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THE VARIATIONS IN LINOLENIC ACID AND GALACTOLIPID LEVELS IN GRAMINEAE SPECIES WITH AGE OF TISSUE AND LIGHT ENVIRONMENT

I. K. GRAY,* M. G. RUMSBY and J. C. HAWKE

Department of Chemistry and Biochemistry, Massey University of Manawatu,
Palmerston North, New Zealand

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Abstract—The increases in total lipid and galactolipid which occur in leaf tissue of increasing maturity appear to be dependent on increasing photosynthetic activity rather than age of tissue. Although the total amount of linolenic acid in leaf tissue increases with increased chlorophyll levels there is very little change in the relative proportions of the component fatty acids in the monogalactolipid, high levels of linolenic acid being maintained under all conditions.

INTRODUCTION

THE lipid composition of leaf tissue from several species of higher plants such as red clover,[1] alfalfa,[2] spinach,[3] runner-bean[4] and potato[5] has been totally or partially determined, and all species appear to have similar lipid compositions which are characterized by high concentrations of mono- and digalactolipids. Further it has been shown that the galactolipids are concentrated in the chloroplasts of photosynthetic tissues[6] and α-linolenic ($\Delta^{9,12,15}$ octadecatrienoic) acid constitutes a very high proportion of the total fatty acids of chloroplasts and is the predominant fatty acid of the galactolipids.[1,2,3]

A possible relationship exists between the levels of chlorophyll and of galactolipids and unsaturated fatty acids in green tissue. For example, green leaves of elder (*Sambucus nigra*) have been found to contain higher galactolipid levels than yellow leaves from the same species,[6] and Nichols has reported similar differences between the dark outer leaves of cabbage and the pale inner leaves and stalk material.[7] Furthermore, Crombie[8] has reported that lipid extracts from green leaves of a variety of plant species contain a higher proportion of unsaturated fatty acids compared with lipids from non-green leaves. It appears that decreased rates of photosynthesis lead to decreases in the concentrations of galactolipids and linolenic acid in leaf tissue[9] and, in studies with lower plant forms, the levels of galactolipids and of linolenic acid were stimulated or depressed by respective light or dark treatment.[10,11]

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* Present address: The Dairy Research Institute (N.Z.), Palmerston North, New Zealand.

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During investigations into dietary factors which may possibly influence the composition of bovine milk lipids it was found that the young leaf tissue of short-rotation ryegrass (*Lolium multiflorum* × *L. perenne*) contained higher levels of lipid than older tissue.[12] The young growth also contained higher proportions of linolenic acid and correspondingly lower levels of linoleic and palmitic acids. Newman,[13] however, has reported that plastids from mature tissue of bush bean (*Phaseolus vulgaris*) contained less chlorophyll and have a higher saturated to unsaturated fatty acid ratio than plastids from young tissue. Similarly it was found that young leaves of the squash plant (*Cucurbita maxima*) have a lower ratio of saturated to unsaturated fatty acids than old leaves.[9]

The aim of the present study has been to investigate the relationship between age of plant tissue, photosynthetic activity and lipid composition with special reference to mono- and digalactolipids and the linolenic acid content of these components. Leaf tissue of perennial ryegrass (*L. perenne*) was chosen for studies on the effect of age on lipid composition and barley (*Hordeum vulgare*) seedlings were used to study the effects of light intensity because of the suitable growth characteristics of this species.

RESULTS

When the lipid content of three leaf regions cut from 40-day-old plants of *L. perenne* was determined separately it was found that the lipid content expressed as a per cent wet weight, increased from the basal (1–10 cm) to the terminal (19–28 cm) region (Table 1). This trend is not evident when lipid content is expressed in terms of per cent dry weight, due to the parallel increase in the dry weight of the tissue. In addition to changes in lipid content, large differences in the fatty acid composition of the leaf regions were observed. There was an increase from 53·9 to 67·0 in the percentage content of linolenic ($C_{18:3}$) acid and corresponding decreases in the proportions of palmitic ($C_{16:0}$) and linoleic ($C_{18:2}$) acids proceeding from the basal to the distal leaf region of the plant. The central leaf region gave values which were consistently between the extreme values of the other two regions, e.g. 64·6 per cent for

Table 1. Lipid and chlorophyll content and the fatty acid composition of the leaf tissue of *Lolium perenne*

Days of growth	Leaf region measured from the base (cm)	Total lipid		Absorptivity/g wet wt. of tissue		Fatty acid composition (moles %)								
		% of wet wt.	% of dry wt.	Chl*a*	Chl*b*	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20*
40	1–10	1·34	9·35	0·28	0·08	tr.	1·3	20·9	1·0	1·4	2·4	17·9	53·9	1·2
40	10–19	1·52	10·12	0·43	0·23	—	0·9	16·6	1·1	1·5	1·3	12·6	64·6	1·4
40	19–28	1·63	9·85	0·51	0·29	—	0·7	15·5	1·4	1·6	1·2	11·7	67·0	0·9
8	Whole leaf 9	1·36	10·5	0·46	0·09	—	0·2	11·9	1·7	1·0	2·2	14·6	68·2	0·2

* Tentatively identified as 20:1.

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$C_{18:3}$, while the fatty acid composition of 8-day-old leaves from plants of *L. perenne* most closely resembled the composition of the 19–28 cm leaf region of 40-day-old plants. In addition to the fatty acids shown in Table 1, trace amounts of other fatty acids ($C_{15:0}$, $C_{17:1}$, $C_{20:0}$, $C_{22:0}$, $C_{24:0}$) were also identified in the leaf tissue.

When barley seedlings (*H. vulgare*) were grown in differing light environments for 7 days the total lipid content of the seedlings increased from 0·60 per cent when grown in the dark, to 1·04 per cent when grown in natural light. These changes were accompanied by increases in the relative proportions of linolenic acid, namely 51·8, 61·6 and 71·1 per cent in dark, low, and natural light conditions respectively. Corresponding decreases in the proportions of palmitic, stearic ($C_{18:0}$), oleic ($C_{18:1}$) and linoleic acids in the total fatty acids were observed (Table 2).

TABLE 2. LIPID AND CHLOROPHYLL CONTENT AND THE FATTY ACID COMPOSITION OF THE LEAF TISSUE OF 7-DAY-OLD *Hordeum vulgare* SEEDLINGS

Light treatment	Total lipid % of wet wt.	Total lipid % of dry wt.	mg/100 g wet wt. tissue Chl*a*	mg/100 g wet wt. tissue Chl*b*	Fatty acid composition (moles %) 12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20*
Dark	0·60	8·2	0·28	0·28	0·3	1·4	19·5	1·0	3·0	6·8	15·1	51·8	1·1
Low light (2–3% of natural light intensity)	0·69	9·6	3·60	1·40	tr.	0·3	17·5	0·9	1·0	2·6	13·7	61·6	2·4
Natural light	1·04	10·4	14·00	4·41	tr.	1·3	12·4	1·6	1·0	1·1	9·4	71·1	3·1

* Tentatively identified as 20:1.

TABLE 3. GALACTOLIPID CONTENT AND THE FATTY ACID COMPOSITION OF MONOGALACTOLIPID ISOLATED FROM THE LEAVES OF *Lolium perenne*

Leaf region (length in cm)	Galactolipid content: % of wet weight GDG*	GGDG†	Total	% of total lipid GDG	GGDG	Total	Fatty acid composition (moles %) 14:0	16:0	18:0	18:1	18:2	18:3
1–10	0·25	0·15	0·40	19·0	11·2	30·2	0·6	3·1	1·3	1·5	5·5	88·0
10–19	0·31	0·19	0·50	20·3	12·5	32·8	0·3	2·8	1·0	1·2	3·9	90·8
19–28	0·35	0·21	0·56	21·2	12·6	33·6	0·7	5·3	1·5	1·4	2·5	88·6
Whole leaf 9	0·28	0·19	0·47	20·9	12·8	33·7	0·2	3·4	0·9	1·9	3·2	90·4

* GDG = galactosyl diglyceride.
† GGDG = galactosyl galactosyl diglyceride.

A separate consideration of the galactolipid components of the lipids from the three leaf regions of ryegrass showed that the total galactolipid content increased from the 1–10 cm to the 19–28 cm leaf region (Table 3). Again these differences are more evident when the galactolipid content is expressed as a percentage of the wet weight of leaf tissue rather than as a percentage of the total lipid, and apply to both the monogalactolipid and the digalactolipid components.

The development of quantitative methods for the isolation of monogalactolipid by thin-layer chromatography made it possible to compare the fatty acid composition of this lipid in different leaf tissues. The notable feature of the fatty acid composition of the monogalactolipid isolated from tissue of varying maturity is the small variation in the proportions of the individual fatty acids (Table 3). The linolenic acid content of the monogalactolipid from the basal, middle and distal leaf regions was 88·0, 90·8 and 88·6 per cent respectively. In contrast, the proportions of this acid and of saturated fatty acids in the total lipid from the same tissues increased and decreased respectively with increasing maturity of the tissue (Table 1). Compared with the fatty acid composition of the total lipid, the monogalactolipid component from the same tissue contained higher proportions of linolenate and lower proportions of linoleate and palmitate.

Although the quantitative determination of the digalactolipid content of leaf tissue based on galactose measurement was reproducible, possible contamination by non-galactose-containing lipids made a comparative study of the fatty acid moieties of this component less satisfactory, and the results have not been recorded in the present report.

TABLE 4. GALACTOLIPID CONTENT AND THE FATTY ACID COMPOSITION OF MONOGALACTOLIPID ISOLATED FROM THE LEAVES OF *Hordeum vulgare* SEEDLINGS

	Galactolipid content												
	% of wet weight			% of total lipid			Fatty acid composition (moles %)						
Light treatment	GDG*	GGDG†	Total	GDG	GGDG	Total	14:0	16:0	18:0	18:1	18:2	18:3	Unidentified
Dark	0·08	0·03	0·11	13·1	5·5	18·6	0·5	7·5	3·8	5·3	1·9	78·0	3·0
Low light (2–3% of natural light intensity)	0·14	0·05	0·19	20·4	7·1	27·5	0·3	3·9	3·2	2·5	1·3	83·8	5·0
Natural light	0·21	0·09	0·30	20·6	8·8	29·4	tr.	4·8	1·3	1·6	1·0	85·6	5·7

* GDG = galactosyl diglyceride.

† GGDG = galactosyl galactosyl diglyceride.

Increased exposure of 7-day-old barley seedlings to light resulted in leaf tissue with an increased concentration of galactolipids (Table 4). As with the monogalactolipid component of *L. perenne* from leaf tissue of different ages, only minor differences in fatty acid composition were found in the monogalactolipid component of the 7-day-old leaf tissue of *H. vulgare* exposed to different light intensities.

DISCUSSION

Although plastids of mature bush bean (*P. vulgaris*)[13] and mature squash (*C. maxima*)[19] leaves have been shown to contain a higher ratio of saturated to unsaturated fatty acids than plastids of immature leaves, in the present investigation the most mature leaf region (19–28 cm) of *L. perenne* was shown to contain higher proportions of unsaturated fatty acids than immature regions (1–10 cm). This reversed relationship may be attributed to the different growth characteristics of the species studied. In *L. perenne*, leaf elongation takes place by cell division at the leaf base so that the older leaf tissue reduces the light intensity received by immature tissue. In squash (*C. maxima*) and bush bean (*P. vulgaris*) however, whole leaves were compared and in these plants immature leaves develop above mature leaves probably resulting in a reduced light intensity in the region of the lower leaf. The finding that an increased concentration of chlorophyll in *L. perenne* and *H. vulgare* leaf tissue was accompanied by an increase in unsaturated fatty acid content is consistent with earlier work on the plastids of bush bean and squash in which it was shown that a high chlorophyll content was paralleled by a low ratio of saturated to unsaturated fatty acids. The observation that the leaves of immature *L. perenne* plants, i.e. 8-day-old growth, and the 19–28 cm region of leaves from plants grown for 40 days have similar fatty acid composition and chlorophyll levels, further suggests that there is a relationship between chlorophyll content and fatty acid composition.

As found previously in photosynthetic micro-organisms,[10, 11] and higher plants,[9] the greatest effect of increased light intensity is to increase the concentration of linolenic acid. The suggestion that linolenic acid may be involved chemically in photosynthesis[11, 14] has not been supported by a recent study on the lipid composition of various photosynthetic systems by James and Nichols.[15] These workers emphasize the contribution which lipids, containing a high concentration of unsaturated fatty acids, make to the non-aqueous environment of the chloroplast. Alternatively the high linolenic acid content may be a reflection of the stimulation of linolenic acid synthesis by non-cyclic photophosphorylation.[16]

The fatty acids of monogalactolipid, which together with digalactolipid constitute the major lipid component of plant tissue, were found to contain mainly linolenic acid. This confirms previous work on photosynthetic micro-organisms and higher plants.[2, 3, 4] However, relatively small changes in the fatty acid composition occurred in the monogalactolipid components of tissues of different chlorophyll content. This suggests that the frequently observed increase in linolenic acid content associated with increased photosynthetic activity is due to an increase in the total galactolipid content of the tissue and not to changes in the constituent fatty acid components of monogalactolipids.

EXPERIMENTAL

Growth of Plants

Perennial ryegrass (*L. perenne*) was grown in a controlled environment growth cabinet as individual clones in a pumice:peat mixture (3:1) in a 12-hr photoperiod with a light intensity of 20,000 lux (obtained from a mixture of ten 125 W reflector-backed fluorescent tubes (TL/33) and ten 150 W incandescent flood-lamps) at light and dark temperatures of 21 ± 1° and 18 ± 1° respectively. Plants were sub-irrigated daily with modified Hoagland's solution diluted 1:1 with water.

Barley (*H. vulgare*, var. Kenia) seedlings were grown in a pumice:peat:sand mixture (3:1:1) in a glasshouse with a controlled environment, in darkness and in high and low levels of natural light at day and night temperatures of 23 ± 2° and 15 ± 2° respectively. Natural day length was extended by incandescent lamps to make a photoperiod of 14 hr. Low light was maintained at 2–3 per cent of the full natural light intensity.

Harvesting of Leaf Tissue

L. perenne plants were grown for 40 days and reached a height of about 28 cm. Leaf tissue was cut at levels 1, 10 and 19 cm above the surface of the pots, the 1–10 cm region

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containing the basal sections and leaf sheaths of the more mature leaves, while the 10–19 cm and the 19–28 cm regions contained leaf tissue of increasing maturity, since in grasses leaf elongation occurs by cell division at the leaf base. Whole leaves were also harvested as a single sample after 8 days' growth.

H. vulgare seedlings were grown for 7 days after germination and the primary leaf was then cut at a point just above the coleoptile.

Extraction of Lipids

Lipids were extracted from freshly harvested leaf material by mascerating the tissue immediately in chloroform:methanol (2:1, v/v) in a Waring blendor using 100 ml solvent mixture for each 100 g fresh leaf tissue. The residue obtained after filtration was similarly re-extracted twice more and the combined solvent extracts were removed *in vacuo* below 50°. The lipid extract was redissolved in chloroform:methanol and non-lipid contaminants removed according to the Folch method[17] by partition with 0·1 M KCl. The Folch wash was repeated twice more and the purified lipid extract finally dried *in vacuo*.

Separation of Lipids and Fatty Acids

Aliquots of total lipid extracts were chromatographed on thin layers of Silica-gel G prepared according to the method of Stahl[18] and developed in solvent systems of chloroform: methanol:water (65:25:4, v/v),[19] toluene:ethyl acetate:95% ethanol (10:5:5, v/v) and chloroform:methanol (185:15, v/v). The latter two solvents were of particular value for the identification and isolation of galactolipids, and a typical separation is shown in Fig. 1. Galactolipids and other lipids were identified by reference to authentic compounds and specific spray reagents[18] and confirmation that the galactolipids have been correctly identified was obtained by paper and cellulose thin-layer chromatography of alkaline and acid hydrolysis products.[4]

A portion of the total lipid extract was saponified by refluxing with 5% KOH in methanol for 4 hr and, following the removal of the non-saponified material, the fatty acids were extracted with light petroleum, dried, and converted to methyl esters by refluxing with 1% H_2SO_4 in methanol for 1 hr.[20] Fatty acids of galactolipids which had been isolated from thin-layer chromatoplates were methylated as described by O'Brien and Benson.[2]

The methyl esters of the fatty acids were analysed by gas–liquid chromatography in a Pye-Argon Chromatograph fitted with a β-ionization detector. Columns (4 ft × 4 mm i.d.) were packed with 10% diethylene glycol adipate on acid-washed Celite (60–80 mesh) or with 20% diethylene glycol succinate on Chromosorb W (60–80 mesh) held at 165°. The identity of the methyl esters was established from the logarithmic values of the retention volumes and the proportions of methyl esters present were determined quantitatively by planimetric measurement of peak areas.[21] Confirmation of structure was obtained by fractionation of the total methyl esters into tri-, di-, mono-unsaturated and saturated fractions on Silica-gel G impregnated with 10% $AgNO_3$.[22] These fractions were eluted and analysed by gas–liquid chromatography.

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Determination of Galactolipid and Chlorophyll

Galactolipid content of the lipid extracts was determined by hydrolysis of the galactolipid component, obtained by preparative thin-layer chromatography, in 1 N-H_2SO_4 for 1 hr at 100°. Galactose was estimated by the orcinol method of Svennerholm,[23] and factors of 4·3 (monogalactolipid) and 2·6 (digalactolipid) used for the conversion of galactose to galactolipid. These conversion factors assume that the mono- and digalactolipids contained two linolenic acid residues.

Chlorophylls were analysed according to the method of Koski *et al.*[24] Semi-quantitative measurement of chlorophyll levels was obtained by measurement of the absorptivities of standard ether solutions at 662 and 644 nm.[25]

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