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CHANGES IN THE CHLOROPHYLLS AND CAROTENOIDS OF LEAVES OF *NICOTIANA TABACUM* DURING SENESCENCE

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Abstract—In addition to chlorophylls *a* and *b*, β-carotene, lutein, violaxanthin and neoxanthin, leaves of tobacco (*Nicotiana tabacum* L cv Virginia Gold) contain antheraxanthin in some harvests In lower leaves, chlorophylls decreased more rapidly than carotenoids during senescence, but both types of pigment decreased at equal rates in upper leaves The chlorophyll *a b* ratio decreased only in post-mature leaves Total carotenoid decreased with age, with the relative proportion of β-carotene increasing in lower leaves Seasonal influences rather than age of leaf determines whether antheraxanthin is present No esterified xanthophylls were found in senescent leaves

INTRODUCTION

CHLOROPHYLLS and carotenoids are essential for normal granal structure[1–5] and photosynthetic activity[4–6] in green leaves The aim of this study was to measure the concentrations of the components of these two classes of pigment during maturation and senescence of tobacco leaf, particularly looking for possible changes characteristic of maturity The chlorophyll *a b* ratio generally decreases during senescence of tobacco leaves though Weybrew and Mann reported that the ratio does not fall appreciably until most of the chlorophyll has already disappeared [8] In addition to the major carotenoids always found in green leaves (β-carotene, lutein, violaxanthin and neoxanthin[9–12]), lutein-5,6-epoxide,[7,13–15] antheraxanthin[16–19] and zeaxanthin[16,18–20] have also been reported

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Goodwin[7] found that quantitative changes occur in carotenoids during senescence in leaves of *Acer Quercus* and *Prunus* and that lutein-5,6-epoxide appeared and β-carotene was lost during senescence in all these species Esters of lutein and violaxanthin have also been found in senescing leaves of several species of deciduous trees[7 21]

Tobacco undergoes sequential senescence[22] appearing as a progressive yellowing of the leaves from the base of the plant upwards In commercial flue-cured tobacco, leaves are harvested at maturity when the first signs of senescence appear but before the leaves turn yellow

RESULTS

Identification of carotenoids

TLC of the lipid fraction on silica gel G/KOH plates distinctly resolved four or five yellow bands These, in order of decreasing R_f value, are denoted fractions 1-5 Fractions 1 2 4 and 5 were identified as β-carotene, lutein, violaxanthin and neoxanthin respectively by comparing spectral absorption maxima with published values in several solvents,[23 28] the extent of any hypsochromic shift in absorption maxima (0 0, 39 and 17 nm respectively) after reaction with dilute HCl[23] and the value of the ratio of peaks III to II[29] Rechromatography of the eluted bands on TLC plates of either Al_2O_3 MgO (fraction 1) or silica gel G/MgO (fractions 2, 4 and 5) confirmed the homogeneity of these fractions

TABLE 1 SPECTRAL PROPERTIES OF FRACTION 3 COMPARED WITH AUTHENTIC ANTHERAXANTHIN AND LUTEIN-5,6-EPOXIDE

Solvent compound	Absorption maxima (nm) *n*-Hexane			EtOH			$CHCl_3$			III/II Ratio (%)	Ref
Fraction 3	420	445	472	424	448	474	431	457	483	51	–
Antheraxanthin	420	444	472		446	474		457	486		23
				421	443	473					30
										50	29
Lutein-5,6-epoxide	416	439	469	416	440	469	425	450	480		23
				417	442	471					30
										86	29
				After treating with dil HCl							
Fraction 3	401	423	449		426	452		434	459		
Antheraxanthin					426	453					23
				405	428	453					30
Lutein-5,6-epoxide				398	421	448					23
				400	420	450					30

The maxima of the absorption spectrum, the hypsochromic shift of 22 nm (Table 1) and the ratio of peak heights III/II of 51%[29] suggested that fraction 3 was antheraxanthin,

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though in some samples peak I was relatively distinct, as in the α isomer lutein-5,6-epoxide (Table 1), reported present in tobacco leaf by Costes[14] However, the observed absorption maxima of fraction 3 before and after treating with HCl and the III/II ratio agree more closely with antheraxanthin than lutein-5,6-epoxide (Table 1), the fraction is unlikely to be a mixture of the α and β isomers since it remains homogeneous upon chromatography on silica gel G/MgO

Changes in the concentration of chlorophylls and carotenoids during ageing

Sampling began soon after leaves were fully expanded and continued until leaves 7 and 13 were a uniform yellow and leaf 19 a pale yellow–green Rate of loss of chlorophyll in leaf 7, initially slow, increased during the final stages of senescence (Fig 1, Table 2) whereas chlorophyll concentration decreased regularly in leaves 13 and 19 The chlorophyll/carotenoid ratio decreased most rapidly in leaf 7 (from *ca* 10 1), decreased less rapidly in leaf 13, and did not decrease significantly in leaf 19

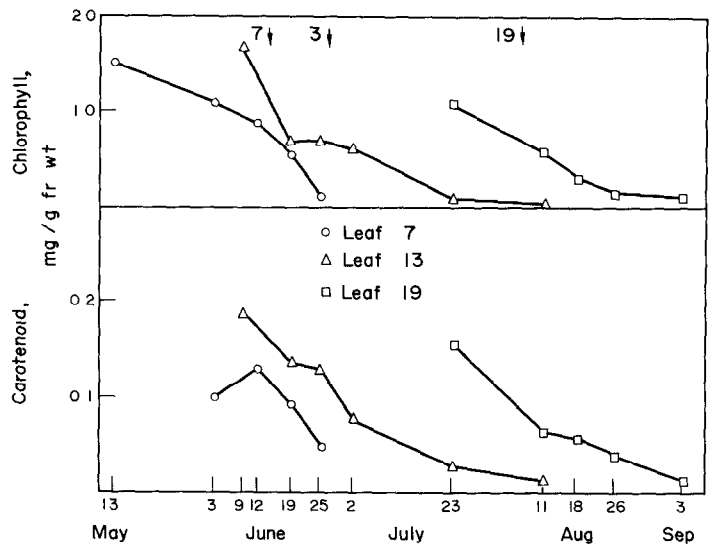

FIG 1 CHANGES IN THE TOTAL CHLOROPHYLL AND TOTAL CAROTENOID CONCENTRATIONS IN *Nicotiana tabacum* LEAVES WITH DATE OF HARVEST

Values are means of analyses made on single leaves from four plants Arrows indicate the dates of estimated maturity for each leaf

Changes in the chlorophyll a b *ratio in tobacco leaf during ageing*

In pre-mature leaf, the chlorophyll *a b* ratio usually ranged between 2 0 and 2 2 It did not decrease until commercial maturity in leaf 7, decreased some days later than this in leaf 13, and in leaf 19, the ratio increased after maturity before finally decreasing These results, however, should be viewed with caution, concentrations of chlorophyll *a* when plotted against chlorophyll *b* show a strong correlation (r = 0 99) with no obvious deviation from linearity as would be expected if the proportions of the two pigments changed markedly as the total pigment concentration falls Furthermore, the low values for the

TABLE 2 CHANGES IN RATIO (W W) OF CHLOROPHYLL

Date	Leaf 7					Leaf 13	
	Chl / Car	E / C	L / E	L / C	V / N	Chl / Car	E / C
2 June	11 5						
3 June		1 3	1 9	2 5	1 0		
9 June						8 8	1 2
12 June	8 1	1 9	1 6	2 9	0 8		
19 June	6 5	2 7	1 2	3 2	0 9	4 4	2 4
25 June	0 7	1 2	3 5	3 0	0 9	6 0	1 5
2 July						8 2	1 0
23 July						3 9,	0 8
11 August						3 5	0 6
18 August							
26 August							
3 September							
r	0 75**	0 50	0 64*	0 83**	0 96**	0 91**	0 84**

Chl -Chlorophyll *a* + Chlorophyll *b* Car - Total carotenoids E Total epoxy xanthophylls C *β*-Carotene

ratios were found only in leaves with low chlorophyll concentrations where errors in determining chlorophyll are likely to be large [31]

Changes in concentrations and proportions of individual carotenoids during senescence

The concentrations of all four major carotenoids decreased during senescence (Fig 2) Loss of lutein violaxanthin and neoxanthin was delayed in leaf 7 until maturity whereas concentrations in leaves 13 and 19 decreased continuously after the first sampling Antheraxanthin was found only in leaves 13 and 19 harvested in August and September

Correlations between the following carotenoid pigments were calculated for each leaf position epoxy xanthophylls/*β*-carotene, epoxy xanthophylls/lutein lutein/*β*-carotene, violaxanthin/neoxanthin (where epoxy xanthophylls were the sum of violaxanthin neoxanthin and antheraxanthin) Most correlation coefficients were greater than 0 9 (Table 2) However, in leaf 7, epoxy xanthophyll concentrations fell close to zero in later harvests whereas *β*-carotene and lutein concentrations remained relatively high, giving lowered coefficients of 0 50 (N S) and 0 64* respectively In leaf 13 also *β*-carotene tended to be retained relative to the epoxy xanthophylls, even though the two were strongly correlated ($r = 0\,84^{**}$) Thus old, lower leaves tended to retain *β*-carotene and lutein during senescence

The proportion of *β*-carotene in the total carotenoids of tobacco leaf generally ranged between 15 and 30% lutein between 40 and 60% and each of the epoxy xanthophylls between 5 and 20% of the total carotenoids (w w) Esterified xanthophylls were not detected in senescent tobacco leaves

DISCUSSION

Goodwin[7] reported three types of change in carotenoids during loss of chlorophylls in senescing leaves of deciduous trees carotenoids decreased either less rapidly (*Acer pseudoplatanus*), at an equal rate (*Quercus robur*) or more rapidly (*Prunus nigra*) than the

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TO CAROTENOID AND OF CAROTENOID FRACTIONS

Leaf 13				Leaf 19			
L/E	L/C	V/N	Chl/Car	E/C	L/E	L/C	V/N
14	17	13					
12	29	12					
14	22	10					
18	17	12					
19	16	11	74	11	20	21	10
23	14	12	97	04	28	12	13
			81	09	21	20	07
			45	15	13	20	09
			88	10	12	12	05
097**	093**	097**	095**	093**	093**	099**	096**

L—Lutein, V—Violaxanthin, N—Neoxanthin Values are usually means of four ratios

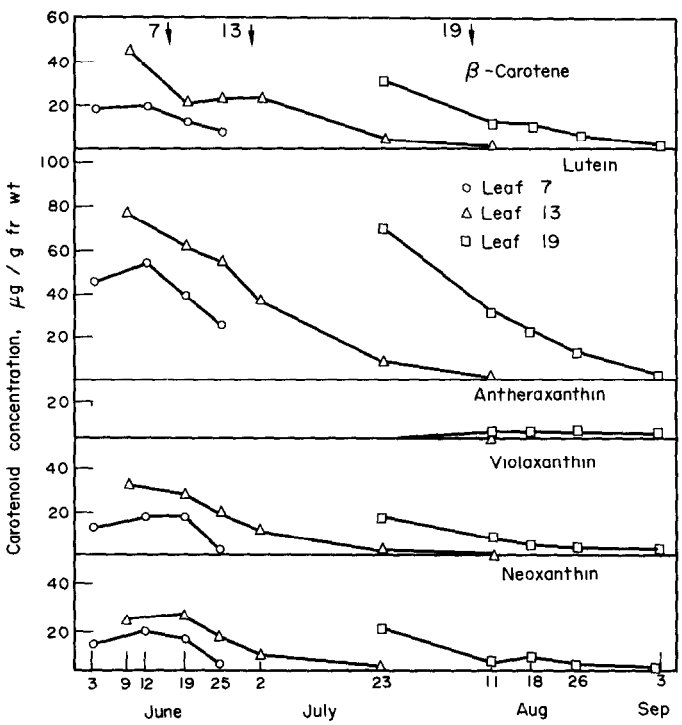

FIG. 2 CHANGES IN CONCENTRATION OF CAROTENOID FRACTIONS WITH DATE OF HARVEST

Values are means of analyses made on single leaves from four plants Arrows indicate the dates of estimated maturity for each leaf

TABLE 3 SYSTEMS FOR TLC

Layer	Solvent	Use
Silica gel G–KOH*	*n*-Hexane diethyl ether (1 9 v v)	Routine initial separation of carotenoids
Silica gel G–MgO (1 1 w/w)	Acetone *n*-hexane (1 4 v v)	Resolution of strongly absorbed xanthophylls[34]
Al_2O_3 MgO (1 3 w/w)	Ethyl acetate *n*-hexane (1 19 v v)	Resolution of carotenes[34 36]

* The silica gel was suspended in 1% (w v) KOH to prevent epoxide-furanoid oxide isomerization All materials for TLC were supplied by E Merck Darmstadt

rate of loss of chlorophylls In the present experiment lower leaves of tobacco resemble *Acer* while younger, upper leaves resemble *Quercus*, thus confirming that there is no common pattern of changes in carotenoid and chlorophyll concentrations during leaf senescence [7] In deciduous leaves β-carotene and neoxanthin disappeared more rapidly than other carotenoids, and lutein-5,6-epoxide, and in some species esterified xanthophylls appeared [7] In tobacco leaf, β-carotene and lutein decreased less rapidly than other carotenoids and esterified xanthophylls did not appear The retention of β-carotene has been observed previously in tobacco [8 32 33] although the balance is apparently modified by nitrogen nutrition [33] Antheraxanthin, instead of the γ-isomer, lutein-5,6-epoxide, appeared during senescence in upper leaves harvested in August and September, though Costes[14] has identified the latter in tobacco In subsequent seasons we have found antheraxanthin in lower leaves, but only in August or later Although we did not detect zeaxanthin when fraction 2 was chromatographed on silica gel G MgO other workers have reported that this pigment accumulates only under special conditions[14 16 19] and the amount present in tobacco leaves could well be below the level detected by the methods we have used The accumulation of antheraxanthin probably reflects an effect of changes in the environment on the equibria of reactions of the violaxanthin cycle [16]

$$\text{zeaxanthin} \rightleftharpoons \text{antheraxanthin} \rightleftharpoons \text{violaxanthin}$$

Both quantitative and qualitative changes in carotenoids that we have observed during sequential senescence in tobacco leaves follow different patterns to synchronous senescence[22] in leaves of deciduous trees,[7] whether the differences are general in the two types of senescence requires further investigation

EXPERIMENTAL

TLC Plates and solvents used are shown in Table 3

Growing conditions and sampling of leaves Tobacco plants (*Nicotiana tabacum* L cv Virginia Gold) were grown through autumn, winter and spring in a glasshouse at not less than 13 Seedlings were transplanted in March into pots containing *ca* 13 kg of soil Fluorescent lamps provided additional light during winter Harvesting of leaves 7 13 and 19 began after each was fully expanded and continued until senescence Pigment concentrations were determined in quadruplicate in single leaves from each of four plants

Isolation and identification of carotenoids Leaves at a range of maturities were harvested, the midribs discarded cut into small pieces and macerated in 90% (v v) acetone for 30 sec using an Ultra-Turrax probe blender (Janke and Kunkel type TP 18 2) taking less than 1 g fr wt of leaf tissue per 10 ml acetone The blend was

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filtered through Whatman No 50 paper Pigments were transferred to diethyl ether washed several times with H_2O and evaporated to dryness in a rotary film evaporator at less than 30°, adding a few ml absolute EtOH to give a dry residue of lipid containing both chlorophylls and carotenoids This was stored under N_2 at −16° until required The dry lipid fractions were dissolved in acetone and applied as a narrow band to TLC plates of silica gel G/KOH (20 × 20 cm) The plates were developed in darkness in glass tanks lined with thick filter paper until the front was *ca* 15 cm from the start line The bands were removed and pigments eluted with suitable mixtures of diethyl ether and acetone The solutions were dried again as above and redissolved for chromatography or for determination of absorption spectra in *n*-hexane, EtOH and $CHCl_3$ Ethanolic solutions were acidified with a few drops of dil HCl to determine the extent of any hypsochromic shift Spectra were recorded with a Beckman DB or Unicam SP 800 spectrophotometer calibrated with a Holmium filter The method of Goodwin[7] was used for testing for esterified xanthophylls

Determination of concentrations of chlorophylls and carotenoids 30 disks 1 3 cm dia (1 6 cm for yellow leaves) cut from each leaf with a cork borer were placed in a boiling tube containing 35 ml 90% (v/v) acetone and held in an ice bath The tissue was macerated for 30 sec with the Turrax blender after solids settled the supernatant solution was filtered through Whatman No 50 paper, the residue macerated again with 20 ml 90% acetone for 10 sec and the blend added to the filter funnel The residue was washed with 90% acetone and the vol of the filtrate adjusted to 100 ml, adding H_2O to give a final concentration of 80% acetone The concentrations of chlorophylls *a* and *b* per g fr wt were determined using the equations of Arnon[37] When total chlorophylls calculated using Bruinsma's equation[38] differed by more than 10%, the result was discarded Extinctions were measured using a Hilger Spectrochem spectrophotometer corrected by measuring the apparent E_{max} of a leaf extract in 80% acetone (663 nm) For determining carotenoids, pigments were transferred from 80% acetone into diethyl ether in a separating funnel as above The epiphase was transferred to a pear-shaped flask, ethanolic washings from the funnel added, and evaporated to dryness The dry residues were stored in darkness at −16° under N_2 For chromatography on silica gel G/KOH plates, the dry residues were dissolved in 1 0 or 2 0 ml acetone and 0 10 or 0 20 ml samples run on duplicate plates After running the gel containing each fraction was scraped from the plates and eluted with ethanol or petroleum spirit by suction on a small sintered funnel into a tube calibrated to 3 0 ml The volume was adjusted to 3 0 ml, the extinction measured using the Hilger spectrophotometer and concentrations per g fr wt calculated from appropriate extinction coefficients[35,39]

Acknowledgement—D M Whitfield held a Research Scholarship from the University of Melbourne

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