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Cuticular Waxes of Developing Leaves and Fruit of Blueberry, *Vaccinium ashei* Reade cv. Bluegem[1]

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Abstract. **An ultrastructural and chemical study of the cuticular waxes from leaves and fruits of rabbiteye blueberry was conducted in North Central Florida between March and November 1977. Leaf epicuticular wax concentration was relatively constant (75-83 $\mu g/cm^2$) for the 40 days of leaf expansion following leaf emergence. Epicuticular wax reached a peak of 136 $\mu g/cm^2$ in mid-May, 3-4 weeks after full leaf expansion. Levels declined thereafter to less than 70 $\mu g/cm^2$ by October. The concentration of fruit surface wax increased from 159 $\mu g/cm^2$, 24 days after full bloom, to 295 $\mu g/cm^2$ at maturity. β-Diketones, the dominant leaf surface wax fraction for the first 100 days (34-54% of total wax) declined to less than 9% in October. Acidic triterpenoids increased from 10-12% to more than 40% of total wax and primary alcohols increased from 10% to 20-30%. Secondary alcohols, initially 22% of total leaf wax, were not detected after the first month. Fruit surface wax was chemically similar to the leaf surface wax with β-diketones increasing to 50-60% of the total. The surface wax on leaves and fruit was made up of a dense network of interlocking branched rodlets (closed tubes). Disappearance of rodlet structure on the leaves coincided with the decline of total wax and β-diketones. These data are discussed with respect to leaf and fruit water relations.**

Plant cuticles and cuticular waxes are important to foliar absorption of herbicides and other chemicals (20, 21). The cuticle also plays an important role in the control of plant water loss. Cuticular developmental studies are required to determine the sequence of foliar wax development, to more accurately predict cuticular wax development response to environmental factors, and to determine how these changes influence the cuticle's role as a barrier to water loss.

Recent studies on epicuticular wax development have emphasized either morphological (2, 9, 12, 25), chemical (7, 8, 11, 20, 22, 28, 29), or gross quantitative changes (2, 3, 6, 11, 27). Some studies have observed epicuticular morphological characteristics and linked these with wax chemistry (18, 30). The primary aim of this study was to characterize blueberry epicuticular wax chemically and follow its development on leaves and fruit quantitatively. The intent was to correlate any chemical or quantitative changes in epicuticular waxes with ultrastructural changes.

Materials and Methods

The leaves and fruit of rabbiteye blueberry 'Bluegem' grown near Gainesville, Florida, were periodically sampled between March 11 and November 20, 1977. Leaf emergence was observed on March 2. Fruit sampling ceased in early June when the fruits were fully ripe. Sample sizes ranged from about 500 leaves and fruits initially to 200-300 from May onwards. Leaf areas were determined using a Lambda Model L1-3000 area meter. Fruit surface areas were calculated from fruit volume which was determined by water displacement.

Scanning electron microscopy (SEM) samples were collected periodically and sections (5 × 5 mm) were cut from leaf and fruit surfaces. These were fastened to plastic Petri dishes using a drop of citrus oil (1) and air-dried in a desiccator. Individual leaf lengths and fruit diameters were recorded. The air-dried samples were reduced to 2 × 3 mm and fastened to aluminum stubs using either silver conductive glue or Avery self adhesive paper tacks. The stubs were coated with about 100 Å of gold-palladium (60:40) on a 'Technics' sputter coater and observed on a JEOL JSM-35 or a Novascan Scanning Electron Microscope operating at 10-25 kV and 80-100 μA current.

Surface wax extraction of leaves and fruit was accomplished by totally immersing and agitating in 500 ml chloroform (55–58°C) for 50 sec. This procedure was previously found not to extract internal lipids. The chloroform-wax solution was filtered, reduced to dryness with a rotary evaporator, and oven dried overnight at 55°C in a preweighed flask. The dried waxes were made up to 1% solution in chloroform and stored in a freezer for subsequent chromatographic analyses.

Intracuticular wax extraction of leaf discs (1.3 cm^2) punched from the surface of chloroform dewaxed blueberry leaves was done in August, 1977. Isolated cuticles were obtained by placing the discs in a digestive zinc chloride-hydrochloric acid solution according to Holloway and Baker (15). The isolated cuticles were washed several times in distilled water to remove cellular debris, oven dried at 55°C, and weighed. The intracuticular waxes of 3 replicates of 59-90 discs each were extracted using the method of Baker and Procopiou (5) and stored in a freezer. The extracted cuticle discs were oven dried (55°C), reweighed, and considered to be mainly cutin.

Thin layer chromatography (TLC) was used to fractionate both epicuticular and intracuticular waxes into their constituent classes. Plates were developed on silica gel G using a benzene: acetic acid (99:1) v/v mixture as developing solvent. Spots were detected by spraying with 0.05% aqueous Rhodamine-6G and observing under both long and short UV light. The spots were identified using Rf values and chromatography with standards (Table 1). Additional tests were used in the identification of triterpenyl acetates and acidic triterpenoids (Table 1). Ultraviolet and infrared spectra were used to confirm the presence of β-diketones.

Preparative thin layer chromatography of duplicate wax samples (10-14 mg) applied as bands on Gelman Instant Thin Layer Chromatography (ITLC) sheets (Type SA) using the benzene-acetic acid solvent system separated the wax into constituent classes. The bands visualized by spraying with 0.05% aqueous Rhodamine 6G, were thoroughly dried, cut

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Table 1. Standards used for identification of wax classes.[z]

Class	Standard	Rf[y]
Fatty acid	Lignoceric acid	0.08
Primary alcohol	Tricontanol	0.15
Secondary alcohol	Hentriacontan-16-ol	0.50
Ketone	Hentriacontane-16-one	0.74
Ester	Triacontylhexadecanoate	0.78
β-Diketone	Hentriacontan-14, 16-dione	0.73
Paraffin	Docosane	0.85
Aldehyde	Sugarcane wax (19) Hexadecanal	0.65
Acidic triterpenoid[x]	Ursolic acid	0.01, 0.01[w], 0.65[v]
Triterpenyl acetate[x]	--	0.35, 0.42[w], 0.65[v]

[z]Standards generously supplied by A. P. Tullock, Prairie Regional Laboratory, Saskatoon, Canada and P. E. Kolattukudy, Washington State Univ., Pullman.

[y]Developed in 99 benzene:1 acetic acid (by volume) mixture.

[x]Positive Lieberman-Burchard test (16).

[w]Developed in 7 benzene:3 chloroform (by volume) mixture.

[v]Developed in 1 chloroform:1 ethylacetate (by volume) mixture.

into individual bands, eluted in boiling chloroform, and filtered. The solvent was removed by evaporation and the quantity of wax in each class was determined gravimetrically. Each extracted band was checked for purity using TLC.

Results

Ultrastructure. The surface of newly unfolded leaves initially appeared amorphous except for a rippled appearance broken by stomata (Fig. 1A). Wax rodlets were beginning to develop on stomatal antechambers (Fig. 1A, arrow). The rodlets were either single or multiple and often branched (Fig. 1B). They developed rapidly during the next 2 weeks to cover the entire leaf surface (Fig. 1C). The degree of branching and interconnecting among the rodlets at that time is shown in Fig. 1D which also shows some flattened plate-like structures (arrows). These delicate rodlets were damaged by the electron beam in the SEM at 20 kV. They could only be safely viewed at 15 kV and for thinner rodlets, 10 kV. These rodlets were never observed to be open tubes (30). This may be the result of electron beam damage. Natural loss of leaf rodlet structure was observed as early as April (Fig. 1E). This progressed through final leaf expansion and aging until all rodlet structures were lost. Fig. 1F shows the loss of structure through rodlet fusion (arrow). By May, much of the rodlet structure had degraded with the exception of a secondary extrusion of new rodlet wax over the stomatal antechambers (Fig. 1G). In November, only amorphous wax remained over which fungal mycelium had developed (Fig. 1H).

Fruit wax development closely paralleled leaf wax development. Rodlets were observed on very immature fruits (Fig. 2A). These fruit had prominent waxy bloom deposits which were highly variable. Fig. 2B and 2C represent samples from April and June. Regions with fully developed rodlets were often adjacent to regions in which rodlets were undeveloped. This surface pattern and the variability in structure are depicted in Fig. 2D and 2E. Thin rodlets appeared to emerge directly from the surface whereas thicker rodlets developed from mounds of amorphous wax (Fig. 2D). Degradation of rodlet structure, similar to that described for leaf waxes, occurred in some areas on the more mature fruits (Fig. 2F, 2G, arrows). The ultrastructure of wax on ripe berries varied from the narrow rodlets (Fig. 2C) to short and stubby rodlets and amorphous wax (Fig. 2H).

Wax quantitative studies. Surface concentration of leaf wax declined initially in March and then rose to a peak of 138 μg/cm² in mid-May (Fig. 3). Total epicuticular wax increased steadily from March until full leaf expansion which occurred between late April and early May (Fig. 3). Both total amount and concentration of surface wax declined 44 and 50%, respectively, between mid-May and mid-August. The concentration of embeded wax and residual cutin in mature leaves was 23 μg/cm² and 206 μg/cm², respectively.

Fruit surface area increased rapidly between April and June (Fig. 3). Wax accumulation initially was at a great enough rate to result in an increase in surface wax concentration in spite of the fruit surface increase. By late April, surface wax concentration reached a peak and was relatively constant thereafter. Wax accumulation per fruit continued until harvest.

Chemical studies – epicuticular wax. β-Diketones were tentatively identified by their TLC rf's, strong absorption in the ultraviolet, λ max at 278 nm in chloroform (17), and a strong band between 1600 and 1650 cm⁻¹ in infrared spectra (KBr) (17). β-Diketones were the major leaf wax fraction for most of the samplings, up to 54% of total wax, and the major fruit wax fraction, up to 62% of total wax (Fig. 4). These and other values expressed as percentages in the text are presented as μg/cm² in Fig. 4 and Table 2. The leaf surface wax concentration increased until it reached a peak in mid-May, coincidental with the peak in total wax. From mid-May to August, β-diketones declined rapidly from 69 to 6 μg/cm² or 1326 μg/leaf to 146 μg/leaf. During the same period, total leaf wax declined from 135 to 63 μg/cm². Thus, most of the reduction in leaf wax concentration was due to the loss of β-diketones. The surface concentration of wax on fruits increased until mid-May and then declined until June (Fig. 4). This decline was apparently due to fruit expansion as the total β-diketone per fruit increased throughout the sampling period from 191 to 909 μg/fruit.

Acidic triterpenoids had characteristic bands at 3420 and 1690 cm⁻¹ in infrared spectra (KBr) (24). Their concentration in the leaf surface wax increased throughout the sampling period and represented 47% of total wax by November (Fig. 4). These triterpenoids were the dominant leaf wax fraction after July. Total amount per leaf was 36.7 μg in March and this increased to 650.4 μg in August and was constant thereafter despite a decline of total leaf wax. Acidic triterpenoids were the second most abundant fruit wax fraction increasing to 22% of total wax in May followed by a decline (Fig. 4). The decline in concentration from 61.0 to 33.8 μg/cm² was apparently due to a reduction in the rate of accumulation as total per fruit remained relatively constant at 213-226 μg during the same period.

Primary alcohols increased from 10% of total surface leaf wax in March to 31% in October. The leaf surface primary alcohol concentration increased until mid-May (Fig. 4), the period at which total wax concentration was greatest (Fig. 3). Thereafter, it remained relatively constant despite a decline in the concentration of total wax. Primary alcohols were a minor fraction of fruit wax (Fig. 4) comprising only 3-13% of total wax. Accumulation rate, however, was sufficient to maintain an increasing concentration despite the rapid rate of fruit expansion.

Secondary alcohols were 22% of total leaf epicuticular wax at the first sampling, 9% at the second, and were not detected thereafter (Table 2). The secondary alcohol content of fruit wax was minor, ranging from 0.7 to 2.6% of total wax (Table 2). Fatty acids, paraffins, aldehydes (Fig. 4) and triterpenyl acetate, esters, and ketones (Table 2) were minor fractions of both leaf and fruit waxes throughout the measurement period.

Chemical studies – intracuticular wax. Wax extracted from cuticle discs was mainly fatty acids with traces of paraffins and primary alcohols. These waxes were of the same proportions

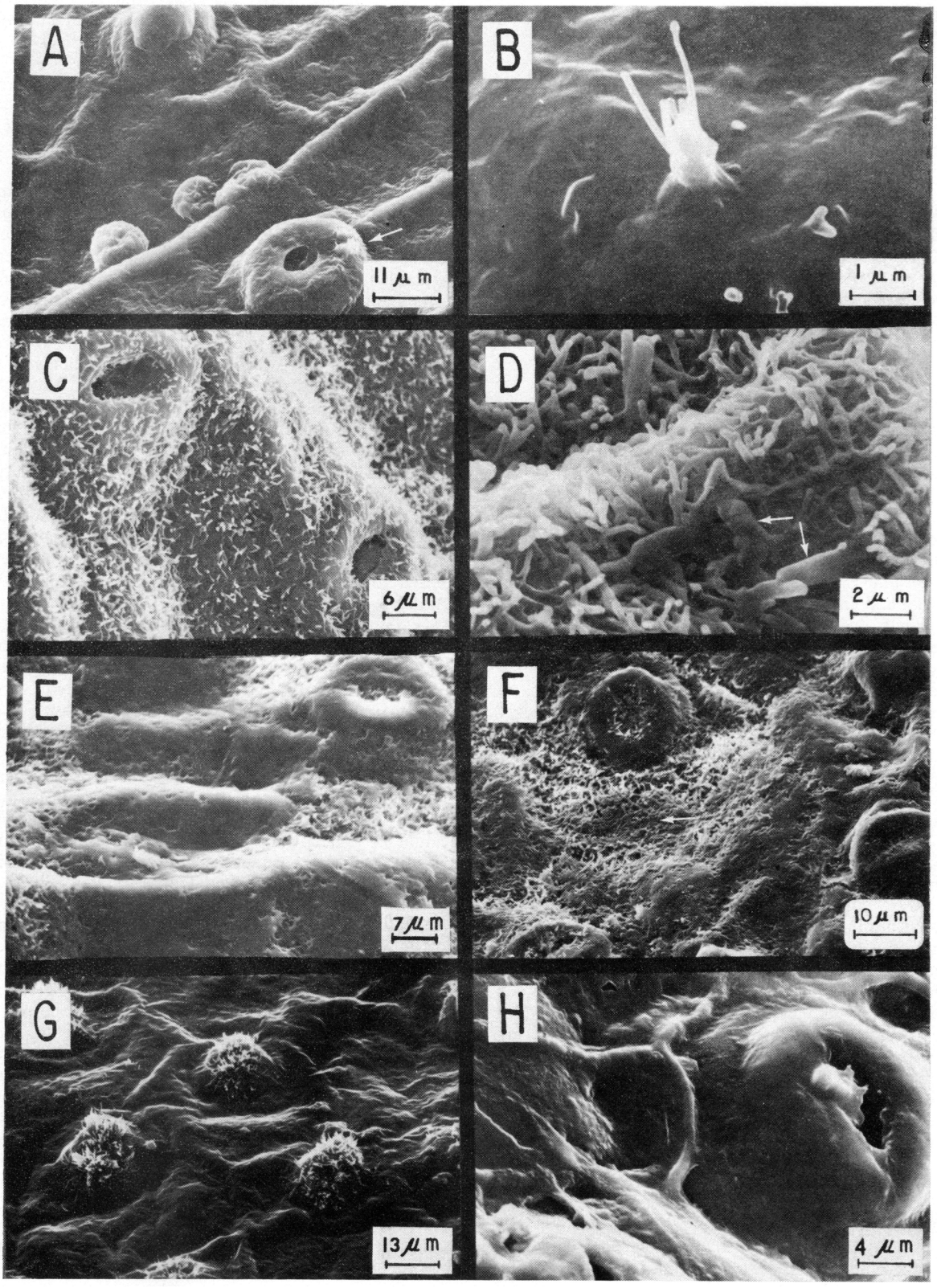

Fig. 1. Epicuticular wax development on the abaxial surface of 'Bluegem' blueberry leaves: A. Sampled March 11, leaf length 1.7 cm; B. Same sample as A; C. Sampled March 26, leaf length 4.1 cm; D. Sampled March 26, leaf length 4.6 cm; E. Sampled April 9, leaf length 5.4 cm; F. Sampled May 14, leaf length 6.0 cm; G. Sampled May 14, leaf length 6.0 cm; H. Sampled Nov. 21, leaf length 6.2 cm.

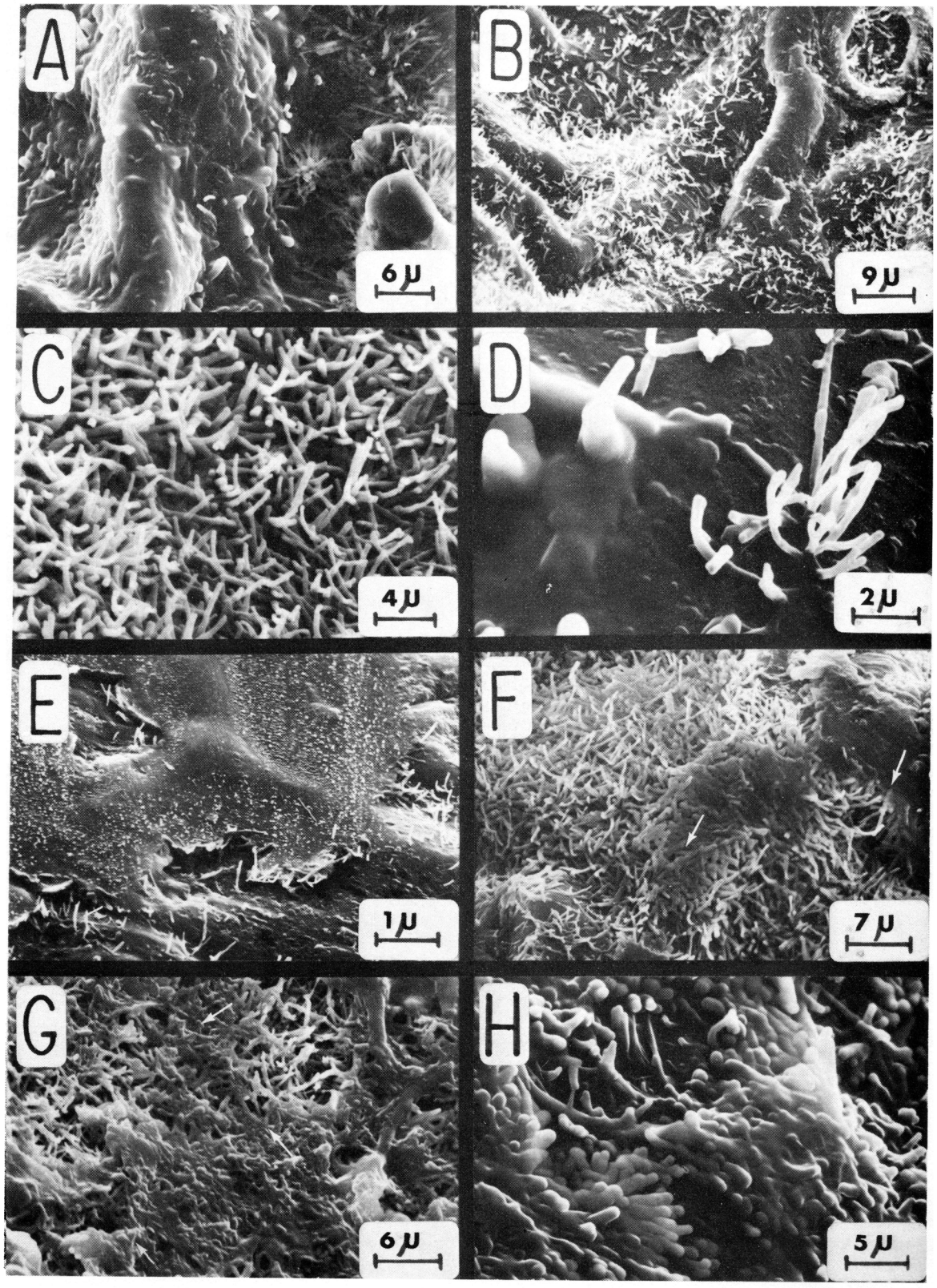

Fig. 2. Epicuticular wax development on 'Bluegem' blueberry fruit: A. Sampled April 9, diam 0.9 cm; B. Sampled April 24, diam 1.0 cm; C. Sampled June 5, diam 1.6 cm; D. Sampled May 15, diam 1.3 cm; E. Sampled May 15, diam 1.3 cm; F. Sampled May 24, diam. 1.6 cm; G. Sampled June 5, diam 1.6 cm; H. Sampled June 5, diam 1.6 cm.

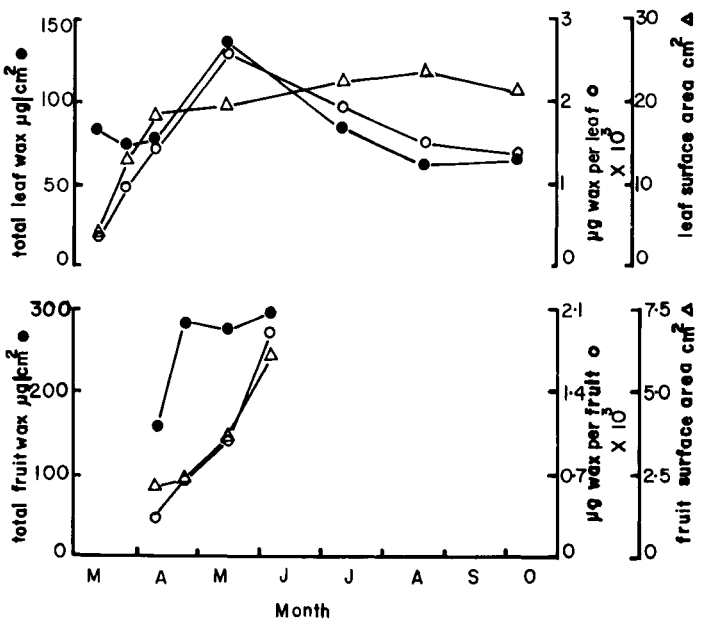

Fig. 3. Changes in the surface area [△], total wax [○] and total wax per unit surface area [●] of 'Bluegem' blueberry leaves and fruits sampled between March 11 and October 6, 1977.

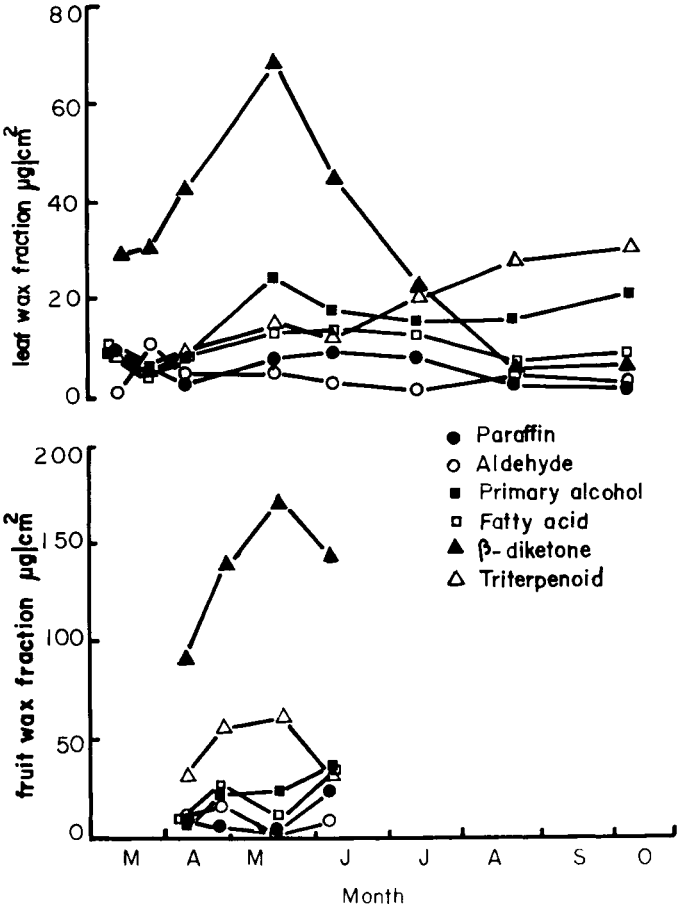

Fig. 4. Changes in some leaf and fruit wax constituents, expressed as $\mu g/cm^2$ surface area, for 'Bluegem' blueberry sampled between March 11 and October 6, 1977.

as the leaf and fruit intracuticular waxes of several citrus cultivars (unpublished data).

Discussion

Palmitate, a precursor of cuticular waxes, is produced *de novo* in chloroplasts (13). The high chloroplast densities observed in the guard cells of blueberry leaves (unpublished data), therefore, may be a contributing factor to the observed initial development of rodlet wax on stomatal antechambers. Earlier wax development above stomatal guard cells has been observed on other plant species that produce rodlet wax (25).

Leaf expansion was essentially complete by mid-April while total surface wax continued to increase until mid-May (Fig. 3). β-Diketones contributed most to total wax, hence changes in β-diketone concentration greatly influenced total wax changes. The marked decline of total leaf epicuticular wax, due mainly to the loss of β-diketones, was partly offset by increases in the concentration of acidic triterpenoids. The continued synthesis of acidic triterpenoid and the new wax rodlets observed on near senescent leaves is contrary to the concept of Schieferstein and Loomis (26) that wax extrusion stopped due to final hardening of the primary cuticle when the leaf was mature. Davis (9) also implied that wax extrusion ceased beyond a critical stage of

Table 2. Concentration ($\mu g/cm^2$) of surface wax minor constituents from leaves and fruits of rabbiteye 'Bluegem' blueberry.

	Wax concentration									
Constituent	Mar. 11	Mar. 26	Apr. 9	Apr. 24	May 15	June 5	July 10	Aug. 21	Oct. 6	Nov. 20
Leaves										
Esters	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.6	0.4
Ketones	0.0	0.0	0.0		0.0	2.7	2.4	0.0	0.0	0.0
Secondary alcohols	18.5	6.6	0.0		0.0	0.0	0.0	0.0	0.0	0.0
Triterpenyl acetate	1.8	1.5	1.0		3.5	5.5	4.8	0.4	0.5	0.0
Fruit										
Esters			0.0	0.0	0.0	15.1				
Ketones			0.0	0.0	0.0	0.0				
Secondary alcohols			0.0	1.9	7.2	2.3				
Triterpenyl acetate			5.9	13.3	1.9	6.8				

growth. Giese (10) postulated that the amount of wax on the cuticle determines the rate of synthesis and extrusion. The ability to replace lost wax, however, must decline as leaves age. The small amounts of newly emerged rodlets, probably β-diketone, on the stomatal antechambers of near senescent leaves with a weathered wax layer may support Giese's hypothesis, as may the increase of triterpenoids. But in each case, production of new wax was insufficient to restore wax concentration to its previously higher levels.

The loss of rodlet structure was coincident with the decrease in β-diketone. Rodlet structure in blueberry was shown to be due to the presence of β-diketones (unpublished data). Von Wettstein-Knowles (30) similarly demonstrated the contribution of β-diketones to wax tube structure in barely *(Hordeum vulgare)*. The fate of the lost β-diketones was not determined. It seems likely that weathering was the prime cause. The susceptibility of rodlets to beam damage in the SEM may indicate their susceptibility to weathering. The longer exposure of leaves than fruits to the atmosphere would account for the greater loss of wax and rodlet structure in leaves.

Andersen et al. (4) showed the time of maximum leaf resistance to water loss of 'Bluegem' blueberries to be coincident with the time of greatest wax levels in our study. A significant positive correlation existed between seasonal changes in leaf resistance in their study and seasonal wax concentration changes in ours. Holloway (14) reported that alkanes were the most hydrophobic of wax classes but that esters, ketones, and secondary alcohols were almost as hydrophobic. The least hydrophobic are sterols and triterpenoids. The contact angle, as a measure of wettability, of wheat leaf surfaces increased with increasing β-diketone content (21). Thus, in blueberry leaf, the loss of β-diketone and the increase of acidic triterpenoids would have undoubtedly influenced both leaf wettability and cuticular transpiration. Possingham et al. (23) suggested that the structural arrangement of wax on grape berries, together with the hydrophobic nature of the surface controls water movement. Thus in blueberry, pathways for water vapor through rodlets would be expected to be quite different from those through amorphous waxes in immature and the more mature leaves. The occlusion of stomatal antechambers in leaves with this rodlet wax (Fig. 1C and 1F) should have considerable influence on stomatal transpiration.

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