, but they may also act as synomones, attracting organisms that prey on the enemies of plants (Gershenzon 1999; Harrewijn *et al.* 2001). Monoterpenoids display both fungicidal and bactericidal properties, the mechanism of which appears to involve membrane disruption, leading to loss of both cell (Harrewijn *et al.* 2001) and organelle integrity (Uribe *et al.* 1991). Membrane disruption has been implicated in plant toxicity as well (Maffei *et al.* 2001) and may account for the difficulty of maintaining tissue cultures for producing these compounds (Brown *et al.* 1986). However, studies of terpenoid effects to date have generally been conducted under artificial conditions, on undifferentiated tissues, isolated cells, or organelles. In addition to the terpenoids, essential oils often contain phenylpropanoids, a diverse group of compounds with equally diverse modes of biological activity (Rosenthal & Berenbaum 1991), but their autotoxicity to plants under natural conditions is unknown.

In this study we assessed the potential for autotoxicity from several monoterpenes and sesquiterpenes and another common essential oil component, the phenylpropanoid myristicin, in two apiaceous species, *Petroselinum crispum* (Miller) Nyman ex A.W. Hill (Apiaceae) and *Pastinaca sativa* L. (Apiaceae) and in the rutaceous species *Citrus jambhiri* Lush (Rutaceae). First, we examined the toxic effects of exogenous applications of pure compounds and then we observed the autotoxic effects associated with release of endogenous essential

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oil known to contain these compounds, among others. Toxicity was assessed by quantifying reductions in photosynthetic capacity as measured by chlorophyll fluorescence imaging.

Materials and methods

Autotoxicity of essential oil components was examined in three species – wild parsnip (*Pastinaca sativa*), parsley (*Petroselinum crispum*), and rough lemon (*Citrus jambhiri*). Differences among these species in containment structures permit comparisons of autotoxicity according to the manner in which the essential oils are stored; parsley and wild parsnip sequester the oils in oil tubes (Hegnauer 1971), whereas in *Citrus* the oil is inside glands (Fahn 1979).

All three species are know to contain substantial amounts of essential oil and several of the essential oil components assayed are shared by all three species, facilitating comparisons of toxicity among species. Myrcene, α-pinene, γ-terpinene, terpinolene, limonene, and caryophyllene occur in all three species tested. Additionally, farnesene occurs in wild parsnip, geraniol and limonene occur in both parsley and rough lemon, and myristicin occurs in both wild parsnip and parsley (Masada 1976; Kubeczka & Stahl 1977; Agricultural Research Service – http://www.ars-grin.gov/duke). To verify the occurrence of these compounds in leaves of the test plants, we performed gas chromatography coupled to a mass spectrometer (Hewlett Packard, Agilent, Palo Alto, CA), and then also performed gas chromatography with flame ionization detection to estimate the relative abundance of essential oil components.

Parsley and rough lemon were cultivated in the University of Illinois Entomology greenhouse. Wild parsnips were transplanted to pots from locations in the vicinity of Urbana (Champaign Co.), IL. Daylength in the greenhouse was maintained at 16 h with 250 W metal halide lamps. Air temperature was controlled at 28 °C during the day and 25 °C at night. All experiments were conducted with vegetative plants. The compounds assayed were β-caryophyllene, (80 %, TCI America, Portland OR), farnesene (mixed isomers, TCI America, Portland OR), myristicin (>85 %, Sigma, St. Louis MO), geraniol (99 %, Aldrich, Milwaukee WI), myrcene (tech., Aldrich, Milwaukee WI), limonene (97%, Aldrich, Milwaukee WI), α-pinene (98 %, Aldrich, Milwaukee WI), γ-terpinene (95 %, TCI America, Portland OR), and terpinolene (racemic 85 %, TCI America, Portland OR). The impact of each of the tested compounds on photosynthesis was assayed 9 to 10 times by topical application for each plant in which the compound occurred. Only compounds that naturally occur in the plant were tested. Further experiments were conducted in which endogenous oils were released by a single razor cut across the leaf (five replications per species).

We employed a pulse-amplitude-modulated imaging chlorophyll fluorometer (IMAGING-PAM model IMAG-L, Heinz Walz GmbH, Effeltrich, Germany) to map reductions in photosynthetic activity across leaf areas associated with topical application of compounds and with razor cuts. The fluorimeter consists of a ring of 96 blue light-emitting (470 nm) diodes that provide measuring, actinic, and saturating pulses of blue light. Images of chlorophyll fluorescence were captured by a video camera. The images provide quantitative spatially resolved data on the quantum efficiency of electron transport through photosystem II (ϕ_{PSII}) Genty *et al*., which, under low oxygen, is closely correlated with rates of CO_2 assimilation (DiMarco *et al.* 1990; Edwards & Baker 1993; Siebke & Weis 1995).

Prior to each trial, a healthy young leaflet still attached to the plant was situated in the fluorescence imager and allowed to light-adapt to a moderate intensity of actinic light (225 μmoles quanta m^{-2} s^{-1} PAR) for at least 12 minutes until uniform and stable levels of ϕ_{PSII} across the leaf were observed. Fluorescence data were subsequently collected following saturating pulses of light (2400 μmoles quanta m^{-2} s^{-1} PAR) at 20-second intervals. The intensity of the light pulses used to make measurements of fluorescence was set at the default and made a negligible contribution to the actinic illumination.

With measurement pulses occurring at 20-second intervals, a 0.5 μL droplet of a compound was assayed by placing it on a leaf surface between major veins. Immediately afterward, an insect pin was inserted through the center of the droplet and into the leaflet; the prick was timed to coincide with a measurement pulse and allowed the compound to enter into the leaf. The effect of the compound was then monitored *in vivo* by recording chlorophyll flourescence of the leaf every 20 seconds for at least 240 seconds. Depending on the spatial extension of the effect, one to four droplets of each compound were assayed for each leaf before light-adapting the next fresh leaf. Pinpricks without compound served as controls. Images of ϕ_{PSII} taken 200 s after treatment were analyzed with SCION IMAGE for Windows software (BETA VERSION, 4.0.2, Scion, Frederick, MD). The area (mm^2) of treatment-induced reduction of photosynthesis was quantified from these images by selecting all area contiguous to the application point with values lower than the surrounding uniform areas of ϕ_{PSII}.

Most of the compounds tested occur in all three species, and for these compounds a two-way analysis of variance was performed with compound and species as main effects (SPSS for Windows version 9.0.0, SPSS Inc., Chicago). This analysis allowed us to determine whether compounds are differentially toxic and whether species differ in sensitivity. Simple one-way analyses of variance were performed for effects of compounds within each species.

To examine the effects of release of endogenous oils, we cut leaflets perpendicular to the major vein. The cut was made by applying downward pressure to the blade rather than by a side-to-side slicing motion, which would have spread the oils away from their locations inside the leaf (the oil tubes associated with veins in wild parsnip and parsley and the oil glands in rough lemon). Fluorescence images were taken every 20 s for 200 s following cutting, and the effect was calculated as the area of reduced photosynthesis per mm of cut (mm^2 mm^{-1}) after 200 s. The effect of razor cuts was analyzed by one-sample t-tests with zero effect as the null hypothesis.

Results

Droplets of compounds rapidly infiltrated the leaves through the pinpricks, creating circular (Fig. 1) or irregular areas of reduced ϕ_{PSII}. Toxicity of the compounds, as measured by area of impaired ϕ_{PSII}, varied dramatically according to the type of compound and species (Fig. 2). As well as highly significant main effects of compound and species, a significant interaction was detected (Table 1), indicating that the relative toxicity of the spectrum of compounds differed among species. Post-hoc Tukey tests revealed that rough lemon was more sensitive to essential oil compounds than the other two species ($P < 0.001$) and that toxicity of the compounds tested in all three species was highest for terpinolene, followed in turn by γ-terpinene and α-pinene ($P < 0.01$). Compounds not displaying significant toxicity were the sesquiterpene caryophyllene, the oxygenated monoterpene geraniol and the non-oxygenated monoterpene myrcene, all three of which were significantly less toxic than terpinolene, γ-terpinene, and α-pinene ($P < 0.01$; (Fig. 2). Additional components tested that were not common to all species suggest a general structure-activity pattern among the terpenes. For example, similar to the sesquiterpene caryophyllene, another sequiterpene, farnesene, was not toxic in wild parsnip (Fig. 2). Limonene, like

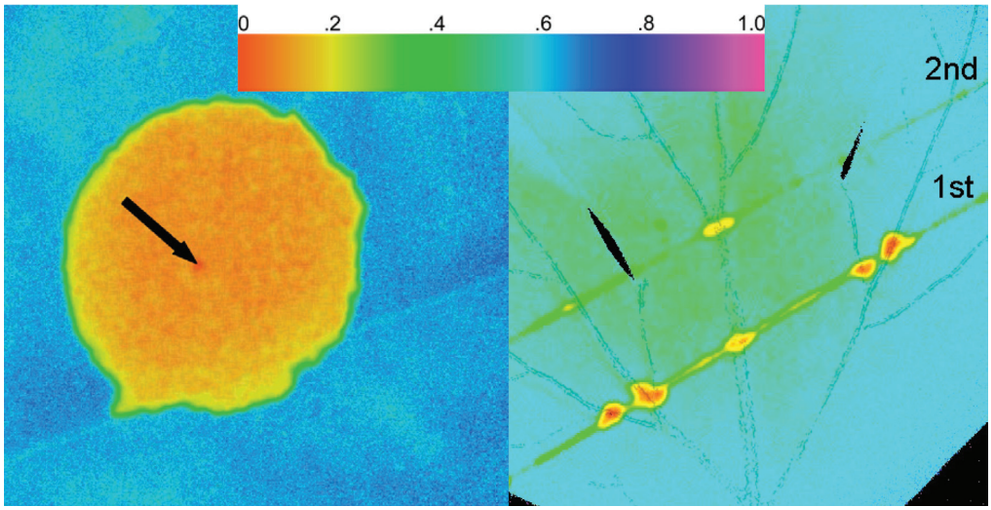

Fig. 1 On the left, effect of γ-terpinolene on ϕ_{PSII} in rough lemon after 200 s (location of the droplet of compound and the pinprick are indicated by the arrow). The effect of razor blade cuts on ϕ_{PSII} in a parsley leaf after 200 s (right). The first and second razor blade cuts were made approximately 40 s apart. Superimposed on the image is the location of major veins in the leaf. The color bar gives equivalent values of ϕ_{PSII}

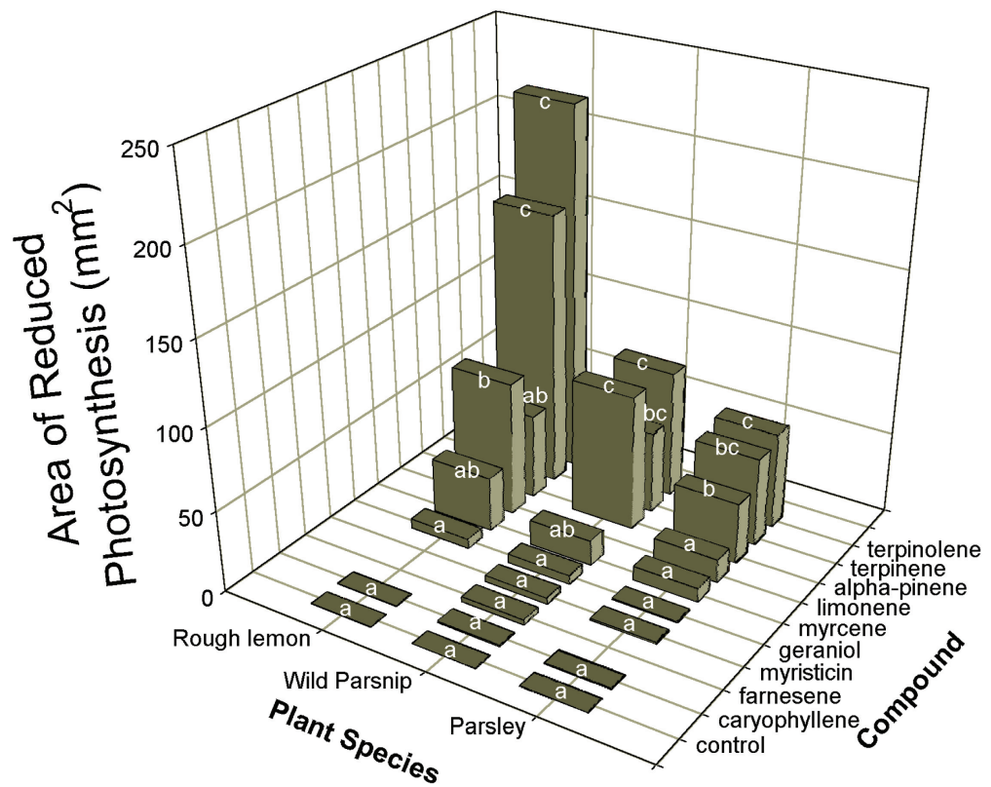

Fig. 2 Mean effects of essential oil components on photosynthetic capacities of wild parsnip, parsley and rough lemon. Controls are pinpricks only. Bars within a species sharing the same letter are not significantly different (critical P value 0.05, Tukey test)

Table 1 Two-way analysis of variance of plant species (wild parsnip, parsley, and rough lemon) and compound (myrcene, α-pinene, terpinolene, γ-terpinene, and caryophyllene) effects on toxicity to foliage

Source	d.f.	Mean square	F	P
species	2	43492	36.8	< 0.001
compound	5	67006	56.8	< 0.001
species × compound	10	16571	14.0	< 0.001
error	151	1179		

most of the other monoterpenes, was toxic in *Citrus*. Myristicin, the only phenylpropanoid tested, was not toxic. Although the fluorescence measurements did not extend beyond 240 s after treatment, the effects of the treatments were permanent, as the areas affected turned brown and brittle within 24 h and never recovered.

Leaves cut with a razor blade exhibited less extensive but nonetheless significant reductions in ϕ_{PSII}. The reductions were greatest for parsley and smallest for rough lemon

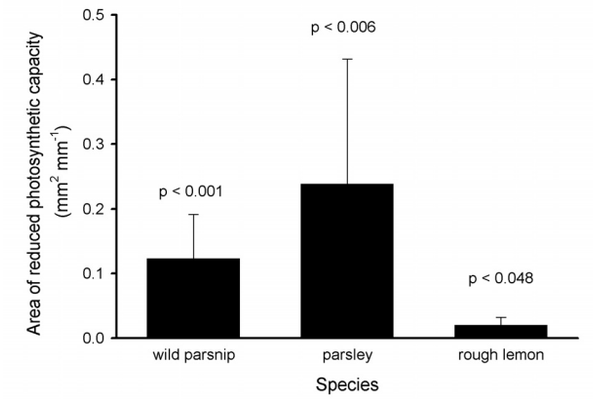

Fig. 3 Effect of razor cut on reduction in photosynthetic capacity of wild parsnip, parsley and rough lemon

(Fig. 3). As in the oil droplet experiment, the effects of the razor cuts were manifested as "fronts" moving rapidly away from the cut. In wild parsnip and parsley, where the essential oils are contained in tubes running inside the leaf veins, the effects were most dramatic in the vicinity of severed veins (Fig. 1). A vein severed mechanically in either of these two species resulted in expulsion of a droplet of oil visible to the naked eye. Subsequent severing of the same vein at a different location produces no droplet, indicating depressurization of the oil tube; this depressurization was temporary and within 24 h the tubes again produced droplets when severed (A. Zangerl, personal observation). This feature was used to confirm the role of oil tube contents in effecting the decrease in photosynthetic capacity. Leaves cut and then after a brief period (~40 s) cut again parallel to, and ~4 mm from, the first slice and either anterior or posterior to it had negligible effects on photosynthesis (Fig. 1).

Mass spectral analysis of a hexane extract from each species used in these experiments confirmed published reports of essential oil composition. Analysis of these extracts by GC with FID revealed that the monoterpenes were far more abundant than the sesquiterpenes in parsley and *Citrus* (based on peak areas, 87 % and 94.5 % of the terpenes were monoterpenes, respectively). In contrast, the sesquiterpenes were far more abundant in wild parsnip, comprising 74.5 % of total terpene peak area.

Discussion

Toxicity of essential oil components assayed in this study was largely limited to a single class of compounds—the monoterpenes. The sesquiterpenes and the phenylpropanoid myristicin had no significant effect (Fig. 2). Although there was significant variation in toxicity of monoterpenes in all three species, there was no clear relationship between other properties of monoterpenes, such as density, boiling point or polarity (e.g., the octanol-water partition coefficients calculated from reversed-phase high-performance liquid chromatography, Griffin *et al*. 1999) and their toxicity. One mode of action of monoterpenes may involve membrane disruption. Maffei *et al*. (2001) found that many monoterpenes disrupt membranes of cucumber root cells (*Cucumis sativus*). The phenylpropanoid myristicin, which is found in several plant families (http://www.ars-grin.gov/duke/) and did not affect ϕ_{PSII} in this study, is not known to disrupt membranes.

If plants adapt to their allelochemicals via mechanisms that make them resistant, one might expect that they would exhibit greater resistance to those compounds that they produce in greater abundance. This was not the case, as the monoterpenes appeared to be as toxic to *Citrus* and parsley, species in which monoterpenes make up the bulk of the terpenes, as to wild parsnip, in which monoterpenes are a minor component of the terpene complement.

Essential oils are typically restricted to a variety of containment structures, including single-celled idioblasts and lactifers and multicellular resin ducts, oil tubes, trichomes, and secretory cavities (Gershenzon & Croteau 1991). The necessity for at least some plant species to sequester monoterpenes is clear from the results of this study; small amounts of some of these compounds rapidly suppressed ϕ_{PSII}, a major component of photosynthetic capacity, over large areas. The nature of the containment of essential oils can also mitigate autotoxicity. In *Citrus* foliage, essential oils are confined to small glands (Turner *et al.* 1998); breakage of any one of these glands released tiny amounts of oil not visible to the naked eye. Consequently, the effect of severing an oil gland in *Citrus* was not nearly as destructive as the severing of an oil tube in parsley and wild parsnip (Fig. 3).

Ordinarily, essential oils are safely confined within their containment structures, but in the course of feeding by herbivores they can be released and add to the damage done by the herbivores. During feeding, leaf-chewing insects are likely to release essential oils much as we did with a razor blade. The effect of insect-feeding may be even more dramatic, however, if oils transferred to the mandibles of an insect are secondarily transferred to other tissues. Although the spatial impact on photosynthesis of releasing endogenous oils was small in this study, leaf-chewing insects frequently produce collections of irregular-shaped holes within a single leaf, leaving far more extensive networks of cut edges than would be produced by removing the same area as a single hole. Thus, the post-attack cost of autotoxicity may be substantial for plants that rely on monoterpenes for defense.

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