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LIPID VARIATION OF THE GREEN ALGA *FRITSCHIELLA TUBEROSA* DURING GROWTH IN AXENIC BATCH CULTURE

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Key Word Index—*Fritschiella tuberosa*; green alga; aging; pigments; triacylglycerol; glycolipids; phospholipids; fatty acids.

Abstract—The lipid and pigment pattern of the chaetophoralean green alga *Fritschiella tuberosa* was studied during different growth periods. Four phases in axenic batch culture are observed. The first phase commences at the end of logarithmic growth and results in a decrease of phosphatidyl inositol and hexadecatetraenoic acid. The second phase coincides with the end of linear growth and is characterized by a decrease in glycolipids, phospholipids and α-linolenic acid, and an increase of linoleic and oleic acid. The third phase starts with the beginning of the stationary phase. During this time glycolipids, phospholipids, and α-linolenic acid decrease and oleic acid, linoleic acid and triacylglycerol increase. The fourth phase begins about one week after the onset of the stationary growth phase. A marked accumulation of triacylglycerol took place, secondary carotenoids were detectable and the long filamentous cells of *Fritschiella* changed into slightly rotund short cells.

INTRODUCTION

Fritschiella tuberosa is a filamentous and branched green alga forming a highly differentiated thallus. It was first described as a terrestial alga [1], but grows well in a synthetic liquid culture medium under axenic conditions [2]. During growth in batch culture, *Fritschiella tuberosa* forms after mitotic divisions thick-walled resting cells (akinetes) and becomes red. The red colour of aged algae is caused by SC† [3] and these have been examined in detail [4, 5].

In addition to *F. tuberosa* [4], the production of SC within the Chaetophorineae has been described for *Trentepohlia aurea* [6] and *Gongrosira papuasica* [7].

The aim of this study was to examine the variation in lipid and pigment content of *F. tuberosa* during different growth periods in batch culture dependent on the nutrient status.

RESULTS

Morphology

The highly differentiated thallus of *Fritschiella tuberosa* in its natural environment is composed of 4 different filament systems [1, 8, 9]. None of them are developed in young aqueous cultures [4]. Germination of zoospores, used as inocula, is followed by mitotic divisions and cytokinesis leading to filaments. By branching, these develop into spherical pellets in aerated cultures.

Initially, branched filaments are composed of long and thin-walled cells each with one extended, parietal chloroplast with a few pyrenoids.

During logarithmic and linear growth, morphology remains unchanged. In aging cultures, *ca* one week after the stationary growth phase sets in, the algal cells form transverse cell walls and shorter cells develop. Under axenic conditions these round off, leading to 'barrel-shaped' forms. The cell walls do not thicken as in the non-sterile cultures. During this growth phase the chloroplast disintegrated and the cells became filled with orange–red cytoplasmic membraneless lipid globules (Weber, A., unpublished results). This morphology remains unchanged for *ca* 4 months then the algae become bleached and die.

Occurrence of secondary carotenoids

The alterations in morphology are accompanied by characteristic changes in the pigment composition. While the alga exhibited a colour typical for green algae during the logarithmic and linear growth phases, it becomes orange–red with the passage of time during the stationary phase. Under the experimental conditions employed the SC are detectable by TLC after 21 days of culture, although at that time the alga still appear green. It was not possible to find a time dependent succession of the different SC (adonixanthin-ester, astaxanthin-esters, echinenone, canthaxanthin and fritschiellaxanthin). From the 21st day onwards, all SC as described by Weber [4] were present.

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† Abbreviations: DGDG, Digalactosyl diacylglycerol; DPG, diphosphatidyl glycerol; MGDG, monogalactosyl diacylglycerol; PC, phosphatidyl choline; PE phosphatidyl ethanolamine; PG, phosphatidyl glycerol; PI, phosphatidyl inositol; SC, secondary carotenoids; SQDG, sulfoquinovosyl diacylglycerol.

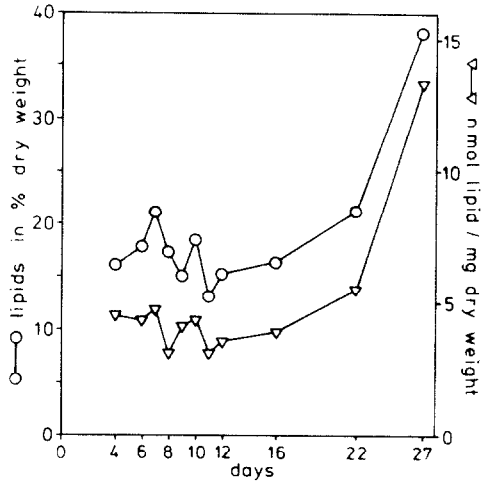

Fig. 1. Changes in total lipid (O—O) and triacylglycerol (▽—▽) content of *F. tuberosa* during growth in axenic batch culture.

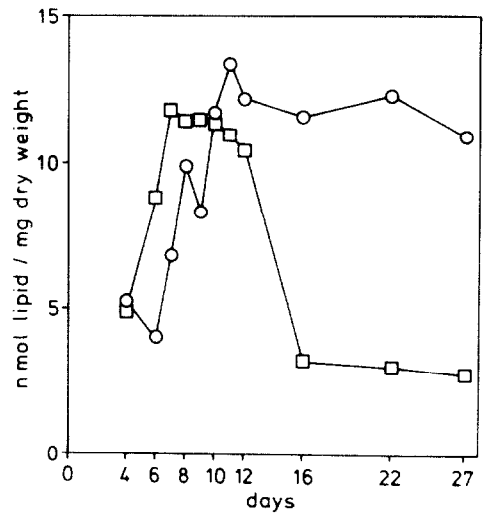

Fig. 3. Changes in PC (O—O) and PI (□—□) content of *F. tuberosa* during growth in axenic batch culture.

Quantitative lipid variation

Total lipid and triacylglycerol. The total-lipid and triacylglycerol content remained almost unchanged during the first 16 days of culture, i.e. during the logarithmic and linear growth phases (Fig. 1). Within this period the calculated means 16.9% S.D. ± 2.3 and 4 nmol/mg S.D. ± 0.6, respectively. A slight increase of both lipids commenced with the beginning of the stationary phase (16 days). After 27 days incubation the total-lipid content had increased 2.2-fold and that of triacylglycerol 3.4-fold. In older algal cultures, still higher values were found [10].

Glycolipids. Beginning with the 7th day of growth (end of logarithmic growth) the concentration of glycolipids increased, although to a different degree (Fig. 2). The 3 glycolipids determined finally reached their highest concentration at the end of linear growth (12th day). At that time the concentration of MGDG was *ca* 44 nmol/mg dry wt and that of DGDG and SQDG was *ca* 14 and 8 nmol/mg, respectively. With the onset of the stationary phase the glycolipids decreased. The concentration of MGDG, comparing the values of the 12th and 27th day, was reduced by *ca* 73%.

Phospholipids. The most noteworthy variation in phospholipids was exhibited by PI and PC (Fig. 3). At the end of the logarithmic growth a maximum in the PI concentration of *ca.* 12 nmol/mg is reached. This concentration was finally reduced by *ca.* 70%.

The concentration of PC reached a maximum of *ca.* 13 nmol/mg at the 11th day. Until the stationary phase this content was only slightly reduced. PG, PE and DPG exhibited their maximal concentration at the end of the linear growth phase (Table 1). During stationary growth phase the concentrations of these phospholipids generally decreased.

Total fatty acids. The most noteworthy variations were exhibited by hexadecatetraenoic acid, oleic acid, linoleic acid, and α-linolenic acid (Fig. 4). Initially α-linolenic acid represented 40–50% of the total fatty acids; during the stationary growth phase its concentration decreased and was reduced by *ca.* 50% by the 27th day of culture.

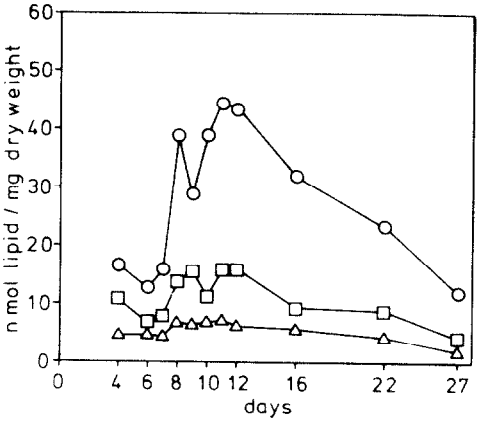

Fig. 2. Changes in MGDG (O—O), DGDG (□—□) and SQDG (△—△) content of *F. tuberosa* during growth in axenic batch culture.

Table 1. Changes in composition of different phospholipids of *F. tuberosa* during growth in axenic liquid batch culture

Days	nmol lipid/mg dry weight PE	PG	DPG
4	3.0	4.1	1.5
6	4.5	4.0	1.8
7	3.8	4.9	1.8
8	4.8	7.3	2.3
9	5.4	6.4	1.9
10	7.0	5.8	2.5
11	5.3	7.4	2.5
12	4.6	6.2	2.8
16	4.6	6.5	2.0
22	4.3	7.3	1.6
27	3.9	4.8	0.3

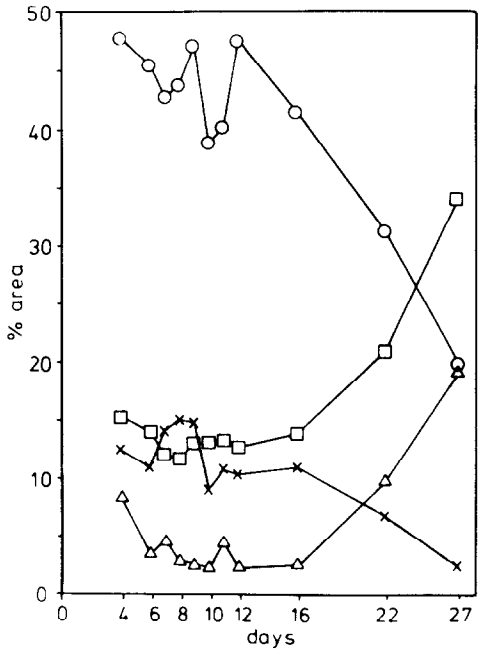

Fig. 4. Changes in total fatty acid composition of *F. tuberosa* during growth in axenic batch culture. (×—×) Hexadecatetraenoic acid, (▽—▽) oleic acid, (□—□) linoleic acid, (○—○) α-linolenic acid.

Variations in the concentration of oleic and linoleic acid ran contrary to the development of the α-linolenic acid during the entire culture time. Until the 27th day the concentration of oleic acid and linoleic acid increased *ca* 6.4- and 2.6-fold, respectively.

Remarkable variations were also exhibited by the hexadecapolyenic acids. They were more pronounced in the hexadecatetraenoic acid than in the hexadecatrienoic acid (Table 2). The hexadecatetraenoic acid represented 11–15% of total fatty acids during logarithmic growth. Parallel to the onset of linear growth its concentration decreased, more markedly during the stationary phase.

Table 2. Changes in palmitic acid and hexadecatrienoic acid content of *F. tuberosa* during growth in axenic liquid batch culture

	% Area	
Days	Palmitic acid	Hexadecatrienoic acid
4	11.4	2.6
6	20.1	4.6
7	18.9	6.3
8	19.9	5.0
9	16.3	4.1
10	21.3	4.8
11	17.6	9.0
12	17.7	7.1
16	20.2	8.0
22	21.6	6.6
27	14.5	6.1

DISCUSSION

During the growth of *F. tuberosa* in axenic batch culture, characteristic changes in the lipid composition, accompanied by variations in morphology and fine structure, are observed. The results indicate important variations in lipids characteristic for chloroplasts [11–15] during the aging of *F. tuberosa* as well as the accumulation of cytoplasmic triacylglycerol.

The gradual depletion of nutrients during batch culture is probably the reason for the variation during this process although the following explanations cannot be neglected: (a) that mutual shading during the growth of algae results in light becoming in effect a limiting factor, or (b) that the cultures regulate themselves by release of auto-inhibitory compounds without the necessity of nutrient salt deficiency at the same time. But as the algal dry wt per volume of nutrient medium increased until the end of the linear growth phase (12th day) [2] and the possibility to induce the formation of orange–red resting cells by varying the nitrate concentration of the nutrient medium [4], the two explanations above might be excluded for the stated culture time.

The changes observed in morphology, pigment pattern and in particular the variation in lipid content during batch culture led to the classification of 4 different phases.

The first phase parallels logarithmic growth (until 8th day of culture) and is characterized by a maximum content in PI and hexadecatetraenoic acid. After this time the concentrations of these lipids decreased. The trigger for these alterations could be a decrease in the manganese concentration of the culture medium as PI is thought to be a phospholipid the synthesis of which is Mn^{2+}-dependent [16]. The manganese-dependent synthesis of fatty acids is known from the biotin–carboxylase activity [17]. But this step of biosynthesis of hexadecatetraenoic acid does not seem to be inhibited in *F. tuberosa* as the concentrations of the other determined fatty acids do not decrease at the same time. Therefore it might be a specific inhibition of the final desaturase system introducing the fourth carbon double bond in the hexadecatetraenoic acid. At the 8th day of culture the concentration of manganese would have reached a level of insufficient for the activation of the transferase system synthesizing PI and the activation of the specific desaturase synthesizing hexadecatetraenoic acid. This hypothesis is supported by the results of Christlieb [18] who found a Mn^{2+}-dependent extended logarithmic growth period in *F. tuberosa*.

The second phase coincides with the end of linear growth (until the 12th day of culture). Up to then a marked increase of the lipid classes characteristic for photosynthetic membranes is observed. The concentration of glycolipids increased and triacylglycerol is found only in small concentrations: glycolipids represented 65 nmol/mg dry wt and triacylglycerol 4 nmol/mg dry wt.

The third phase runs parallel with the beginning of stationary growth and lasts *ca* 1 week. This period commences with a decrease of glycolipids, phospholipids, α-linolenic acid, and an increase of oleic acid, linoleic acid, and triacylglycerol. The reason for these changes could be a decrease in nitrate concentration of

the culture medium [19]. As there was always diacylglycerol present in all tested total lipid extracts, the activity of glycerolphosphoryl acyltransferase, leading to 1,2-diacylglycerol, should then not be regulated by nitrate but the diacylglycerol acyltransferase, catalysing triacylglycerol from 1,2-diacylglycerol, should be activated in the absence of nitrate (see the interconversion between major classes of plant glyceride [16, 20]). So the biosynthetic pathway of plant glycerides might be still working during supposed nitrogen deficiency up to the level of 1,2-diacylglycerol. This product is not introduced into the glyco- and phospholipid pathway but used for the synthesis of triacylglycerol.

The increase of oleic acid and linoleic acid might be caused by a similar regulated mechanism. The desaturase system, introducing the third carbon double bond in α-linolenic acid could be inhibited by the supposed low level of nitrate, thus precursors of α-linolenic acid, namely oleic and linoleic acid, are accumulated.

A fourth phase begins *ca* 1 week after the onset of the stationary phase. From this time on, continuation of the assumed nitrate-dependent inhibition of glyco- and phospholipids results in a marked accumulation of triacylglycerol. The synthesis of primary carotenoids is shifted to that of secondary carotenoids; these are exclusively ketonic carotenoids. The formerly long filamentous cells of *F. tuberosa* are changed by mitotic divisions into rotund short cells leading to resting cells, akinetes.

However, the aging of *F. tuberosa* in batch culture certainly is not regulated exclusively by the manganese and nitrate concentration of the nutrient solution. But it must be pointed out that it is quite difficult to distinguish between an acute or physiological mineral salt deficiency and the existence of simultaneous limitation of an organism by two or more nutrients is at present an area of great controversy.

EXPERIMENTAL

Organism and culture conditions. An axenic strain of *F. tuberosa* (cf. [2]) was cultured in 250 ml of an inorganic aerated medium [21] in a light–dark cycle of 14:10 hr at 4000 lx and a temp. of 18 ± 1°. A zoospore suspension was used as inoculum. Algae were harvested at different growth phases by filtration (membrane filter 11301, Sartorius). Samples taken after 4, 6, 7, and 8 days growth (log growth phase), after 9, 10, 11, and 12 days (linear growth phase), and 16, 22, and 27 days (stationary phase) were used (cf. [2]). Dry wt determinations (as basis for the analysis of lipids) and sterility tests were routinely performed as described elsewhere [2].

Pigment analysis. Quantitative and qualitative analyses were done using the methods of ref. [4].

Lipid analysis. Lipids were extracted from wet algae with $CHCl_3$–MeOH (2:1) [22]. H_2O-soluble impurities were removed using 0.1 M KCl [23]. The evapd $CHCl_3$ layer was redissolved in $CHCl_3$–MeOH (1:1) containing 0.005% 2,6-di-*tert*-butyl-4-methylphenol. Extracts were immediately analysed or stored under N_2 at −20°.

Separation of lipid classes. The polar lipids were separated by TLC using the system of ref. [24] and precoated Si gel plates HPTLC 60 F_{254} (Merck, 5642). Separation of neutral lipids was carried out using the system of ref. [25] and Si gel 60 HR (Merck, 7744) plates. Lipids were identified by co-chromatography with standards and by specific spray reagents [26–28]. Lipids were also detected with rhodamine 6G [24] or 2′,7′-dichlorfluorescin [29]. The following quantitative determinations of the different lipid classes were performed: (1) total-lipid content gravimetrically using an 'electronic' microbalance (Sartorius); (2) triacylglycerol enzymatically using a test-kit of Boehringer (125032); (3) glycolipids by the method of ref. [30]; (4) phospholipids by the method of ref. [31] using the modification described in [32].

Analysis of total fatty acids. Transmethylations of total lipid extract were performed in C_6H_6 with H_2SO_4–MeOH [33]. Purification of fatty acid Me esters followed the procedure of ref. [34]. Me esters were separated by $AgNO_3$-TLC [29]. Hydrogenation of Me esters was done as described in ref. [35].

GLC analysis. Separation of Me esters was performed with a FID instrument, equipped with a column (3m × 2mm) packed with 10% DEGS on Gas Chrom Q (80–100 mesh) at 185° isothermal and a N_2 flow-rate of 14.5 ml/min. Me esters were identified by their R_t as compared to those of standards and by ECL values. Quantitative determinations were done by absolute calibration with palmitic acid.

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