Long-Term Effect of Diuron on Chlorophyllous Callus of *Bromus erectus:* Lipid Composition

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The selection of plants resistant to photosynthetic herbicides is limited by the low rate of success in obtaining a photosynthetic callus. An attempt was made to induce diuron tolerance with chlorophyllous tissue strains of *Bromus erectus:* growth, chlorophyll contents and total fatty acids were studied during four months of photoheterotrophic growth in the presence or absence of 6 μM diuron. The presence of the herbicide, which reduced the fresh weight by 40%, provoked increases in chlorophyll levels by 2- or 3-fold, and in galactolipids (especially DGDG) which were enriched in linolenic acid content. These results, closely related to already described characteristics of herbicide-resistant material, suggest that callus of *Bromus erectus* could constitute an interesting photosynthetic *in vitro* material.

Introduction

It has been demonstrated that diuron and atrazine inhibit PS II electron transfer in higher plants as well as in algae. Fluorescence induction experiments [1], and trypsin treatment of either thylakoids [2] or PS II particles [3] have indicated that these herbicides are bound at the site of the secondary electron acceptor Q_B on the D 1, a 32 kDa polypeptide [3], and cause a decrease in the oxydation/reduction potential of Q_B, which then becomes inaccessible to reduction by the primary acceptor Q_A [4].

In *Spinacia oleracea* and in a cyanobacterium *Aphanocapsa,* a direct correlation was demonstrated between the binding of diuron and the inhibition of electron transfer [5]. In *Chlorella* and chloroplasts isolated from higher plants, it has been shown that the first biochemical changes induced by diuron, were to depress drastically the fatty acid biosynthesis, especially in galactolipids [6]. Similar observations on galactolipid metabolism held true in *Euglena gracilis,* during the first weeks of photoheterotrophic growth in the presence of 25 μM diuron [7].

In cells tolerant to herbicides ("adapted" cells), the modifications described were: an increase of the level of chlorophyll in mixotrophically grown cells of *Chenopodium album* L. [8]; a restoration of the galactolipid amounts in *Euglena gracilis,* which became close to the values of control, although significantly different whether or not the diuron was present in the culture medium [9]. Using *in vitro* culture techniques the selection of plants resistant to photosynthetic herbicides was limited because of the difficulty to obtain photosynthetic callus, especially from Graminaceae [10]. Chlorophyllous tissue strains of *Bromus erectus* had been obtained [11], which were able to evolve photosynthetic oxygen [12], as were triazine-resistant biotypes [13]. This material was then used in an attempt to induce a diuron resistance in photoheterotrophic conditions of growth, the same way it have been done in *Euglena gracilis* [14–16].

In *Euglena,* the acquirement of the diuron resistance was accompanied by lipid modification [7], which then characterized the resistant strains [9]. Accordingly, growth, chlorophyll contents and total fatty acids were studied in the callus from *Bromus erectus,* treated with 6 μM diuron, during a four month photoheterotrophic growth. Details in the fatty acid composition within the different classes of polar lipids have been studied at the stage of growth corresponding to 4 months, because of the two following observations: (i) the cal-

Abbreviations: Chl, chlorophyll; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS II, photosystem II.

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lus had the most homogeneous state; (ii) the chlorophyll contents per g fresh weight were the closest between the control and adapted callus.

Materials and Methods

Callus culture

Callus cultures were established from seedlings of *Bromus erectus* Huds. (Graminaceae) [11]. Every two months, inoculums constituted of three fragments of 50 mg of callus, were cultured in Petri dishes containing 15 ml of modified medium of Murashige and Skoog [11], supplemented with 2,4-dichlorophenoxyacetic acid (2 mg/l). The culture conditions were standardized to photoperiods of 16 h of light (30 $\mu E \cdot m^{-2} \cdot s^{-1}$) and a temperature of 26 ± 1 °C, as used for chlorophyllous callus of *Melilotus albus* [17]. After 5 years of subcultures, parts of a given callus, were inoculated on the same medium supplemented (adapted callus) or not (control callus) with 6 μM of diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea), dissolved in isopropanol (final concentration reaching 0.04%), and added after autoclaving. Some of the data presented are relative to the changes occurring during this first two-month period of contact with diuron.

The two types of callus, control and adapted, were then subcultured regularly every two months during a 14-months period. The most of the biochemical study presented here refer to data of control and adapted callus tissues kept on the same medium from the 14th to the 18th months, using the greenest homogeneous parts of the callus.

Chlorophyll extraction and assay

The callus (1 g) were homogenized in about 20 ml of acetone–water (90:10 v/v), the suspension centrifuged at 3500 × *g* during 5 min. The extract was analyzed immediately in a double-beam spectrophotometer (CARY 219). A spectrophotometric method of controlled pheophytinization was used for the determination of both chlorophylls and pheophytins [18].

Extraction of total fatty acids and lipid classes

For total fatty acids 200 mg of callus were directly homogenized in 8 ml methanol then maintained at 100 °C for 20 min.

For lipid classes, 1 g of callus was homogenized in 15 ml NaCl (2%), then maintained at 100 °C for 20 min. Lipids were subsequently extracted with about 110 ml of a mixture of chloroform, methanol and water in the ratio (8:4:3 v/v/v). After a strong manual mixing, the emulsion was centrifuged at 1000 × *g* for 10 min. The recuperated chloroformic phase was evaporated at 35 °C, then stored under nitrogen at −20 °C till the separation of the different lipid classes and the analysis.

A spot of chloroformic solution of total lipids was deposited on silica gel TLC plate (Kieselgel 60, Merck). The lipids were separated by two-dimensional chromatography using, for the first dimension, a mixture of chloroform, methanol, water (65:25:4 v/v/v), and for the second dimension a mixture chloroform, acetone, methanol, acetic acid, water (50:20:10:9:4 v/v/v/v/v). The spots corresponding to the different lipid classes were characterized under ultra-violet light, after a spraying with rhodamine 6G (0.02% w/v). They were scraped off the plate (stored at −20 °C) and resuspended in 8 ml sulfuric acid/methanol (2.5% w/v).

Esterification and methylation of fatty acids

For all fatty acid analyses, 20 μg of 19:0 (nonadecanoic acid, Sigma) was added as internal standard, primarily to the esterification. Sulfuric acid was added in the proportion of 2.5% w/v either to the methanolic extract of total fatty acids, or directly as sulfuric acid/methanol in the tubes containing silica gel impregnated with a defined lipid class. The tubes were maintained for one and a half hour at 70 °C. After the addition of 2 ml petroleum ether and 1 ml of water, the methyl esters were collected in the upper phase. The fatty acid methyl esters (FAME) from total fatty acids or from each lipid class, were analyzed with a gas chromatograph (Delsi 200) using He-U as carrier gas in a capillary column (Carbowax 20 M, 0.35 mm × 25). The separation was realized at 180 °C for 20 min then 210 °C for 1 h 30; the detector was of flame ionization type.

Quantification and identification of fatty acids

The quantitative analysis was based on the known quantity (20 μg) of 19:0. The FAME were identified by comparison of the retention times

with those of known standards. The identification was also based on a precise determination of fatty acids specific of *Euglena,* already reported [19]. The peak areas were integrated (Enica 21 integrator).

Unsaturation indexes

The insaturation index (U.I.) of a given lipid class was calculated by the average number of double bonds per fatty acid chain (whatever its number of C atoms be), according to the following formula [20]:

$$\text{U.I.} = \frac{\sum_{n=0}^{n=6} (\text{sum of fatty acids comprising } n \text{ double bonds}) \times n}{100}$$

Results and Discussion

Growth and chlorophyll contents

After two months of growth, the fresh weights and chlorophyll contents of control callus were studied as a function of the concentration of sucrose. The assayed concentrations varied from 0 to 30 $g \cdot l^{-1}$, concentrations currently used for heterotrophic *in vitro* cultures [21]. Growth and chlorophyll content increased linearly with the sucrose concentration, for 30 $g \cdot l^{-1}$ the growth was twice that for 10 $g \cdot l^{-1}$ and tissue concentration in chlorophyll was 5-fold greater (results not shown). The concentration of 30 $g \cdot l^{-1}$ of sucrose was therefore retained for the subsequent study.

The mean fresh weights of six callus, adapted or not, increased linearly for four months (the control growing from 50 mg to 670 mg, and the adapted material from 50 mg to 580 mg). Between zero and one month, the growth of callus was accompanied by a dilution of the chlorophyll contents. The concentration of 18.5 nmol per 100 mg fresh weight became 3.8 in the control, and 10.7 in the adapted material. Then, after two months of growth the tissue concentration in chlorophyll was 2- to 3-fold greater in adapted callus than in control. Therefore, the growth of control tissues was probably more heterotrophic than that of adapted callus. For the two first months of growth, the Chl *a*/Chl *b* ratio was very low in adapted callus (about 2 instead of 3.5 in control), indicating that the adapted callus became more enriched in chlorophyll *b*, the PS II antennae being perhaps reinforced by diuron effect. A striking greening then occurred from month two to month four, especially in the adapted material in which the chlorophyll content reached the value of 26.5 nmol per 100 mg fresh weight against 18 in control. After the third month, the Chl *a*/Chl *b* ratio of adapted tissues reached the same value than this of control and then continued to increase till the end of the fourth month. It had been previously demonstrated that the decrease of the Chl *a*/Chl *b* ratio was accompanied by a simultaneous increase of the ratio of xanthophyll/β-carotene in different plants resistant to triazine *Chenopodium album* L., *Amaranthus retroflexus* L., *Solanum nigrum* L., *Brassica campestris* L., and *Brassica napus* L. [22].

Changes in the content of total fatty acids after a first two-month contact with diuron

The concern of this study was to follow the lipid changes accompanying the adaptation to diuron. Therefore, the analysis of fatty acids was performed in three different samples: A, an initial control callus; B, a part of it cultivated in control conditions for two months; and C, another part of it cultivated also for two months, but in the presence of diuron for the first time. The results (Table I) indicated that a first exposure to diuron induced a forty percent reduction in the amount of total fatty acid. This drastic decrease affected each type of fatty acids: the 18:3 was nevertheless the most reduced (the ratio 18:2/18:3 equal to 3.3 in

Table I. Contents of fatty acids (in μg/g fresh weight) in an initial callus A, and after a two-month period of growth in the absence, callus B, or in the presence of 6 μM of diuron for the first time, callus C.

	UI	16:0	16:1	18:0	18:1	18:2	18:3	Sum
A	1.69	247.50	7.50	8.76	28.40	619.18	206.85	1118.19
B	1.62	268.23	6.44	8.01	48.94	645.82	192.95	1170.39
C	1.63	156.68	3.95	4.86	22.70	388.05	100.79	677.03

the control reached 3.8 under the effect of diuron). Such changes were not accompanied by any variation in the unsaturation index.

Total fatty acid distribution in control and long-term adapted callus

Control and long-term adapted callus, having been reinoculated every two months for 20 months, were studied without any reinoculation each month during four months. The fatty acid distributions, expressed as percent of total fatty acids stayed remarkably constant in control as well as in adapted callus. The comparison between the two materials indicated that the diuron treatment affected mainly the distributions in 18:2 and 18:3: a 18 percent decrease in 18:2 was simultaneous with a 47 percent increase in 18:3. The other fatty acids: 16:0, 16:1, 18:0 and 18:1 remained relatively unchanged.

Fatty acid distributions in the different lipid classes of control and adapted callus

After four months of growth of the two types of material, the callus tissues became homogeneous. It was the stage at which the chlorophyll contents per g of fresh weight were maximal and the closest to each other. The fatty acid quantities (expressed as μg per nmol chlorophyll, results not shown) still indicated differences between adapted and control callus. The adapted material displayed increases of the 16:0 by 2-fold, of the 18:2 by 1.4-fold and of the 18:3 by 2.8-fold. The ratio 18:2/18:3 then decreased from 3.2 in control to 1.6 in the adapted callus. In spite of these changes, the membrane fluidity [23] remained identical, since the unsaturation indices remain identical at about 1.65. These results indicated that the diuron did not modify the desaturation of linoleic in linolenic acid in adapted callus. Comparable effect had already been described in *Chenopodium album* resistant to atrazine [22], which acts, as diuron, at the level of the herbicide-binding Q_B protein [24]. A reduction of heterotrophic characteristics of growth had been correlated with an increase of 18:3 and a decrease of 18:2 in isolated cells of *Chenopodium rubrum* [25]. Accordingly, the observed decrease of the ratio 18:2/18:3, simultaneous with the increase of chlorophylls level in adapted callus appeared as clues of autotrophic growth.

At this stage of growth, after four months, when the differences were the weakest, we performed the detailed analysis of both the distributions of the lipid classes and of the fatty acids in these classes. Fig. 1 indicates, for either control or adapted material: (i) the repartitions of the different lipid classes, represented by the horizontal bars; and (ii) for each lipid class, the fatty acid quantities (expressed in μg per g fresh weight), represented by histograms.

Relative proportions of all lipid classes varied, except PE and PA. The diuron adapted material displayed decreased proportions of PG and MGDG (2.6- and 2-fold, respectively), and increased proportions of PC and DGDG (1.4- and 1.3-fold, respectively). Quantitatively, the total fatty acids (expressed in μg per g fresh weight) of all lipid classes, except PG and MGDG, were reinforced in adapted callus. For PE, PA, PC and DGDG, the increases were respectively: 2.25-, 2.8-, 3.3-, and 3-fold.

The fatty acid distributions, in each of the lipid classes, varied quantitatively as well as qualitatively (Fig. 1). Per comparison with control, in the PG of adapted callus the 16:0 and 18:1 increased and the 18:2 decreased, while the 18:3 remained unchanged. In PA and PE, classes in which the fatty acid distributions remained qualitatively almost identical in the two types callus, the fatty acids contents were doubled in adapted callus. In PC, the major phospholipid class, the treatment induced striking increases in the quantities of 16:0 and 18:2 (by 3-fold) and of 18:3 (by 6-fold). Important qualitative changes were noticeable between the galactolipids of control and adapted material: (i) in the MGDG of adapted material the ratio 18:3/18:2 became superior to one; (ii) in DGDG, on the contrary, this ratio became very low. Such a change in the balance of unsaturated species was still accompanied by an important increase of the fatty acid quantities. As a matter of fact, the levels of 16:0, 18:1, 18:2 and 18:3 were respectively multiplied by factors 5, 10, 2 and 3.5.

The percentages of 18:3 in galactolipids were nevertheless much weaker in callus (35 to 50%) than in leaves (75 to 95%), suggesting that callus could present characteristics of reserve tissues [26]. If such an hypothesis held true callus might be rich in neutral lipids. As a matter of fact, preliminary assays indicated that neutral lipids were as abun-

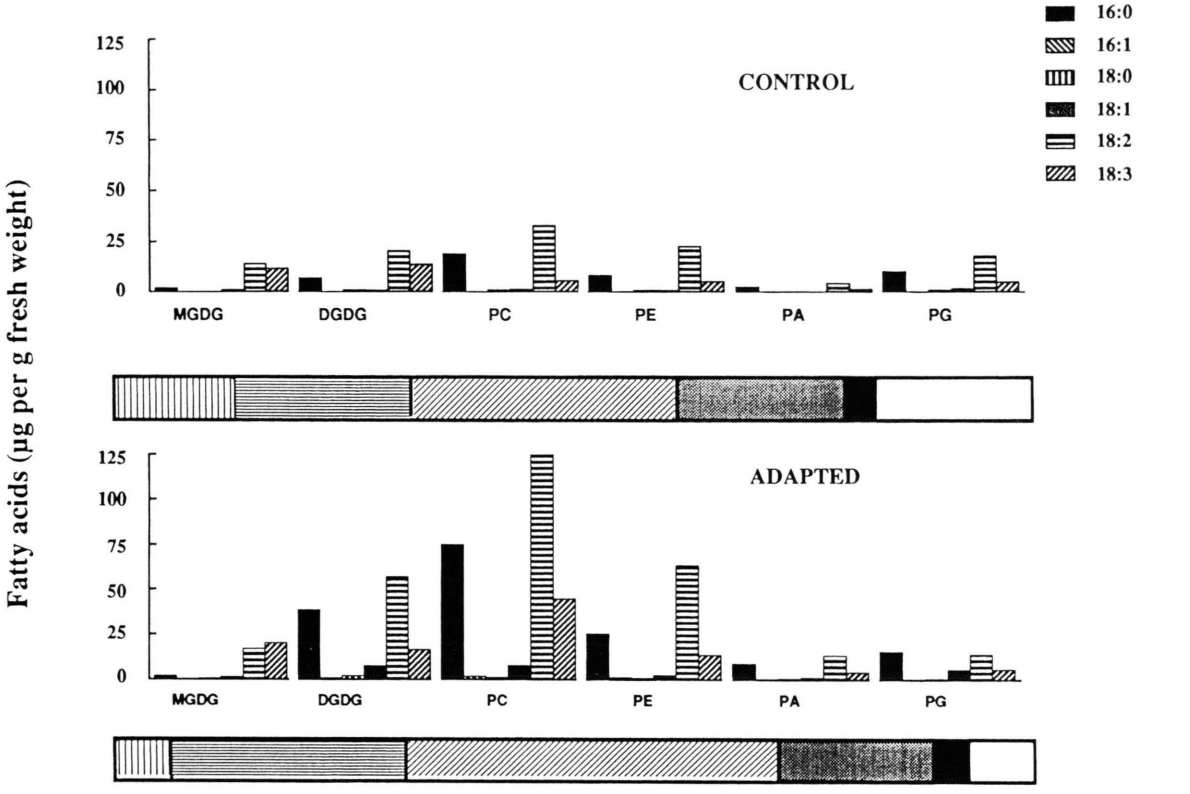

Fig. 1. Variations, in control and adapted callus grown for four months, in the fatty acids of the different lipid classes. Horizontal bars represent the distributions in percentages of the classes: MGDG, ▥; DGDG, ▤; PC, ▨; PE, ▩; PA, ■ and PG, □. Histograms represent the fatty acid contents (16:0, 16:1, 18:0, 18:1, 18:2 and 18:3) in each class, expressed as µg per g fresh weight.

dant as polar lipids in control material, while in adapted callus the neutral lipids represented only one eighth of polar lipids. In adapted callus, these neutral lipids were then twofold reduced when the polar lipids were increased by a factor four: data indicating a remarkable richness in membranal lipids and possibly in photosynthetic activity because of the increase in 18:3 in galactolipids (see Fig. 1).

Conclusion

In callus of *Bromus erectus* the chlorophyll content represented from 5 (in the control) to 10 percent (in the adapted material) of that measurable in the leaf of open-door grown plants (about 3000 nmol/g fresh weight: A. Regnault and F. Piton, unpublished data). The compositions in polar lipids of both control and adapted callus indicated very high proportions of phospholipids (68 percent); values very different from those of photosynthetic tissues in which the phospholipids ratio never exceed 35 percent [26]. The other polar lipids of callus consisted in 13 percent of MGDG and 19 percent of DGDG in control, against 6 and 25 percent of MGDG and DGDG, respectively, in adapted callus. In 18:3 angiosperms, the ratio in percentages of 18:2/18:3 in MGDG and DGDG, varied from 2–6/96–75 to extreme values of 8/50. In the studied callus of *Bromus erectus*, these ratios were very different: because of the drastic increase in 18:2, they became equal to 50/41 in MGDG and 49/32 in DGDG in control tissues, and changed to 41/49 in MGDG and 34/37 in DGDG in the adapted tissues in which 18:3 accumulation was favorized.

The adapted callus, presenting a factor of tolerance to diuron of two [12] and exhibiting two other recognized characteristics of herbicide resistance: enrichment in chlorophyll and in linolenic acid, could constitute an interesting photosynthetic *in vitro* material.

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