Plant Physiol. (1988) 88, 1297–1302
0032-0889/88/88/1297/06/$01.00/0

Characteristics of Five New Photoautotrophic Suspension Cultures Including Two *Amaranthus* Species and a Cotton Strain Growing on Ambient CO_2 Levels[1]

Received for publication April 15, 1988 and in revised form July 25, 1988

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ABSTRACT

Suspension cultures of cotton (*Gossypium hirsutum*), *Amaranthus cruentus*, *A. powellii*, *Datura innoxia*, and a *Nicotiana tabacum-N. glutinosa* fusion hybrid were adapted to grow photoautotrophically under continuous light. The cotton strain grew with an atmosphere of ambient CO_2 (about 0.06 to 0.07% in the culture room) while the other strains required elevated CO_2 levels (5%). Photoautotrophy was indicated by the requirement for CO_2 and for light for growth. The strains grew with doubling times near 14 days and had from 50 to 600 micrograms of chlorophyll per gram of fresh weight. The cells grew in small to moderate sized clumps with cell sizes from 40 to 70 micrometers (diameter). Like most photoautotrophic cultures described so far the ribulose 1,5-bisphosphate carboxylase (RuBPcase) activity levels were well below those of mature leaves. The phosphoenolpyruvate carboxylase levels were not elevated in the C_4 *Amaranthus* species. The cells showed high dark respiration rates and had lower net CO_2 fixation under high O_2 conditions. Dark CO_2 fixation rates ranged from near 10 to 30% of that in light. Fluorescence emission spectra measurements show that the cell antenna pigments systems of the four strains examined are similar to that of chloroplasts of green plants. The cotton strain which was capable of growth under ambient CO_2 conditions showed the unique properties of a high RuBPcase activation level in ambient CO_2 and a stable ability to show net CO_2 fixation in 21% O_2 conditions.

The first higher plant tissue culture described as being photoautotrophic, *i.e.* growing with CO_2 and light as the sole carbon and energy source, respectively, was that of Bergmann (3) who grew *Nicotiana tabacum* suspension cultures. Since that time only about ten different species have been grown photoautotrophically in liquid medium (reviewed by Horn and Widholm [11], Newmann and Bender [20]). That agar or agarose not be present in the medium for strict photoautotrophy was demonstrated by McHale (16) who showed that these substances either could be used themselves or contained impurities to support callus growth in CO_2-free air with no sugar added.

In general, the photoautotrophic cultures described thus far have much higher dark respiration rates than mature levels and lower RuBPcase[2] activity which often leads to RuBPcase to PEPcase activity ratios near one. These differing characteristics are not really surprising since photoautotrophic cultured cells are growing and dividing unlike a mature leaf. Thus, the cultured cells should be compared to developing leaves which do have high respiration rates and a lower ratio of RuBPcase to PEPcase activity (1, 14). Probably because of these characteristics all photoautotrophic cultures described thus far require elevated CO_2 levels, usually 1 to 5%, for growth and have CO_2 compensation concentrations higher than that of mature leaves (reviewed in 11, 20).

To date, no species with the C_4 photosynthesis pathway has been grown photoautotrophically. Photoheterotrophic, green cultures of the C_4 species, *Gisekia pharnaceoides* (25), *Portulaca oleracea* (12, 13) and *Froelichia gracilis* (15) have been initiated and used in photosynthesis studies, but these cultures were grown with 2 to 2.5% sucrose in the culture medium.

Photoautotrophic cultures can be used for many purposes including the selection of mutants resistant to photosynthetic herbicides, for screening for herbicidal activity, and for herbicide mechanism of action studies. The photoautotrophic cultures may be valuable for producing desired compounds if chloroplasts are involved in the biosynthesis. Studies of chloroplast development and breakdown can be readily studied during the reversible transition to and from photoautotrophy as controlled by the culture medium and conditions. The large numbers of cells and the readily controllable conditions may permit the selection of mutants with alterations in different photosynthetic components. Suspension cultures always provide materials well adapted to labeling studies and rapid medium manipulation and easy extraction.

In this report we describe the initiation and characterization of five new photoautotrophic cultures including two from C_4 species and one which grows on ambient CO_2 levels.

MATERIALS AND METHODS

Origin of Suspension Cultures. The photoautotrophic cotton (*Gossypium hirsutum* cv Stoneville 825) culture designated COT-P was initiated as described previously (4). The photoautotrophic cultures of *Datura innoxia* (denoted DAT-P) and a *Nicotiana tabacum-Nicotiana glutinosa* fusion hybrid (denoted NTG-P) were derived from the heterotrophic line of *D. innoxia* initiated by Ranch and Giles (22) and the fusion hybrid T3g1C (9),

[1] Supported by funds from the McKnight Interdisciplinary Photosynthesis Research Program, the SOHIO:UI Center of Excellence in Crop Molecular Genetics and Genetic Engineering, and the Illinois Agricultural Experiment Station.

[2] Abbreviations: RuBPcase, ribulose 1,5-bisphosphate carboxylase; MS, Murashige and Skoog (18) basal medium; NAA, naphthaleneacetic acid; PEP, phosphoenolpyruvate; PEPcase, phosphoenolpyruvate carboxylase; RuBP, ribulose 1,5-bisphosphate.

respectively. Cultures of the following six *Amaranthus* genotypes were also initiated: *A. cruentus* (ACR-P), *A. hybridus* both wild-type and triazine resistant, *A. powellii* both wild-type (APO-P) and triazine resistant and *A. retroflexus*.

Amaranthus seeds were surface sterilized for 30 min in 20% Clorox, rinsed twice in sterile water, and then placed in a MS (18) liquid medium lacking plant growth regulators on a 80 rpm reciprocating shaker with 5 μE m^{-2} s^{-1} continuous illumination at 27 to 28°C. After 3 d the flasks were set under 60 μE m^2 s^{-1} illumination and when the cotyledons were fully expanded and some primary leaves had emerged the roots were removed and the rest of each seedling was chopped and placed on 0.8% agar solidified MS medium with 1.81 μM 2,4-D. After 28 d incubation in the dark the callus which formed was placed into the same medium without agar and incubated on the reciprocating shaker with transfers every 10 d.

Development of Photoautotrophic Cultures. The *Amaranthus* and *Nicotiana* heterotrophic cultures in early log phase (50 mL volumes) were placed in 125 mL flasks on a gyratory shaker (130 rpm) under 300 μE m^{-2} s^{-1} of fluorescent light at 30°C. With reduced transfer frequency the cultures became yellow or green within 1 to 4 transfer cycles in 2 to 12 weeks. These cells were then transferred into GR^{S3} medium (MS with 3% soluble potato starch [Sigma] instead of sucrose, 7.0 μM kinetin and 0.5 μM picloram). When the Chl levels reached 30 to 100 μg Chl mg^{-1} fresh weight, the cells were transferred to GR^{S1} medium which is GR^{S3} with 1% rather than 3% starch. These continuous mixotrophic cultures were transferred monthly. A mixotrophic *D. innoxia* culture was initiated by placing green callus grown on GR^{S3} with 0.8% agar into GR^{S1} liquid medium.

Photoautotrophic suspension cultures were initiated from these mixotrophic cultures by inoculating them into KTP^0 medium which is KT^0 medium (10) modified as follows: picloram at 0.3 μM and the RT vitamin mixture (10) is filter sterilized. The flasks were partially wrapped with Al foil to reduce the effective irradiance to 15% and a 5% CO_2 in air gas mixture was blown into each flask (10). The shading was gradually removed during the initial 4 to 10 week culture period in carbohydrate free medium. The cells were transferred to fresh medium when dark green growing tissue was visible.

The COT-PA line was initiated by culturing the COT-P cells in flasks without the usual 5% CO_2 atmosphere. When the cells were almost all dead the 5% CO_2 atmosphere was reinstated to rescue the live cells. This cycle was repeated many times until cells capable of surviving in ambient CO_2 conditions were selected.

All of the photoautotrophic cultures studied here had been grown under photoautotrophic conditions for about 18 months before analysis.

For the culture cycle measurements 1 g fresh weight cells were transferred into 50 mL of KTP^0 liquid medium in 125 Erlenmeyer flasks and incubated under continuous light for 4 weeks (COT-P, APO-P, ACR-P) or 6 weeks (COT-PA, DAT-P, NTG-P) before reinoculation. All were grown with a 5% CO_2 in air atmosphere except for COT-PA which had no gas blown through the flask. The CO_2 level in the culture room was 600 to 700 μL/L.

Cell fresh weights were determined after collecting the cells on Miracloth filters under vacuum.

Chl Measurement. Chl was extracted in 80% acetone (v/v) with a glass tissue homogenizer and the concentration determined spectrophotometrically using the formula of Arnon (2).

IR Gas Exchange Analysis. Steady state CO_2 exchange rates in light (400 μE m^{-2} s^{-1}) were measured with an UNOR-2 (Bendix Corp.) IR gas analyzer in a open system at 26°C. Ten mL cell suspensions, containing about 300 μg Chl, were placed in a 50 mL side arm tube with a porous tipped glass aerator submerged in the suspension. Humidified gas mixtures (350 μL CO_2 L^{-1} and 2 or 21% O_2 in air) were bubbled through the stirred suspensions at the rate of 100 mL min^{-1}. The tube was covered with Al foil for the dark measurements.

$^{14}CO_2$ Fixation. One mL of cell suspension containing about 30 μg Chl were incubated with 5 mM $NaH^{14}CO_3$ (1 μCi/μmol) and 50 mM Mes (pH 4.5) in the reaction mixture to simulate the pH of the culture medium. The assays were carried out in light (400 μE m^{-2} s^{-1}) or darkness (vials wrapped with foil) as described by Rogers *et al.* (23) in triplicate.

PEPcase and RuBPcase Assays. The enzyme assays were carried out generally as described by Rogers *et al.* (23). A thick slurry of cells were pipetted into liquid N_2 and ground in a prefrozen mortar and pestle. The homogenate was mixed with the extraction buffer containing 50 mM Hepes (pH 7.3), 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM D-isoascorbate, 5 mM DTT, and 2% (w/v) PVP. Aliquots were taken for Chl determination. The homogenates were centrifuged for 15 s in a microfuge at 5000*g* at 4°C. The supernatants were assayed immediately to obtain the 'initial' RuBPcase activity by injection of 50 μL into airtight, serum capped, scintillation vials containing in 0.45 mL volume: 0.1 M tricine (pH 8.1), 10 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (3 μCi/μmol), and 0.5 mM RuBP. PEPcase assays were carried out in an identical way except that 5 mM PEP (4 μCi/μmol) was substituted for RuBP and 2 mM DTT, 0.2 mM NADH, and 5 units of Sigma malate dehydrogenase were added.

'Total' RuBPcase was measured after 100 μL of supernatant was activated by incubation for 5 min at 25°C with 100 μL of 50 mM tricine (pH 8.1), 50 mM $NaHCO_3$, and 10 mM $MgCl_2$.

The enzyme assays were carried out at 26°C for 1 min when they were terminated by injection of 0.2 mL 6 N acetic acid. The samples were dried at 60°C, 0.5 mL of 0.5 N HCl was added to dissolve the residue, 5 mL scintillation fluid was added, and the acid stable ^{14}C determined by scintillation spectrometry.

Fluorescence Emission Spectra Measurement. A laboratory built spectrofluorometer with exciting light from a Kodak 4200 projector with the light filtered by two Corning blue filters (CS 4-71 and CS 5-56) was used. Emission was detected with a photomultiplier tube protected from exciting light with a red filter (CS 2-61) and a monochromator. Data was stored and analyzed by a Biomation 905 waveform recorder and a LSI-11 computer (see Blubaugh [5] for details). The spectra presented here have not been corrected for the spectral sensitivity of the S-20 photomultiplier EMI 9558, and of the Bausch and Lomb monochromator (B&L 33-86-45, blazed at 750 nm). The slit widths were 1.0 nm. Since the exciting light intensity was saturating, the spectra are that for the so-called P (or F_m) level, where the quinone electron acceptor Q_A of PSII is in the reduced state (8).

RESULTS AND DISCUSSION

Photoautotrophic Culture Initiation. Photoautotrophic suspension cultures were successfully initiated from all of the mixotrophic cultures tested except the two atrazine resistant *Amaranthus* mutants and the two wild-type *Amaranthus* species, *A. retroflexus* and *A. hybridus*. One of the wild types corresponding to the atrazine resistant lines, *A. powellii*, did successfully form a photoautotrophic culture, while the other, *A. hybridus*, did not.

The mixotrophic cultures from which the photoautotrophic cultures were initiated were grown in a medium containing soluble potato starch (Sigma catalog No. 2004). The type of starch used was important since soluble potato starch prepared by the improved Lintner method (Sigma catalog No. S2630) was very toxic. Starch was used instead of sucrose because we expected that a less available carbon source would promote greening and studies had demonstrated slower growth and more rapid greening in media where starch replaced sucrose.

The mixotrophic and photoautotrophic cultures showed light sensitivity since sudden increases in light intensity killed the cells especially during the transition from mixotrophic to photoautotrophic conditions. The flasks with photoautotrophic medium had rubber stoppers which caused less shading than the inverted paper cups of the mixotrophic flasks. This decreased shading led to an increase in incident light of 6 to 7 times as determined by light measurements. Self shading, where the cells in a dense culture reduce the effective irradiance per cell by shading each other, might also be important as indicated by experiments where small inoculation densities resulted in no growth, unless additional shading was imposed. In addition, we found that the COT-PA culture would not grow if the usual inverted paper cup which is normally placed over the top of the culture flask was replaced by a small transparent cap. Despite repeated attempts to adapt the COT-PA cells to unshaded conditions, they would not survive unless a carbon source such as soluble starch was also present.

Photoautotrophic Growth. The five newly initiated suspension cultures studied here (COT-PA, APO-P, ACR-P, DAT-P, NTG-P) had been growing for at least 18 months with 6 week subculture intervals. The COT-PA cells were grown under ambient CO_2 conditions in the culture room (about 600–700 $\mu L\ CO_2\ L^{-1}$) but the other strains required higher CO_2 levels (5% used normally). The cells were grown in medium lacking sucrose with minimal levels of organic compounds (0.95 mg/L of a vitamin mixture, 1 mg/L NAA, 0.2 mg/L kinetin, 1.2 g/L Hepes, 0.73 mg/L picloram, and 29.2 mg/L EDTA). Even if all of these organic compounds are metabolized by the cells this should not be sufficient to maintain continuous growth. The growth of four of the new strains and that of COT-P (4) which was used as a control, showed that decreasing the usual CO_2 levels decreased growth greatly and that light was absolutely required for growth (Table I). This evidence indicates that these suspensions are indeed photoautotrophic *i.e.* utilize light energy to fix CO_2 for growth.

The growth rates calculated from a series of growth studies give doubling times near 14 d. The COT-P and APO-P cells did have doubling times near 8 d in experiments shown in Table I where a lower than normal quantity of cells was used as inoculum. The cells generally grew in variable sized clumps, usually with fewer than 30 loosely packed cells, with the mean cell size being in the range from 40 to 70 μm in diameter (Fig. 1). The cells have numerous chloroplasts and no clearly different features which would distinguish each cell type at least at the light microscopic level. Figure 1 shows COT-PA, COT-P, APO-P, and DAT-P cells as representative examples.

Photosynthetic Characteristics. The five newly developed cultures were studied during two successive culture cycles of either 4 or 6 weeks while growth and the parameters listed below were measured at 2 week intervals. Only representative data from one culture cycle is presented to save space. Data was also collected for the previously characterized COT-P strain (4) as a control for comparative purposes.

Table I. *Requirement for CO_2 and Light for Growth of Photoautotrophic Cultures during a 28 d Incubation*

Cell Strain	Initial Fresh Weight	Net FW increase		
		Normal[a]	Low CO_2[b]	Dark[c]
	g		*g*	
COT-PA	0.16	2.2	0.80	0
COT-P	0.22	2.3	0.88	0
APO-P	0.26	4.4	0	0
DAT-P	0.63	3.0	0.77	0
NTG-P	1.1	3.8	0.50	0

[a] 5% CO_2 blown through flasks except for COT-PA where flask was grown under ambient CO_2 condition with inverted waxed paper cup over the flask top. [b] All grown under ambient CO_2 condition with inverted waxed paper cup sealed with Parafilm over the flask top. [c] All grown under the 'normal' conditions in flasks wrapped in aluminum foil to exclude light.

The five new strains contained the following levels of Chl listed as the ranges found in different growth experiments and at different times during the culture cycle in $\mu g\ Chl\ g^{-1}$ fresh weight; COT-PA, 218 to 607; APO-P, 114 to 294; ACR-P, 148 to 231; DAT-P, 101 to 226; and NTG-P, 47 to 237. The level measured in COT-P used as a control, varied from 263 to 639 $\mu g\ Chl\ g^{-1}$ fresh weight which is similar to the values reported previously for this strain (4). These Chl levels are similar to most of the values reported for other photoautotrophic cell lines (reviewed in 11 and 20). The exceptions would be two different soybean strains which now contain near 2000 $\mu g\ Chl\ g^{-1}$ fresh weight (23, 24). These soybean strains gradually increased in Chl content so the strains described here may do likewise.

When CO_2 exchange was measured with an IR gas analyzer under culture medium conditions, all strains showed high dark respiration at time zero and after 4 weeks of growth (Table II). The values ranged from 8.8 to 98.0 $\mu mol\ CO_2\ mg^{-1}\ Chl\ h^{-1}$. These values are similar to those generally found with photoautotrophic cultures including COT-P (4), but are higher than that usually found in mature leaves. For example, Kisaki *et al.* (14) showed that the dark respiration rates of mature leaves of greenhouse grown tobacco (*Nicotiana tabacum*) were 8 $\mu mol\ CO_2\ mg^{-1}\ Chl\ h^{-1}$ or less. Forrester *et al.* (7) reported dark respiration rates for mature soybean leaves from growth chamber plants of no more than 14 $\mu mol\ CO_2\ g^{-1}$ fresh weight h^{-1} which would be near 5 $\mu mol\ CO_2\ mg^{-1}\ Chl\ h^{-1}$ assuming about 3 mg Chl g^{-1} fresh weight.

Net CO_2 fixation of from 2.4 to 28.5 $\mu mol\ CO_2\ mg^{-1}\ Chl\ h^{-1}$ was measured in all strains in light in a 350 $\mu L\ CO_2$ and 2% O_2 atmosphere. The CO_2 fixation rate in light decreased greatly in an atmosphere of 21% O_2 and in many cases was negative. Only the COT-PA cells showed net fixation in 21% O_2 at both times shown and this also occurred with these cells at other measurement times as well. Previously the COT-P cells did not show net CO_2 fixation at either 2% or 21% oxygen conditions with the low CO_2 concentration used (4) while soybean cells did show net fixation at most times even with 21% O_2 (23).

The decrease in net CO_2 fixation found with increasing O_2 levels is usually considered to be due to the oxygenase activity of the C_3 fixation enzyme, RuBPcase-oxygenase. The increased oxygen would compete with CO_2 and directly cause less CO_2 fixation and, in addition, stimulate photorespiration through production of phosphoglycolate (21). Photorespiration, in turn, releases CO_2 which would additionally decrease the net CO_2 fixed. Studies with photoautotrophic tobacco cells have shown that increased O_2 concentrations decrease net photosynthesis and growth and that increased CO_2 levels can revvrse the inhibition of photosynthesis but not of growth (17). These results are consistent with our data and indicate that photorespiration is occurring in these cells.

When fixation of $^{14}CO_2$ was measured under both dark and light conditions under the culture medium pH of 4.5 (Table III), the dark fixation varied from 1.3 to 13.9 $\mu mol\ CO_2\ mg^{-1}\ Chl\ h^{-1}$ and light fixation was from 9.0 to 47.8 $\mu mol\ CO_2\ mg^{-1}\ Chl\ h^{-1}$. In general, the dark fixation was less than 24% that in light except for the NTG-P strain, where the dark values are from 28 to 33% of the light values. The COT-P values determined here are similar to those of Blair *et al.* (4) when a 5 min incubation time was used.

The RuBPcase activity was low in all strains with the initial activity (that measured before *in vitro* activation with CO_2 and MG^{2+}) being from about 6 to 64 $\mu mol\ CO_2\ mg^{-1}\ Chl\ h^{-1}$ (Table IV). Mature spinach leaves grown in a growth chamber have

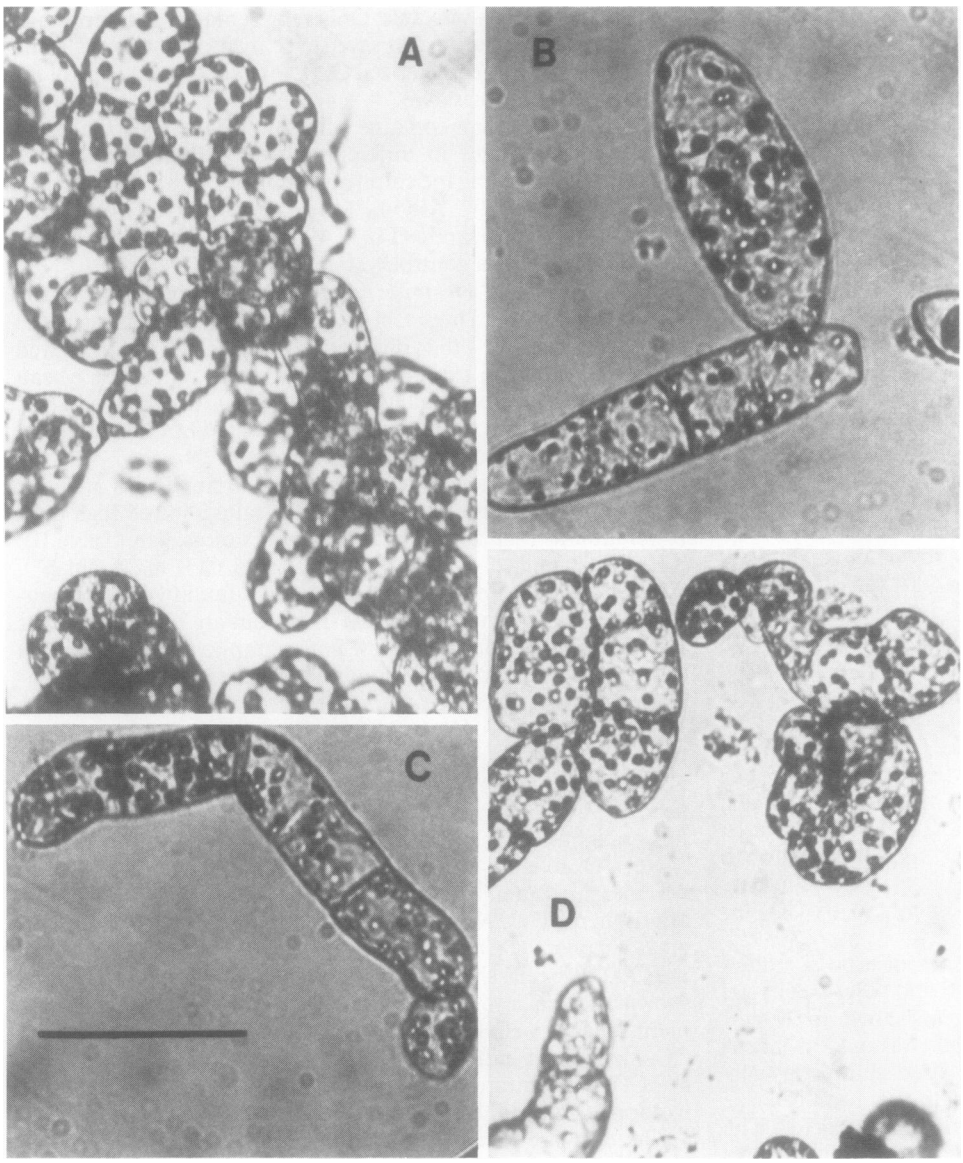

FIG. 1. Phase contrast microscope pictures of A, COT-PA cells 6 weeks after transfer; B, COT-P cells 4 weeks after transfer; C, DAT-P cells 4 weeks after transfer; D, APO-P cells 6 weeks after transfer. The size bar represents 100 μm.

total RuBPcase activity of about 500 μmol CO_2 mg^{-1} Chl h^{-1} with about 86% activation (*i.e.* the initial activity is about 86% of the total). The activation levels in the photoautotrophic cultures described here ranges from 16 to 64%, which is similar to other reports where low activation levels were noted in photoautotrophic cultures.

The PEPcase levels of the photoautotrophic strains ranged from low levels near 10 μmol CO_2 mg^{-1} Chl h^{-1} for COT-PA, COT-P, and APO-P to near 20 for ACR-P, to 40 for DAT-P and near 80 for NTG-P. These values can be compared to levels near 80 μmol mg^{-1} Chl h^{-1} for growth chamber grown spinach leaves and the COT-P cells measured previously (4). The reason for the low PEPcase activity measured in COT-P cells in the present studies is not known. It is possible that the cells might have changed since the previous assays were run (4). This conclusion is supported by recent data obtained with the COT-PA cells and a coupled enzyme assay which gave PEPcase activity levels near 20 μmol CO_2 mg^{-1} Chl h^{-1} (C Roeske, JM Widholm, unpublished data).

The fluorescence emission spectra of the photoautotrophic cells (SB-P, DAT-P, COT-P, and COT-PA) obtained at room temperature showed a peak at 685 nm (Fig. 2) which has been suggested to originate in the antenna Chl *a* molecules of PSII (19). At 77 K the fluorescence emission spectra showed three bands near 685 (F685), 695 (F695), and 740 (F740) nm. Both the F685 and F695 nm bands originate in the antenna Chl *a* molecules of PSII, F685 in the so-called CP-43 complex, and F695 in the so-called CP-47, the antenna that feeds energy to the reaction center II complex. F740, that is composed of at least two subbands, originates in the antenna Chl *a* of PSI. The 77 K spectra, shown here, are similar to those found in green algae (8) and chloroplasts of higher plants (6). Our results with the photoautotrophic cells suggest that their antenna systems are similar to those of other green plants. Minor differences in the ratio of F685–F695 of different cell lines may be due to slightly different experimental conditions and/or slightly different ratios of antenna systems within them.

CONCLUSIONS

The five new photoautotrophic suspension cultures described here have the usual characteristics of photoautotrophic cultures, high dark respiration, low RuBPcase activity, low RuBPcase activation levels, and low Chl levels which would all cause these cultures to have net photosynthesis rates which are relatively low in comparison to mature leaves. The reason for some of these

Table II. *Net CO_2 Exchange Rates of Photoautotrophic Suspension Cultures Measured by IR Gas Analysis at Time Zero and After 28 d of Growth*

Cell Strain	Time after Transfer	CO_2 Exchange		
		Dark 2% O_2	Light 2% O_2	Light 21% O_2
	d	*μmol CO_2 mg^{-1} Chl h^{-1}*		
COT-PA	0	−37.3	+16.6	+1.53
	28	−35.6	+20.4	+4.08
COT-P	0	−16.3	+9.8	0
	28	−48.5	+17.5	−6.62
APO-P	0	−8.8	+7.7	+4.41
	28	−39.9	+7.7	−6.13
ACR-P	0	−16.6	+2.43	0
	28	−33.6	+10.1	+4.02
DAT-P	0	−56.0	+28.5	ND[a]
	28	−78.8	+25.8	+10.1
NTG-P	0	−21.6	+10.0	+2.37
	28	−50.0	+7.8	−6.88

[a] Not determined.

Table III. *$^{14}CO_2$ Fixation by Photoautotrophic Suspension Cultures Measured at Time Zero and after 28 d of Growth*

Cell Strain	Time after Transfer	$^{14}CO_2$ Fixation		
		Dark 2% O_2	Light 2% O_2	$\frac{\text{Rate dark}}{\text{Rate light}} \times 100$
	d	*μmol mg^{-1} Chl h^{-1}*		%
COT-PA	0	3.87	16.1	24
	28	3.90	19.4	20
COT-P	0	4.1	27.9	15
	28	9.1	37.9	24
APO-P	0	1.76	9.0	20
	28	6.1	28.0	22
ACR-P	0	1.29	13.5	10
	28	3.88	38.1	10
DAT-P	0	1.69	27.9	6
	28	5.4	47.8	11
NTG-P	0	8.8	31.6	28
	28	13.9	41.7	33

Table IV. *PEPcase and RuBPcase Activities in Photoautotrophic Suspension Cultures at Time Zero and After 28 d of Growth*

Cell Strain	Time after Transfer	Enzyme Activity			RuBPcase activation $\left(\frac{\text{initial}}{\text{total}} \times 100\right)$
		PEPcase	RuBPcase (initial)	RuBPcase (total)	
	d	*μmol mg^{-1} Chl h^{-1}*			%
COT-PA	0	11.8	36.2	56.4	64
	28	14.3	29.4	51.9	57
COT-P	0	8.4	28.7	49.6	58
	28	9.7	14.3	65.0	22
APO-P	0	12.4	8.7	20.8	42
	28	13.1	33.1	78.9	42
ACR-P	0	20.1	16.7	41.1	41
	28	22.1	50.0	98.9	51
DAT-P	0	37.9	6.2	35.6	17
	28	41.3	25.2	94.1	27
NTG-P	0	78.3	6.4	40.2	16
	28	87.6	64.2	214	30

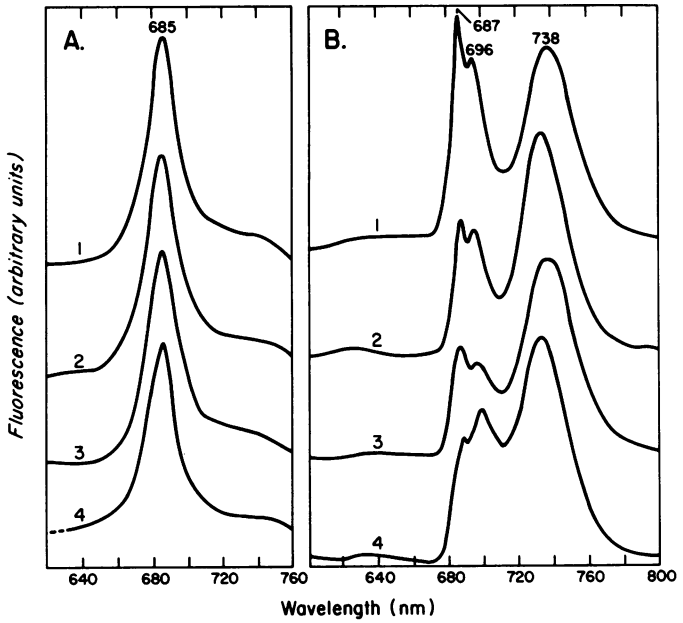

FIG. 2. Fluorescence emission spectra from SB-P (1), DAT-P (2), COT-P (3), and COT-PA (4) cells measured at room temperature (A) and at 77 K (B).

differences may be the continuous cell division in the cell cultures relative to that of the mature leaves (23).

The photoautotrophic cultures initiated from the C_4 species APO-P and ACR-P were not appreciably different from the other photoautotrophic strains from C_3 species. In particular, the PEPcase activities were not higher than the other species studied here, as they would be in the whole plants. No $^{14}CO_2$ fixation product analysis studies were done, however, to determine the CO_2 fixation efficiency of the RuBPcase *versus* the PEPcase activities *in vivo*. Previous studies usually show that the $^{14}CO_2$ is fixed into the products expected for the proportion of RuBPcase and PEPcase measured *in vitro*.

The unique COT-PA strain which grows on ambient CO_2 levels (about 600–700 μL/L in the culture room) does not have any clearly different characteristics as measured in these studies except for the reproducible ability to show net CO_2 fixation in 21% O_2 (Table II) and a relatively high stable RuBPcase activation level (about 60% as shown in Table IV).

While it is clear that the carbon fixation enzyme makeup of these cells is different from that of mature leaves it appears that the PSII antenna pigment composition is similar to that of leaves of green plants.

These five strains are continuing to grow with repeated subculturing for a period of at least 1 year after completion of these studies, so they appear to be capable of continuous growth under these conditions. During this period we have been able to remove the Hepes and all of the vitamins except thiamine from the culture medium and have found that the Chl levels of the COT-P, COT-PA, DAT-P, and NTG-P had remained constant while the APO-P and ACR-P levels had increased to 1700 and 875 μg Chl g^{-1} fresh weight, respectively (C Goldstein, JM Widholm, unpublished data).

Further studies are needed to determine if different culture conditions might affect the PEPcase levels of the C_4 species cultures and to determine why the COT-PA and not the other cultures can grow on ambient CO_2 levels.

Acknowledgments—We wish to thank Cindy Goldstein for some cell samples, and W. L. Ogren for discussion and for facilities for the enzyme assays.

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