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DUVATRIENEDIOL, ALKANES, AND FATTY ACIDS IN CUTICULAR WAX OF TOBACCO LEAVES OF VARIOUS PHYSIOLOGICAL MATURITY

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Abstract—4,8,13-Duvatriene-1,3-diol found in the leaf wax of *Nicotiana tabacum* was of highest concentration in the wax from young leaves, and quantitatively decreased in importance with leaf age. Tobacco plants in flower had less duvatrienediol than those that were less mature; however, the total wax content did not change with leaf age. Air drying leaves reduced the duvatrienediol concentration. Changes of total fatty acids and *n*-alkanes with leaf age and air drying were also examined.

INTRODUCTION

An understanding of the surface lipids of leaves is important in studies of deposition and penetration of chemical agents such as herbicides, insecticides, and air pollutants. The cuticle, which is the outermost layer of the leaf, is essentially cutin impregnated with wax. Cutin consists of cross-esterified polymerized hydroxy fatty acids, and the wax is mainly a mixture of hydrocarbons, alcohols, aldehydes, wax esters, and fatty acids [1, 2]. Many other chemical constituents have also been isolated from waxes but the exact chemical composition depends on the tissue and species in question. A number of cyclic compounds have been isolated from plant cuticular lipid fractions and the pentacyclic compounds are generally the most common [2]. Ursolic acid [3] and oleanolic acid [4] in apple fruits and grape berries respectively constituted up to 50% of the cuticular wax. In 1962 a new diterpene, 4,8,13-duvatriene-1,3-diol (I), was isolated from a hexane extract of aged tobacco [5] and more recently it was shown that this diterpene is a component of the cuticular wax [6, 7]. Leaf surface lipids are readily extracted, without much contamination from internal lipids, by washing leaves in chloroform for 10 to 30 sec [8], and with this technique 98% of the duvatrienediol was extracted from tobacco leaves [6]. Furthermore, in young leaves the concentration of duvatrienediol was higher than either the *n*-alkanes or fatty acid components. In the present paper we describe the effect of physiological age and air drying on the concentration of duvatrienediol, *n*-alkanes, and fatty acids in tobacco leaf wax.

RESULTS AND DISCUSSION

Burley tobacco plants were grown for 10 weeks at which time one set of plants was air-dried as whole plants and another was processed immediately. Leaves from all plants were separated according to stalk position into the top one-third, middle one-third, and bottom one-third. Bottom leaves of these plants were just showing yellow color. The total surface wax content of fresh leaves was 1.2 to 1.4 mg/g fr. wt (Table 1) and of air-dried leaves 7.3–8.0 mg/g dry wt (Table 2). Young leaves had slightly higher wax levels than those of older leaves but they are *ca* 50% greater than those previously reported for Burley tobacco [9].

The largest component in the wax fraction from top fresh tobacco leaves was 4,8,13-duvatriene-1,3-diol at 47% of the total wax fraction (Table 1). These upper leaves were immature and not fully expanded. Fully expanded, mature, fresh leaves from the middle stalk position contained only 17% duvatrienediol. The overmature, bottom leaves which had started to yellow, had an even lower level of duvatrienediol of 8.8%. The duvatrienediol concentration in the wax of dried tobacco leaves was only 6% in top leaves and 1% in bottom leaves (Table 2). The duvatrienediol concentration was much lower in leaves from bottom stalk position than those from top position, and air drying markedly reduced the concentration of this diterpene in leaves from all stalk positions.

The cuticular wax fraction from fresh (Table 1) and air-dried tobacco (Table 2) contained about 2% fatty acids and the content was the same for all stalk positions. The major acid in fresh and air-dried tobacco was palmitic acid (Table 3). Palmitic acid is also the major fatty

Table 1. Relative % duvatrienediol, *n*-alkane and fatty acid in cuticular wax of fresh tobacco leaves

Leaf position	Cuticular wax (mg/g fr. wt)	*n*-Alkane (%)	Fatty acid (%)	Duvatriene-diol (%)
Top	1.4	17.6	1.9	47.2
Middle	1.2	14.6	1.8	16.8
Bottom	1.3	9.8	1.9	8.8

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Table 2. Relative % duvatrienediol, *n*-alkane and fatty acid in cuticular wax of air-dried tobacco leaves

Leaf position	Cuticular wax mg/g dry wt	*n*-Alkane %	Fatty acid %	Duvatriene-diol %
Top	8.0	16.7	1.9	6.2
Middle	7.4	16.6	1.6	7.4
Bottom	7.3	18.2	2.1	1.2

Table 3. Percentage composition of cuticular fatty acids isolated from fresh and air-dried tobacco leaves

Tissue	Leaf position	Carbon number 14	16	18:0	18:1	18:2	18:3	20	22	26
Fresh	Top	8.0	31.2	21.6	24.0	6.8	5.7	2.7	--	--
	Middle	7.8	27.5	19.7	19.3	12.4	10.1	3.2	--	--
	Bottom	6.5	33.1	15.1	20.4	10.6	8.2	6.1	--	--
Air-dried	Top	11.0	33.4	10.6	7.5	5.8	19.0	6.0	3.1	3.6
	Middle	12.6	41.4	8.6	5.4	4.0	12.5	7.4	4.6	3.5
	Bottom	14.3	31.3	12.1	8.9	5.7	16.0	5.2	2.7	3.8

acid of the somatic lipids of dried tobacco [10, 11] but in those of fresh tobacco it is linolenic acid [10]. Linolenic acid was only a minor fatty acid in the wax of fresh tobacco leaves but was the second most important in the wax of air-dried leaves (Table 3). Oleic, stearic, and linoleic acids, which were major fatty acids in the wax from fresh tobacco decreased in quantity with air drying. The fatty acid composition changed slightly with leaves from various stalk positions. In fresh tobacco arachidic acid was the fatty acid with the longest carbon chain but in dried tobacco behenic and cerotic acids were detected at levels between 3 and 4%. Fatty acids with a carbon number larger than 18 have been observed in total lipid extracts from dried tobacco [12].

The most common paraffins of plant epicuticular waxes are *n*-alkanes ranging in carbon number from 25 to 33 [2]. Tobacco wax, however, is unusual in that only 50 to 60% of the paraffin hydrocarbons are of the *n*-series [13, 14, 15]. *Iso*- and *anteiso*-homolog paraffin hydrocarbons have been found in tobacco leaf wax in high concentrations [14, 15]. In the present investigation, only the *n*-alkanes were determined (Table 4) and upper leaves contained the highest levels. Hentriacontane was the major *n*-alkane in the wax of both fresh and air-dried leaves from all stalk positions. Other important *n*-alkanes were, in decreasing order of concentration: tritriacontane, nonacosane and heptacosane. Alkanes with carbon numbers below C_{24} or above C_{34} were not observed. Even-number hydrocarbons were observed; however, the quantities of these alkanes were insignificant.

In another series of experiments tobacco plants were harvested after 10 and 14 weeks of growth and again were divided into top, middle, and bottom leaves, and

Table 4. Percentage composition of *n*-alkanes in surface wax of fresh and air-dried tobacco leaves

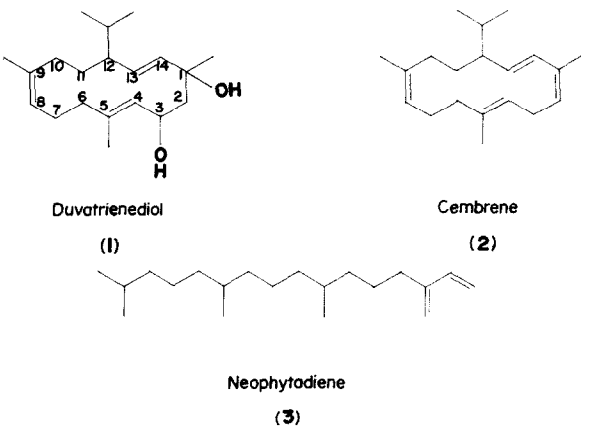
Tissue	Leaf position	Carbon number 27	29	31	33
Fresh	Top	5.4	7.1	47.0	40.5
	Middle	5.7	8.3	48.2	37.8
	Bottom	5.7	8.2	51.9	34.2
Air-dried	Top	4.8	8.5	45.1	41.6
	Middle	5.3	8.7	46.8	39.2
	Bottom	6.6	9.1	49.3	35.0

Table 5. Effect of age and stalk position on the levels of duvatrienediol and *n*-alkanes in fresh tobacco.

Leaf position	Plant age (weeks)	Alkane C_{31} μg/g fr. wt	C_{33} μg/g fr. wt	Duvatrienediol μg/g fr. wt	% Total wax
Top	10	116	100	661	46.2
	14	90	103	476	26.4
Middle	10	84	66	201	17.7
	14	73	62	103	6.0
Bottom	10	66	43	115	9.5
	14	56	32	14	1.2

the cuticular wax was analyzed for duvatrienediol (Table 5). The duvatrienediol concentration was much lower in the cuticular wax from 14-week-old tobacco plants than from 10-week-old plants. The 14-week-old plants were in full flower, and the young, upper leaves contained 30% less duvatrienediol than the nonflowering 10-week-old plants. The bottom leaves of 14-week-old plants were completely yellow and contained very small amounts of duvatrienediol. Apparently duvatrienediol is catabolized as leaves mature and become senescent; however, at present the metabolic end product is unknown. It is very unlikely that the decrease in duvatrienediol is due to chemical decomposition or autooxidation since this diterpene was quite stable on exposure to air under laboratory conditions for up to 30 days. Also duvatriene loss due to weathering can be ruled out since the plants were grown in the greenhouse and were watered from the bottom. Duvatrienediol has a carbon skeleton identical to that of neophytadiene (II) and cembrene (III). Neophytadiene has been isolated from tobacco plants [16, 17]. Present attempts to isolate neophytadiene from the cuticular lipid fraction were unsuccessful.

The solubility of duvatrienediol in organic solvents may provide clues about possible cuticular lipid organization. The compound is soluble in *n*-hexane in limited amounts, but in methanol it is soluble even at −40°. This property was utilized for separating the alkanes from duvatrienediol. Extraction with methanol, however, yielded only 20–30% of the duvatrienediol extracted with chloroform. The poor efficiency of extraction can be explained if duvatrienediol, and possibly other polar lipids, are embedded in non-polar lipid films. The biological role of duvatrienediol is unknown, but since this diterpene accounted for almost half of the wax in young tobacco leaves, it is only natural to assume that it must be involved in controlling the water balance of the plant. Certainly the large amount of duvatrienediol makes the



cuticle of tobacco leaves appear to be unusual in structure. Cutler (personal communication) suggested that duvatrienediol might be the same compound which had plant growth inhibitory properties that he and Cole [18] extracted from rapidly expanding tobacco leaves. However, diterpenes of this nature are not known to be plant growth regulators.

EXPERIMENTAL

Nicotiana tabacum L. var. Burley-21 was grown in the greenhouse and nutrients were supplied in the conventional manner. Seeds were germinated in a high-humidity growth room, and the seedlings were kept in this room until transplanted to the greenhouse where they were held under these conditions until harvesting. Leaves were divided into three groups according to stalk position, top one-third, middle one-third, and bottom one-third. The greenhouse plants were analyzed green (fresh) or air-dried under semi-controlled conditions in growth chambers. Air drying of the whole plant, cut at the soil line, was carried out in the dark for 4 months at 27°.

Extraction of cuticular wax. Surface fatty acids and alkanes were extracted using a slightly modified procedure after ref. [19]. Fresh tobacco leaves (*ca* 30 g) were taken from plants, weighed, and dipped into 800 ml $CHCl_3$ for 30 sec. Excess solvent was allowed to drain for 10 sec, using a wire gauze. With dried tobacco, about 10 g dry wt of leaf material was dipped into 800 ml $CHCl_3$ for 30 sec. As soon as extractions were completed 100 μg heptadecanoic acid and 1 mg *n*-eicosane were added as standards for quantitative determination of fatty acids and alkanes, respectively. To determine total surface wax, the $CHCl_3$ extract was taken to dryness under red. press. to constant wt and corrections were made for added standards.

Analysis of fatty acids and alkanes. The $CHCl_3$ extract was saponified in 3 N KOH in MeOH at 65° for 30 min. After cooling, M H_3PO_4 was added to bring the pH of the hydrolysate to *ca* 4. Both the fatty acids and alkanes were extracted from the hydrolysate 5 × 5 ml portions of hexane. The combined hexane extracts were reduced to *ca* 5 ml. Trimethyl-(α,α,α-trifluoro-*m*-tolyl) ammonium hydroxide (TMTFTH), prepared from trifluoro-*m*-toluidine, was used as a reagent for extraction and methylation of fatty acids [20]. 0.5 M aq TMTFTH (100 μl) was added to the combined hexane extract shaken and then centrifuged for 3 min. The alkanes remained in the hexane layer. For fatty acid analysis *ca* 3 μl of the TMTFTH solution, which contained total saponifiable fatty acids, were sandwiched between methylpropionate–MeOH (1:2) in a 10 μl syringe and injected directly into a GLC. The column was either 15% DEGS coated on 100/120 mesh Anakrom or 10% EGSS-X on Gas Chrom P. The column was operated isothermally at 180° or programmed from 135° to 200° at 4°/min after an initial hold for 6 min. The alkanes which remained in the hexane layer were separated on a column of 1% OV-101 on Gas Chrom Q using temp programming from 100° to 300° at 6°/min.

Analysis of duvatrienediol. The $CHCl_3$ extract can be directly injected into a GLC for quantitative determination of duvatrienediol [6]. For further purification the $CACl_3$ extract (10 g fr. wt equivalent) was placed on top of a Si gel column (Si gel 60, 70–230 mesh, 1.8 × 9 cm) prewashed with hexane. After the sample was applied, the column was washed with hexane until all the alkanes were eluted as monitored by GLC; *ca* 600 ml of hexane were required. Primary alcohols and other similar lipids were eluted with 300 ml hexane–$CHCl_3$ (1:1) followed by 300 ml hexane–$CHCl_3$ (1:2). Duvatrienediol and lipids with similar polarity were eluted with 400 ml of hexane–$CHCl_3$ (1:3) and analyzed either free or as butylboronic acid derivatives on a column of 1% OV-101 coated onto Gas Chrom Q. Injection port and detector temps were 250 and 300°, respectively and the column was programmed from 100° to 300° at 6°C/min with an initial hold of 4 min. Butylboronic acid derivatives of duvatrienediol were formed by adding excess butylboronic acid in pyridine and heating the mixture for 15 min at 100°.

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