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Methyl Salicylate Secretory Cells in Roots of *Viola arvensis* and *V. rafinesquii* (Violaceae)

W. JOHN HAYDEN and JOHN CLOUGH

ABSTRACT

The aromatic roots of *Viola arvensis* and *V. rafinesquii* were studied in order to determine the chemical nature and anatomical localization of their volatile compounds. Gas chromatography and mass spectroscopy revealed a single detectable volatile compound, methyl salicylate. Light microscopy and differential staining with Sudan III indicates the source of this compound to be enlarged secretory cells located in the endodermis, an unusual position for such cells in roots of angiosperms. The secretory endodermal cells are sporadic, but are more frequent in primary roots than in secondary roots and the lower portion of the hypocotyl. It is hypothesized that secretory endodermal cells are restricted within *Viola* to subgenus *Melanium* where the methyl salicylate confers protection against herbivores and/or pathogens.

Viola arvensis Murray and *V. rafinesquii* Greene, known as pansies or field pansies, are widespread in eastern North America; both are members of *Viola* subgenus *Melanium* Ging. (Melchior and Becker 1925). While *V. arvensis* is naturalized from Eurasia, opinions have differed over the provenance of *V. rafinesquii*. Some consider *V. rafinesquii* to be native to North America (Brainerd 1911, Radford et al 1968, Strausbaugh and Core 1970-1977), yet others treat it as a minor variant of *V. kitaibellana* R. & S., adventive from north Africa (Fernald 1938, 1951). Both species are winter annuals of frequent occurrence in the Richmond, Virginia area.

While identifying species of violets in a plant systematics class, it was noticed that roots of both *V. arvensis* and *V. rafinesquii* possess the odor of oil of wintergreen (methyl salicylate). Radford et al (1968) note the presence of this odor in *V. arvensis*, but not for *V. rafinesquii*; curiously, this information concerning root odors in these species is reversed from that presented in Fernald (1951). Confusion in the floristic literature notwithstanding, salicylic acids have long been known in species of subgenus *Melanium*. For example, Wherry (1927) reported methyl salicylate in *V. rafinesquii* (based solely on olfaction), and Hegnauer (1962-1969) cites several European reports, all from the 19th or early 20th century. Aromatic constituents of plants are often localized in special secretory cells (Esau 1965). Such cells, however, have not been reported in accounts of the anatomy of violet roots (Freidenfelt 1904, Kaczmarek 1915, Melchior and Becker 1925, Metcalfe and Chalk 1950). The present study of *V. arvensis* and *V. rafinesquii* was undertaken, therefore, to

confirm the chemical identity of their root-born aromatic compounds and to determine the location of these compounds within the root tissue.

MATERIALS AND METHODS

Specimens. A total of seven populations located in and around the Richmond, Virginia area were sampled for chemical and anatomical studies at various times from April to June, 1988. Voucher specimens, deposited in the University of Richmond Herbarium (URV), are as follows: *Viola arvensis*, Clough 63 & 70; *V. rafinesquii*, Clough 60, 61, 62, 64 & 65.

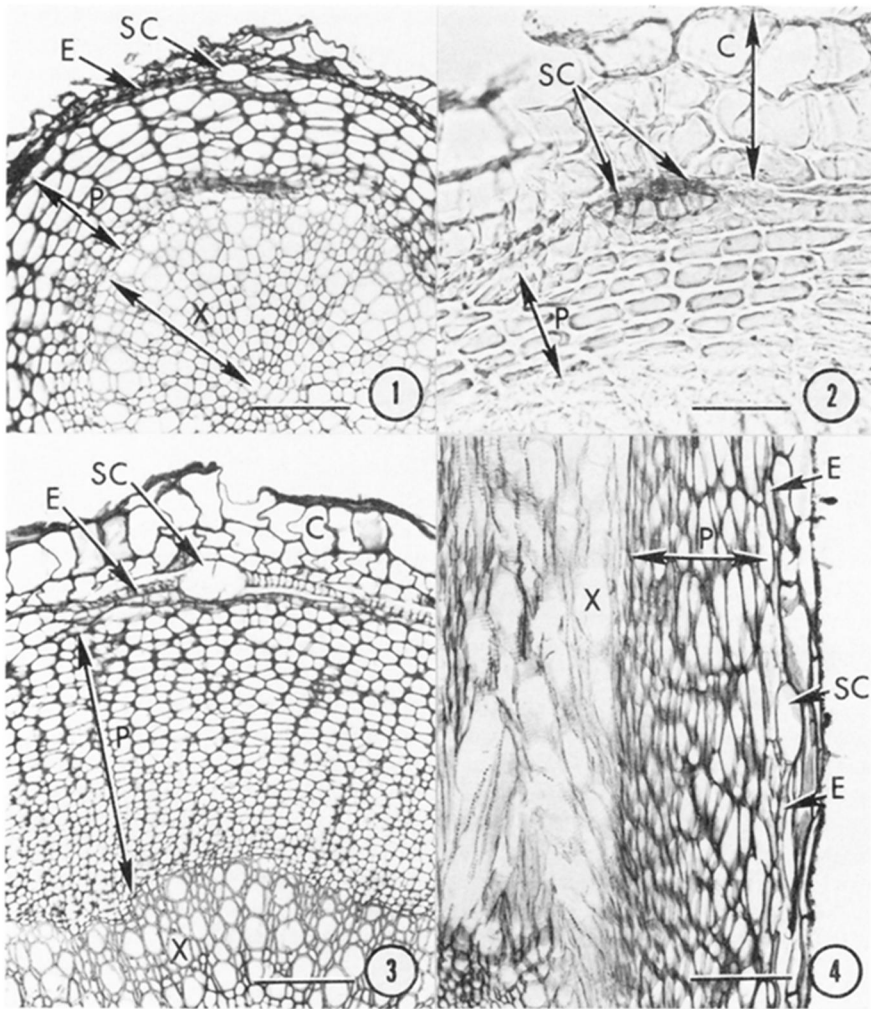
Chemical analysis. Samples for gas chromatography and mass spectroscopy were either freshly collected roots or roots that had been sealed in plastic bags and frozen at -20°C . For each analysis, a quantity of roots ranging from 0.25 to 0.5 g was sealed in a 1.5 ml vial and heated at 100°C for 2 minutes to liberate volatile compounds from the tissue. Ten μl of head space gases were injected into a Hewlett-Packard Model 5890 Gas Chromatograph fitted with a cross-linked methyl silicone high performance capillary column with a film thickness of $0.33\ \mu\text{m}$, internal diameter of 0.20 mm, and length of 12 m. The gas chromatograph used was interfaced with a Hewlett-Packard model 5970 mass selective detector. Volatile compounds were identified by comparison of retention times and mass spectra with those of authentic samples.

Anatomical studies. Root tissues were studied from both permanent and temporary preparations. Permanent slides were made from primary roots, secondary roots, and hypocotyl by means of the paraffin embedding technique (Johansen 1940). This technique includes fixation in FAA₇₀ (formalin—acetic acid—70% alcohol, 5:5:90 by volume), dehydration in tertiary butanol, embedding in paraffin, sectioning at $10\ \mu\text{m}$ on a rotary microtome, staining with a combination of Heidenhain's iron-alum hematoxylin and safranin, and mounting in Permout. Since the paraffin technique employs solvents that dissolve lipids from tissues, temporary sections were also prepared in a manner avoiding lipid solvents. Temporary sections were prepared from freshly collected primary roots which were frozen on the stage of a Hacker Instruments Cryo-Histomat and sectioned at $30\ \mu\text{m}$ on a sliding (sledge) microtome; these cryotomed sections were then stained for 10 minutes in water containing a few drops of Sudan III (saturated solution in 100 percent ethanol) and mounted in glycerine. Photomicrographs were prepared with a Nikon Optiphot brightfield microscope and UFX camera system on 35 mm Kodak Technical Pan film developed with Kodak HC110 developer at dilution F.

OBSERVATIONS

Chemistry. Roots of all populations in the study yielded a single detectable volatile compound. Authentic samples of pure methyl salicylate (oil of wintergreen) produce the same retention times and spectra as those from the violet roots, thus confirming the presence of this compound in the roots of *Viola arvensis* and *V. rafinesquii*.

Anatomy. (Figures 1-4). Primary roots and hypocotyls of both species were studied after the initiation of secondary growth. Secondary xylem is clearly distinguishable from primary xylem by virtue of its wider tracheary



Figures 1-4. Primary roots of *Viola arvensis* and *V. rafinesquii*. 1. *V. arvensis*, Clough 63, cross section, paraffin-embedded root, bar = 100 μm ; 2. *V. arvensis*, Clough 63, cross section, cryotomed root, bar = 50 μm . 3. *V. rafinesquii*, Clough 64, cross section, paraffin-embedded root, bar = 100 μm . 4. *V. rafinesquii*, Clough 64, longitudinal section, paraffin-embedded root, bar = 100 μm . C = cortex; E = endodermis; P = phloem region; SC = secretory cell(s); X = secondary xylem.

elements and sclerified prosenchyma. Pericycle, primary and secondary phloem are not easily distinguished in the roots studied. Most phloem elements exhibit thickened nacreous walls in fresh cryotomed sections. The vascular cylinder is bounded by endodermis readily distinguishable by thicker walls and smaller radial dimensions than cells on either side. Tissues external

to the endodermis exhibited various degrees of degradation, as is common in late developmental stages of soil-collected roots. Epidermis was generally absent, only fragments of exodermis remained, and the persistence of cortex was variable. Secondary roots were structurally similar to primary roots except that all tissue regions were smaller in secondary roots.

Study of paraffin-sectioned slides (Figures 1, 3, 4) yielded presumed secretory cells only in the endodermis. These endodermal secretory cells are radially 2-4 times wider than ordinary endodermal cells, and occur singly or in groups of 2-5 contiguous cells. Their occurrence is sporadic in primary roots. Similar cells are also present but much less frequent in secondary roots and the extreme lower part of the hypocotyl. Upper regions of the hypocotyl are devoid of secretory cells. The endodermis stains selectively with Sudan III in freshly prepared sections that have not been exposed to lipid solvents. In ordinary endodermal cells only the walls accumulate the stain, and only lightly so. In contrast, the larger secretory cells stain darkly with Sudan III (Figure 2), confirming the presence of lipid deposits.

DISCUSSION

Walls of endodermis cells in fresh cryotomed sections consistently stained lightly with Sudan III stain, whereas the stain was primarily located in the cytoplasm of the secretory cells. Given the age of the roots studied and the thickness of the ordinary endodermal cell walls, their light staining with this lipid-soluble stain may reasonably be attributed to suberin lamellae (Esau 1965).

This study confirms previous reports of the presence of methyl salicylate in roots of *Viola* subgenus *Melanium*, and, moreover, it provides the first evidence that this compound is located in enlarged secretory cells of the endodermis. Previous studies of violet root anatomy (Freidenfelt 1904, Kaczmarek 1915, Melchior and Becker 1925, Metcalfe and Chalk 1950) have involved species of other subgenera; these studies neither mention nor illustrate endodermal secretory cells. It thus seems that methyl salicylate secretory cells may be a feature unique to roots of subgenus *Melanium*. From the present study little can be said concerning the sub-cellular localization of methyl salicylate in roots of these violets. Other fixation, sectioning and imaging techniques could be profitably applied to the question.

A wide diversity of secretory cells are known in the roots of angiosperms (Guttenberg 1968). However, endodermal cells bearing deposits of essential oils are unusual, having been reported previously for *Lobelia siphilitica* L. (Campanulaceae), and three tropical genera of Clusiaceae, *Garcinia* L., *Rheedia* L., and *Xanthochymus* Roxb. (Guttenberg 1968). Species of *Viola* subgenus *Melanium* can now be added to the record of plants bearing this unusual anatomical structure.

A large and growing body of information ascribes protective functions to many of the diverse secondary compounds found in plants (Levin 1976). Singleton and Kratzer (1973) have noted deleterious effects of salicylates on herbivores, and Feldman and Hanks (1968) have demonstrated that salicylic acid increases in *Citrus* cultivars in response to infection by the nematode

Radopholus similis. It seems reasonable to hypothesize that methyl salicylate in the roots of *V. arvensis* and *V. rafinesquii* confers protection against soil-born pathogens or herbivores.

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Book Review

Beal, Ernest O. and John W. Thieret, 1986. Aquatic and Wetland Plants of Kentucky. Kentucky Nature Preserve Commission Scientific Journal and Technical Series, Number 5, Frankfort, Kentucky 40601. vii + 315 pages, illustrated with line drawings. \$20.00 softback.

An inexpensive book with readable and clear illustrations treating a difficult group of plants is indeed remarkable. The authors bring much experience and scholarship to this work on aquatics.

There are few surprises with the authors' working definition of aquatic and wetland plants and habitats. The taxa are mostly those one expects. The keys work well! There is even a welcome addition of a special key to vegetative specimens of "true aquatics." An excellent glossary and bibliography are also provided.

The strictly alphabetical arrangement of families and genera seems awkward, e.g. in the treatment of Hydrocharitaceae one finds a brief family description and a fine key to the genera. The next entry following the key is *Hydrocotyle* (Apiaceae). One must look elsewhere to find the generic description and key to species of *Elodea*.

Family and generic descriptions are brief but adequate. There are no species descriptions but very helpful notes on occurrence, habitat, distribution, nomenclature and selected references are provided. Following the systematic section is an atlas with county distribution maps for each species verified as occurring in Kentucky. Different symbols are used for specimens and for literature reports.

The illustrations by Sara Fish Brown are clear and useful although, as always, not as numerous as one would like.

The index (without page numbers) is really an index to common names and synonyms. Accepted names of taxa used in the text are not included. This may well be a source of persistent error for those of us who refer to an index without reading its introduction. This reduces the use of this manual as a reference.

This book is a most welcome addition to the growing number of aquatic plant manuals and a major step toward a modern and model treatment of the flora of Kentucky.—JIM MASSEY, HERBARIUM, UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL, NORTH CAROLINA 27599-3280.