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DETERMINATION OF FURANOCOUMARINS ON THE LEAF SURFACE OF *RUTA GRAVEOLENS* WITH AN IMPROVED EXTRACTION TECHNIQUE

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ABSTRACT.—A novel technique, involving a brief dipping of *Ruta graveolens* leaves into H_2O near its boiling point, removed from the surface amounts of furanocoumarins one to three orders of magnitude greater than those removable by conventional extraction with organic solvents at room temperature. Tests failed to reveal any significant leakage from the plant interior due to cell disruption. Evidence is presented that the greatly enhanced recoveries are largely the result of damage by hot H_2O to the cuticle, which releases the associated coumarins. This technique may have more general application to other surface compounds in this and other species.

Ruta graveolens L. (Rutaceae) has been notorious since at least the first century of the Christian era for its ability to produce erythema and pustular eruptions on human skin coming in contact with it (1). In the present century, the lesions were characterized as a photophytodermatitis induced by exposure to uv and due to the presence in this species of linear furanocoumarins—psoralen and some of its derivatives (2). Although these coumarins have long been known as constituents of the species (3–5), little is known of their localization within tissues.

Accumulation of secondary metabolites on the plant surface has not been extensively studied. The presence of flavonoids on the surface has been shown (6). About 30 occur in *Populus balsamifera* (7), and in some species the amounts are very high—up to 18% of the dry wt of *Dodonaea viscosa* and 28% in *Flourensia resinosa* (6). Occurrence of bergapten and xanthotoxin in the leaf surface wax of *Daucus carota*, where they may act synergistically in stimulating oviposition by the carrot fly, *Psila rosae*, was reported by Städler and Buser (8) and confirmed by Ceska *et al.* (9). The latter group have also reported the occurrence of crystals of furanocoumarins on the surface of parsnip roots infected by a fungus (10). Nothing is known about *R. graveolens* coumarins in this context. The original aim of the present work was to study the localization of the three dominant furanocoumarins of *R. graveolens*—psoralen, xanthotoxin, and bergapten (2)—in the leaves and stem of this species, with special attention to the surface.

Waxes exist on the surfaces of plants, and we agree that, if they are only on the surface, extraction times can be prolonged without concern about eventual leakage from epidermal cells. For extraction of surface waxes, Tulloch and his associates used hexane for different times depending on the species, ranging from 10 sec (11,12) to 45 sec (13,14). Researchers working with water-soluble flavonoids used shorter times, and they have recommended "rinsing" or "washing" with MeOH (15) or Me_2CO (16–18). In attempts to determine the amounts of a substance localized on a plant surface, an important point to be resolved is whether selective extraction from the surface can be achieved; i.e., can the substance be completely removed by methods gentle enough that they do not induce leakage from the interior cells? This question of selectivity appears not to have been sufficiently considered in past studies.

We wish to report here what we believe to be a new approach involving a very short immersion (a single brief dipping) of the plant parts in H_2O near its boiling point. Amounts of furanocoumarins between one and three orders of magnitude greater than those removable by cold MeOH or Me_2CO have been recovered by this technique, unaccompanied by any evidence of cell damage or ensuing leakage from the interior cells.

RESULTS AND DISCUSSION

On the leaf surface there is visible macroscopically a gray coating that was removable by gentle rubbing against filter paper. Extracts of these filter papers were examined qualitatively by tlc, which revealed spots corresponding to authentic samples of the expected furanocoumarins: psoralen, xanthotoxin, and bergapten. Their existence on the surface and removal with the filter paper were confirmed by hplc analysis.

In attempts at quantitative removal of these coumarins from the surface, we first examined two organic solvents known to remove flavonoids from the surface: cold MeOH and Me_2CO, in both of which coumarins are readily soluble in vitro. Extraction times of 5 and 60 sec were used in each case, as the exact rinsing times used in other laboratories were uncertain. The rinsing or washing procedures used previously were supplanted here by a dipping technique in an effort to achieve more uniform removal of substances from both sides of the leaf simultaneously. Knowing that these solvents can rapidly enter the tissues, we further tried to reduce the extraction time to a minimum of one short dipping (ca. 1 sec), but as such brief exposures to either organic solvent removed little of the coumarins, we explored the effect of brief dipping into H_2O just below the boiling point.

Table 1 shows the amounts removed by the latter technique compared to dipping in

TABLE 1. Comparison of Extraction Procedures for Removal of Furanocoumarins from *Ruta graveolens* Leaf Surface.

Solvent	Time of Immersion (sec)	Amount removed (μg/g fresh wt)				Ratio of coumarins extracted (P:X:B)
		P[a]	X[b]	B[c]	Total	
Hot H_2O . . .	< 2	220	440	57	717	3.9:7.7:1
MeOH	5	0.21	1.2	0.26	1.7	0.81:4.6:1
	60	2.0	5.5	0.89	8.4	2.2:6.2:1
Me_2CO	5	1.1	1.4	0.38	2.9	2.9:3.7:1
	60	2.6	5.5	1.7	9.8	1.5:3.2:1

[a]P, psoralen.
[b]X, xanthotoxin.
[c]B, bergapten.

the organic solvents. The shortest dipping in 96° H_2O released a total of ca. 720 μg/g of furanocoumarins (fresh wt), with a psoralen-xanthotoxin-bergapten ratio (P:X:B) of 3.9:7.7:1. In sharp contrast, MeOH removed less than 2 μg/g in 5 sec (P:X:B = 0.81:4.6:1), and scarcely more than 8 μg/g even after 60 sec (P:X:B = 2.2:6.2:1). Me_2CO was only slightly more efficient, removing a total of 9.8 μg/g (P:X:B = 1.5:3.2:1) after 60 sec. In all cases xanthotoxin predominated, with bergapten almost always recovered in the smallest amounts. The ratios of extraction efficiencies of hot H_2O (1 sec), Me_2CO (5 sec), and MeOH (5 sec) (W:A:M) were: psoralen 1000:5.7:1, xanthotoxin 390:1.3:1 and bergapten 220:1.5:1. The corresponding W:A:M ratios for the 60-sec solvent extractions were psoralen 110:1.3:1, xanthotoxin 80:1.0:1, and bergapten 63:1.9:1. A single dipping in hot H_2O thus exceeded in efficiency that of 5 sec in MeOH by factors of ca. 220–1000, and of 60 sec in MeOH by ca. 60–110. The corresponding factors for Me_2CO are, for 5-sec extractions, ca. 150–300, and for 60-sec extractions, 33–85.

Immersion in any of these solvents for as long as 60 sec seemed inadvisable owing to the possibility of cell damage. However, in the case of the brief dipping in almost boil-

ing H_2O three criteria were used to establish that the epidermal cells suffered minimal, if any, damage. No evidence of cell disruption was shown by tests on *R. graveolens* involving plasmolysis followed by deplasmolysis. These tests were repeated on red onion, *Allium cepa* L. (Liliaceae) because the red vacuolar anthocyanins permitted ideal observation conditions by rendering easily visible the border between the vacuole and cytoplasm. After plasmolysis, in each case, the sugar solution used was substituted under the microscope by H_2O, and deplasmolysis was observed, proving that the membranes had not been damaged and that the cells remained alive. In addition, vital staining was done with epidermal peels of *R. graveolens* after H_2O dipping. Neutral red stain in aqueous solution penetrated the vacuoles of the peel when placed on a standing droplet of the dye for 10 min. Preferential capture of the stain, as evidenced by red coloration of the vacuole, is proof that the tonoplast was undamaged and the cells were alive 10 min after the dipping experiment. Finally, the possibility of cell damage with ensuing leakage of cell sap was examined by conductance measurements of the medium after successive immersions of a *R. graveolens* leaf in H_2O at 23°, and in the same H_2O, boiling, for varying times. The conductance of the H_2O (L = 7.0 μS) into which the leaf had been immersed was, after 10 min at 23°, 7.7 μS and after 2 sec, 1 min, and 10 min at boiling temperature, 8.3, 40, and 259, respectively. The very slight increase in conductance after 2 sec in boiling H_2O is a clear demonstration that only insignificant amounts of cell sap could have been released by the somewhat less severe conditions of our extraction procedure.

The most probable explanation of the differences in the proportions of the three furanocoumarins dissolved in these three solvents would appear to be simple solubility differences, although this has not been further investigated. But why did the hot H_2O dissolve up to two or three orders of magnitude more furanocoumarins than the organic solvents, a difference that can be only partially explained by a temperature effect on the solubilities of these coumarins? To examine this point further, we resorted to scanning electron microscopy of the surfaces. Dry leaves, leaves dried after dipping in almost boiling H_2O, and leaves dried after 5-sec dipping in MeOH or Me_2CO were examined, and the results are shown in Figure 1. The dried surface (Figure 1A) consists of various-sized particles of some substance(s) forming patches. Most of these particles disappeared after immersion in MeOH (Figure 1B) or Me_2CO (not shown). The very smooth area lacking these particles (Figure 1C) was observed after one brief dipping in almost boiling H_2O. The texture of the cell wall visible through the cuticle (arrows) indicates a much thinner layer covering the cells in Figure 1C than in 1A and 1B. Under a light microscope it was possible to observe peels of this "over-cuticle layer," comparable to the structure called by some the lamella or cuticular membrane (19), consisting of the continuous layer (a sheet) and patches upon it (Figure 1D). MeOH and Me_2CO removed only the patches, but almost-boiling H_2O damaged the layer below (preventing the sheet from being peeled away as in the control), and, it seems probable, extracted the coumarins associated with this layer. These effects could account to a large extent for the much greater effectiveness of hot H_2O in removing the coumarins.

Despite the greatly enhanced recoveries of furanocoumarins by hot H_2O extraction as compared to cold organic solvent extraction, there is no present proof that even this technique led to quantitative recovery of these compounds from the leaf surface. This point is being explored further with a view to optimizing the extraction time.

Although our finding that furanocoumarins are present on the leaf surface is of interest in itself, we wish to reserve discussion on this point for a more extensive paper (20), since the focus of the present work is methodological. Also of interest is the possibility that brief immersion in hot H_2O may greatly facilitate extraction of exudates other than furanocoumarins from the plant surface. Our hplc analyses have given some indication of this, but we have not pursued the point. We hope that others will do so.

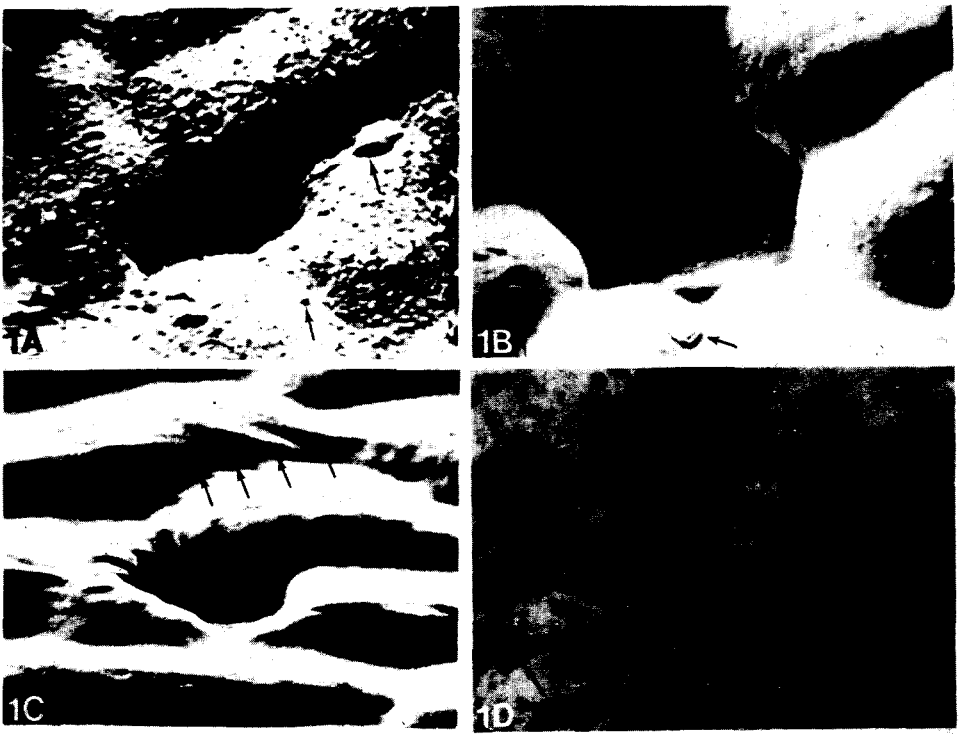

FIGURE 1. A–C, Scanning electronograms of *Ruta graveolens* leaf surface. A, Allowed to dry. Many different-sized structures (arrows) on the thick ground layer which is suggested by the epidermal cell wall borders (not easily visible). B, Dipped 5 sec in MeOH and then allowed to air-dry. Fewer crystal-like structures visible on surface (arrow). C, Dipped 1 sec in almost-boiling H_2O and then allowed to air-dry. The smooth surface of the plant leaf and visible contours (arrows) of the cell walls below suggest a thinner layer covering them. D, Peel of *Ruta graveolens* epidermis placed in xylene with visible edges (arrows) of two cracks in the overcuticular sheet on which are different-sized crystal-like structures (arrowheads).

EXPERIMENTAL

PLANT MATERIAL.—Five *R. graveolens* plants growing outdoors from seeds provided by the Warsaw Botanical Garden, Poland, were investigated in July 1987. A voucher specimen has been deposited in the collection of the Agriculture Canada Biosystematics Research Centre, Central Experimental Farm, Ottawa. Furanocoumarins on the leaf surface were determined in samples consisting of five leaves, each from a different plant. These leaves were mature, bore no trichomes, and had a bluish cast due to a substance easily removable from the surface. No mechanical damage was observed macroscopically or under an epi-light microscope.

REMOVAL OF SURFACE DEPOSITS.—Rubbings of the leaf surfaces against filter paper to remove the grayish deposits were done gently enough so that no green color, indicating release of chlorophyll, appeared on the paper. The filter paper was extracted in two changes of boiling H_2O (10 min each) followed by three washings in warm Me_2CO. Examination of hand-cut cross sections of these rubbed leaves under a light microscope revealed no traces of tissue damage; only the layer above the cuticle was thinner. Nor was any damage to the leaf surface seen in an epi-light microscope. Tlc showed the existence of the expected three furanocoumarins in these rubbings.

QUANTITATIVE FURANOCOUMARIN ANALYSIS.—For quantitative determination of coumarins extracted from the leaf surface, samples as described above were weighed and then dipped into ca. 100 ml of almost-boiling H_2O (ca. 96°) that had been removed from a hotplate for 5 sec after boiling. Other samples were dipped for either 5 or 60 sec into MeOH or Me_2CO at room temperature.

The aqueous extract of the leaf material or the residue from the MeOH or Me_2CO extract resuspended in H_2O was transferred to a liquid-liquid extractor and continuously extracted with Et_2O until well after

inspection of the supernatant under 366 nm uv revealed no visible fluorescence. The Et_2O extract was then allowed to evaporate at room temperature. The residue was partitioned between 60% aqueous MeOH and two changes of isooctane to remove fatty material, and the aqueous phase was concentrated to dryness under an air jet without heat. The residue was taken up in 2–3 ml of EtOAc and stirred with a somewhat smaller volume of 0.5% aqueous KOH to eliminate acidic components. One such extraction was normally sufficient as judged by absence of color in any subsequent extract, and, after a washing with H_2O, the EtOAc solution was transferred to a side-arm test tube and concentrated to dryness under an air jet. The residue was sublimed at 180°, <1 Torr, and the sublimate, in Me_2CO, was finally concentrated to dryness in a 1.5-ml microcentrifuge tube. After being dissolved in an appropriate volume of MeCN, the sublimate was submitted to hplc on a Waters Nova-Pak 7.5-cm reversed-phase C_{18} column developed in 25% aqueous MeCN (21). Quantitation was accomplished by comparison of peak areas representing psoralen, xanthotoxin, and bergapten, which separated well in this system, against a plot of peak area vs. weight of each coumarin, prepared with reference to purified authentic samples.

TESTS FOR CELL DAMAGE.—Three tests were conducted to determine whether cell damage resulted from exposure of the leaves to almost boiling H_2O: examination of plasmolysis and deplasmolysis, vital staining with neutral red (22), and conductance measurements of the aqueous extract.

The plasmolysis experiment was done on two species in parallel—*R. graveolens* leaves and, for easy comparison, red *A. cepa*. After a single dipping into almost-boiling H_2O, peels of ca. 0.5 cm^2 were removed with forceps and placed on a standing droplet of 25% w/v sugar solution. One minute later a cover glass was placed over it, and observations were made under a light microscope (× 300) of the central part of the peel. When all the cells had become plasmolyzed, the sugar solution was removed with filter paper from one side of the cover glass with simultaneous replacement by H_2O from the other side. The process of deplasmolysis was then observed.

For vital staining the peel of *R. graveolens* leaf, after a single dipping in almost-boiling H_2O, was placed on a standing droplet of a 5 ppm solution of neutral red (Sigma Chemical Co.) for 10 min, then covered with a cover glass and observed under a light microscope.

Conductance measurements were done on a Yellow Springs Instrument Co. Model 32 meter with a no. 3401 conductance cell (K = 1/cm) filled to capacity with 24 ml of medium—initially, deionized H_2O at 23°. A leaf was submerged 10 min in a beaker and conductance measured. This was followed by successive immersions of the leaf for 2 sec, 1 min, and 10 min in the same aqueous medium at the boiling point. Before measurement of the conductance, the medium was cooled to 23° and made up to 24 ml with deionized H_2O if necessary.

SCANNING ELECTRON MICROSCOPY.—Some of the leaves were allowed to air dry, as a control. Others were dipped once into almost-boiling H_2O or into either MeOH or Me_2CO for 5 sec and then allowed to dry. A conventional procedure for gold coating was followed by observations in a Cambridge Stereoscan 600 instrument.

LIGHT MICROSCOPY.—On the surface of a leaf three sides of a 5-mm square were cut in the epidermis with a razor blade. The free edge was gently lifted with forceps and the epidermis peeled back to give a strip successively containing undamaged cells, damaged cells, and, eventually, the cuticle followed by the overcuticular sheet alone. The peels were each put into a drop of xylene or histological clearing agent on a slide to prevent possible loss of H_2O-soluble constituents and were covered by a cover glass.

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