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Photoautotrophic Growth of Soybean Cells in Suspension Culture

I. ESTABLISHMENT OF PHOTOAUTOTROPHIC CULTURES[1]

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ABSTRACT

Highly chlorophyllous photomixotrophic callus was visually selected from callus originating from soybean (*Glycine max* (L.) Merr. var. Corsoy) cotyledon. Suspension cultures initiated from this callus became photoautotrophic under continuous light with an atmosphere of 5% CO_2 (balance air). Dry weight increases of 1000 to 1400% in the 2-week subculture period have been observed. The cellular Chl content ranged from 4.4 to 5.9 micrograms per milligram dry weight which is about 75 to 90% of the Chl content in soybean leaves under equivalent illumination (300 micro-Einsteins per square meter per second).

No growth can be observed in the dark in sucrose-lacking medium or in the presence of 0.5 micromolar 3-(3,4-dichlorophenyl)-1,1-dimethylurea, a concentration which does not inhibit heterotrophic growth (on sucrose). Photoautotrophic growth has an absolute requirement for elevated CO_2 concentrations (>1%). During the 14-day subculture period, growth (fresh weight and dry weight) is logarithmic. Photosynthesis quickly increases after day 4, reaching a peak of 83 micromoles CO_2 incorporated per milligram Chl per hour while dark respiration decreases 90% from day 2 to day 6. The pH of the growth medium quickly drops from 7.0 to 4.5 before slowly increasing to 5.0 by day 14. At this pH range and light intensity (200–300 microEinsteins per square meter per second), no O_2 evolution could be detected although at high pH and light intensity O_2 evolution was recorded.

In recent years, several reports concerning photomixotrophic and photoautotrophic cell cultures of higher plants have increased the possibility of studying photosynthesis using cultured cells (2). The majority of the photoautotrophic cell lines, however, exhibit low growth rates when compared to photomixotrophically or heterotrophically grown cells (12, 13). The lone exception to this is a recent paper by Peel (8) which described asparagus cells growing photoautotrophically in turbidostat culture with cell-doubling times of 3 d. For ease of experimentation, photoautotrophic cells growing rapidly in batch culture would appear to be a useful system. In this paper we describe a novel system in which highly chlorophyllous soybean cells are grown photoautotrophically with high growth rates. These cells have been growing continuously without exogenously supplied sucrose for the last 12 months.

MATERIALS AND METHODS

Culture Method. Cotyledons of soybean (*Glycine max* L. Merr. var. Corsoy) were induced to form callus on a medium containing 1% sucrose, 1 mg/l naphthaleneacetic acid, 0.2 mg/l kinetin, a complex array of vitamins (Table I), and the basic Murashige and Skoog salts (6), designated KT medium. By visually selecting the greener parts of the callus a Chl-enriched SB-M[2] cell line was established. This cell line was used to obtain photoautotrophic growth by the following procedure. Suspension-cultured SB-M cells (0.4 g fresh weight) were placed in 250-ml flasks containing 80 ml of KT medium lacking sucrose (KT^0) and containing 5 mM Hepes buffer (pH 7.0). The flasks were plugged with rubber stoppers in which two 16- or 17-gauge needles 7.6 to 8.9 cm long had been placed. To each of these needles was glued a small Millipore filter unit (type HA) containing a 0.45-μ filter. The rubber stopper-needle-filter units were autoclaved separately from the medium. A polypropylene plastic tube was connected at one end to the filter with a hose clamp and the other end to a gas humidifier which in turn was connected to a tank containing pressurized 5% CO_2 (balance air). The flow rate of the gas was 11.25 cc/min·flask. Eight flasks were generally attached to each humidifier by branching the tubing. The flasks were agitated at 130 rpm on a New Brunswick gyratory shaker under a light intensity of 200 to 300 $\mu E\ m^{-2}\ s^{-1}$.

Table I. *Complex Vitamin Mixture in KT Medium*

Components were mixed into a stock solution 1000× more concentrated than those shown and frozen in 1-ml aliquots. Modified from (5).

Vitamin	Final Concentration
	mg/l
Nicotinamide	0.2
Pyridoxine HCl	0.2
D-Biotin	0.1
Choline Cl	0.1
Ca pantothenate	0.1
Thiamine HCl	0.1
Folic acid	0.05
p-Aminobenzoic acid	0.05
Riboflavin	0.05
Cyanocobalamin	0.00015

$^{14}CO_2$ Incorporation. The incorporation of CO_2 was followed by providing 0.21 μCi $NaH^{14}CO_3$ in 57.1 μmol $NaHCO_3$ and injecting this into a serum cap-sealed scintillation vial containing 1 ml of the cell suspension. Vials were either wrapped in aluminum foil (dark controls) or not wrapped before being placed on the gyratory shaker for 30 min under a light intensity of 230 $\mu E\ m^{-2}\ s^{-1}$. The serum caps were then removed and 50 μl concentrated HCl were added to remove all remaining CO_2 in the reaction volume. Ten ml of scintillation cocktail (xylene:Triton X-114, 3:1, with 6 g L^{-1} 2,5-diphenyloxazole) were then added and the samples were counted for radioactivity.

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[2] Abbreviations: SB-P, photoautotrophic soybean cells; SB-M, photomixotrophic soybean cells; SB-H, heterotrophic soybean cells.

Chl Determinations. Chl content was determined by grinding tissue collected on Miracloth filters in a mortar and pestle using a grinding medium of 80% acetone, 20% water. The equations of Arnon (1) were used.

DCMU Studies. In studies involving DCMU, the compound was first dissolved in 95% ethanol and then introduced into sterile flasks where the ethanol was allowed to evaporate. The sterile growth medium and cells were then introduced aseptically.

Respiration and pH Measurements. The cells were collected by vacuum filtration on Whatman No. 1 qualitative filter paper and the pH of the filter medium was determined. One hundred mg fresh weight of cells were then mixed with 3 ml of the same medium in an electrode chamber of a YSI model 52 biological oxygen monitor. A linear O_2 uptake was established after 5 min of stirring and this value was then used as the dark respiration rate.

RESULTS

After transfer from sucrose-containing KT medium into photoautotrophic conditions (5% CO_2, no sucrose, continuous light), SB-P cells grew slowly for the first two subcultures (161 and 116% fresh weight increase, respectively) and then the growth accelerated (Fig. 1). The mean Chl concentration increased from 200 to 280 μg/g fresh weight (2.54–3.56 μg/mg dry weight) in SB-M cells to 450 to 600 μg Chl/g fresh weight (4.4–5.9 μg/mg dry weight) in SB-P cells after four transfers. For the first several months, the growth rate fluctuated. Improvements in the apparatus, specifically, gluing the filter units into the needles and using hose clamps, apparently stimulated growth. Fresh and dry weight increases of 1000 to 1400% in 14 d are now common. Mean doubling times are thus calculated to be 3.58 to 4.05 d. The Chl content on a dry weight basis is nearly twice that of asparagus cells in the turbidostat culture (8), and is approximately 75 to 90% of the Chl level (on a dry weight basis) found in growth chamber-grown soybean leaves with equivalent light intensity.

Several control experiments provide evidence that these cells are growing photoautotrophically. First, no cell growth was observed in flasks with KT^0 medium when placed in the dark. This was true with or without an elevated CO_2 atmosphere. Also, no growth was observed with photoautotrophic cells when 0.5 μM DCMU was present (Table II). This concentration of DCMU inhibits SB-H cells by only 9% (Fig. 2). The SB-P cells bleached after 3 to 4 d and by 14 d had disintegrated. In comparison, SB-M cells in the presence of DCMU grew as much as the SB-H cells growing in the dark in medium without DCMU present. SB-H cells were inhibited by DCMU by only 9% (fresh weight), in close agreement with Figure 2. It is important to note here that the DCMU experiment of Table II uses KT medium with 1% (27.5 mM) sucrose for both SB-M and SB-H cells. This concentration of sucrose is growth-limiting but higher concentrations would induce bleaching and reduce the ability of the photomixotrophic cells to perform photosynthesis.

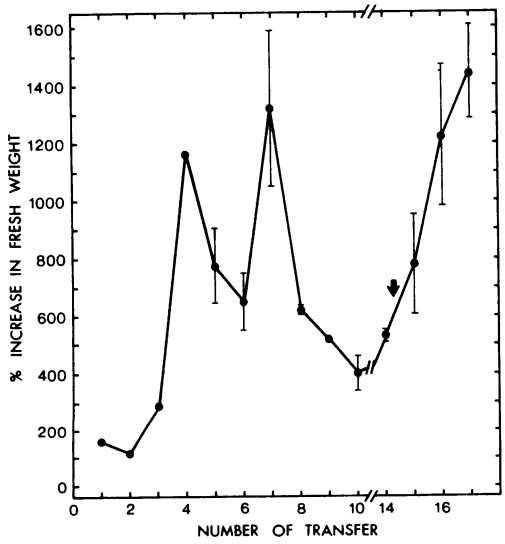


FIG. 1. History of growth of SB-P cells. Transfer 1 was on January 18, 1982. Each subculture period was 14 d with 0.4 g fresh weight of cells being inoculated into 80 ml of KT medium. The arrow indicates when the system was tightened by gluing filter units into the needles and utilizing hose clamps. Most points are the average of four flasks with SD shown.

Table II. *Effect of DCMU on Growth and Chlorophyll Level in SB-P, SB-M, and SB-H Cells*

DCMU (0.5 μM) was introduced as described in "Materials and Methods" and the cells were incubated for 14 d. Inocula: SB-P, 0.39 g fresh weight, 33.4 mg dry weight, 503.2 μg Chl·g^{-1} fresh weight; SB-M and SB-H, 0.39 g fresh weight, 26.5 mg dry weight, 196.8 μg Chl·g^{-1} fresh weight. SB-H flasks were wrapped in aluminum foil and the SB-M and SB-H cells were grown in KT medium containing 1% sucrose.

Cell Type	Treatment	Fresh Weight	Dry Weight	Chlorophyll
			% change	
SB-P	−DCMU	+927.4	+947.3	−24.7
	+DCMU	−100.0[a]	−100.0[a]	−100.0[a]
SB-M	−DCMU	+842.5	+1049.3	+50.4
	+DCMU	+600.0	+680.6	−17.3
SB-H	−DCMU	+495.7	+677.2	−100.0[b]
	+DCMU	+440.2	+558.1	−100.0[b]

[a] Cells deteriorated to such an extent that fresh weight, dry weight and Chl could not be accurately determined.

[b] Cells completely bleached in the dark.

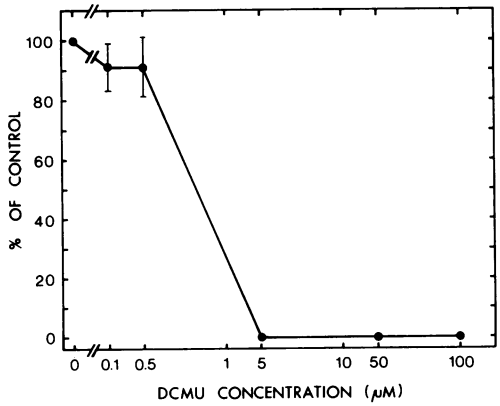
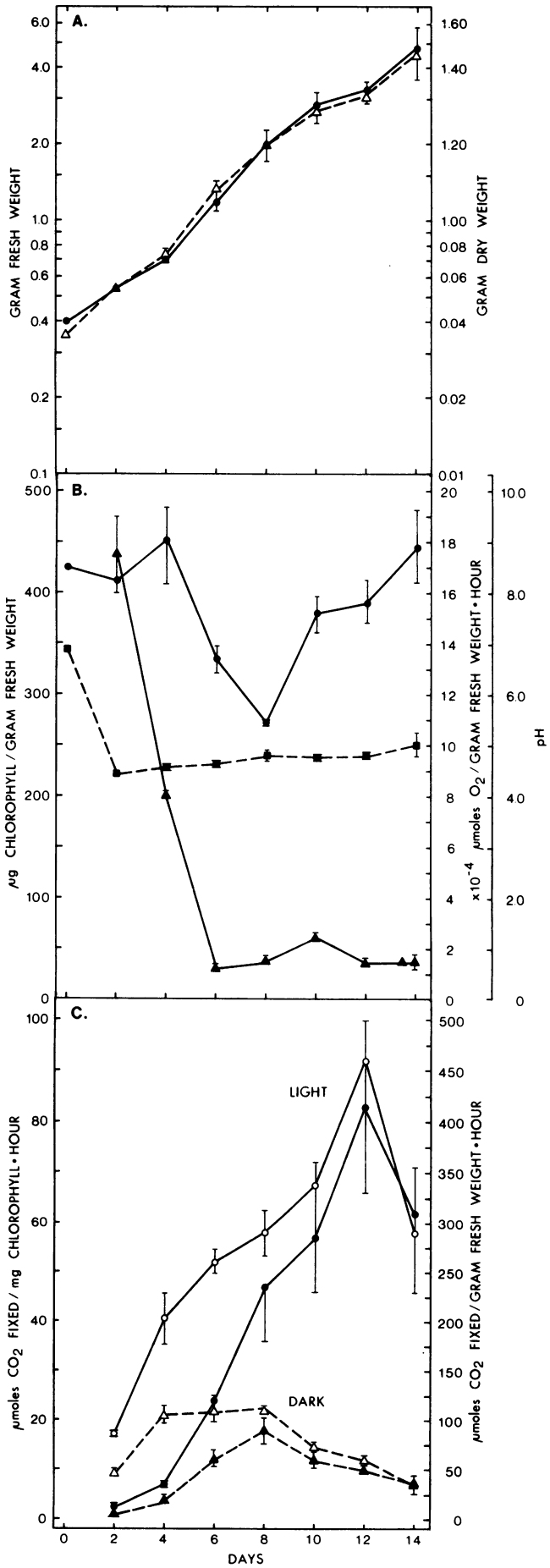


FIG. 2. Effect of DCMU on heterotrophic growth (fresh weight) of SB-H cells in KT medium containing 1% sucrose. DCMU was introduced as described in "Materials and Methods." SD are shown.

The SB-P cells would not grow in the atmospheric level of CO_2 but would grow at 5% CO_2. One per cent CO_2 did not support continuous growth although some increase in dry weight was initially observed. Experiments are in progress to determine the optimum CO_2 concentration. Omission of the Hepes buffer did not alter the growth rate. Omission of the complex vitamin mixture (Table I) resulted in a slowing of growth but complete cessation of growth did not occur until the fourth subculture (data not shown).

The fresh and dry weight of SB-P cultures increase at an exponential rate throughout the 14-d growth cycle, *i.e.* with no apparent stationary phase (Fig. 3A). The fresh weight to dry weight ratio decreases from 10.5 at day 1 to 8.85 at day 6 before rising back to 10.5 by day 14.

Dark respiration was initially quite high (Fig. 3B) but by day 6



had dropped to only 8% of its day 2 value. The Chl content of the cells dropped from 425 to 273 $\mu g\ g^{-1}$ fresh weight between days 4 and 8 before recovering (Fig. 3B). A drop in Chl content is always observed in both photoautotrophic and photomixotrophic cells sometime between days 2 and 8 with continuous Chl accumulation thereafter. The pH of the culture medium quickly dropped from 6.9 to 7.0 to near 4.5 (Fig. 3B). We have observed that this pH drop occurs within a few hours after subculture. After this initial drop, the pH gradually rises throughout the growth period to a final pH of near 5 at day 14. Increasing the buffer concentration from 5 to 25 mM Hepes (or Mops) at pH 7.0 or 7.5 is severely detrimental to continued growth of the cells (M. Horn and J. Widholm, unpublished data).

Light-stimulated CO_2 incorporation as determined by the use of $NaH^{14}CO_3$ increased progressively with time and reached maximum activity (83.1 $\mu mol\ mg^{-1}$ Chl h^{-1}) at 12 d (Fig. 3C). When put on a fresh weight (or dry weight) basis, the increase in CO_2 fixation started before day 4. CO_2 fixation in the dark also increases by day 4 or 6 but could not keep pace with the larger increases in light-stimulated CO_2 fixation, especially after day 8. The light to dark CO_2 fixation ratio did not peak during the culture period but increased until day 14 (light/dark = 8.3).

DISCUSSION

After 8 months of continuous rapid growth on medium containing no sucrose or alternative carbon source, we conclude that the SB-P cells are growing photoautotrophically. Lack of growth in the dark, in the presence of 0.5 μM DCMU, or at CO_2 concentrations of 1% or less further supports this conclusion. The system designed for the photoautotrophic growth of these cells is relatively simple and inexpensive.

Growth of the SB-P cells stabilized dramatically after subculture 14 (Fig. 1) when the system was tightened by gluing the filter units into the needles and using hose clamps. Growth may be dependent upon the positive internal pressure we have measured to exist inside the flasks (1.01 atm) which increases the partial pressure of CO_2 over the medium. At present, 1000 to 1400% increases in cell fresh and dry weight after 14-d growth periods are common. These growth rates are much higher than any previously reported for batch suspension cultures and may reflect the system design rather than the plant species involved. We initially attempted to grow these cells photoautotrophically using the double-tiered flask system of Husemann and Barz (4) but were unsuccessful.

The DCMU concentration used in Table II (0.5 μM) was the highest concentration that did not inhibit heterotrophic growth on 1% sucrose appreciably (Fig. 2). This is of interest since Yamada and Sato (12) found no inhibition of heterotrophic Scotch broom or amur cork tree cultures with 100 μM DCMU, a concentration 500 times higher. The results in Table I indicate that photosynthesis is essential for SB-P cell growth in medium lacking sucrose and that the SB-M cells use photosynthesis to support as much as one-third of their growth. Good growth without the presence of Hepes buffer or vitamins ensures that neither is a significant source of carbon for the SB-P cells.

In contrast to the usual growth kinetics found with heterotrophic batch cultures, the typical 'S' growth curve was not seen with respect to fresh and dry weight increases in SB-P cells. Instead, growth was exponential throughout the 14-d growth period (Fig. 2A). The only indication of an impending stationary phase is seen in a decline of the light-dependent CO_2 fixation (Fig. 2C) by day 14. This exponential growth may be due to the constantly renewed

FIG. 3. Characteristics of SB-P cell growth following transfer. A, Fresh weight (●——●) and dry weight (△- - -△). B, Chl (●——●), dark O_2 uptake (▲——▲), and pH (■——■). C, CO_2 fixation on a per g fresh weight basis (●——●, ▲- - -▲) and on a per mg Chl basis (○——○, △- - -△). SD are shown.

carbon supply (CO_2) in contrast to heterotrophic cultures in which the sucrose concentration would decline continuously during the growth period. Recent work concerning optimization of the KT^0 culture medium has shown that neither total nitrogen nor phosphate is in growth-limiting amounts (Horn and Widholm, unpublished data). The same is true for Mg and Ca.

The $^{14}CO_2$ fixation measurements described here were conducted under the actual growth conditions. The CO_2 fixation rates on a Chl basis are comparable to other reports which use an optimum light intensity and reaction mixture (7, 9, 10). Inasmuch as our cells have a higher Chl content/g fresh weight (or dry weight) than previous reports, the CO_2 fixation rates on a fresh weight basis are higher than those previously reported for photoautotrophic cells. This may help explain why the growth rates are also higher than those previously reported (4, 9, 12). The measured ^{14}C fixation accounts for nearly 40% of total dry weight accumulation during the early days of the growth period and a much higher percentage during the latter part. Inasmuch as carbon comprises 42% of the carbohydrate (CH_2O) molecule, SB-P carbon fixation rates easily account for the measured dry weight increase.

Comparison of respiratory patterns of SB-P cells and heterotrophic *Nicotiana glutinosa* cells (3) shows that both have high initial levels of O_2 uptake but that the rate falls more rapidly in SB-P cells with time. One possibility is that near the end of the 14-d growth cycle starch formation occurs which the cells can use after being subcultured. Preliminary results, however, reveal no detectable starch in 14-d-old cells.

When assayed in their growth medium at pH 4.5 to 5.0 and at a light intensity which supports rapid growth (230 $\mu E\ m^{-2}\ s^{-1}$), the cells did not exhibit net O_2 evolution even with added bicarbonate. They would evolve O_2 if measured under unusually high pH (>7.5) and high light intensity (500 $\mu E\ m^{-2}\ s^{-1}$) which has been the method used by other investigators (9, 11, 13).

Although the cells described in this report had been selected for a high Chl level before initiating photoautotrophic growth, the presence of such high Chl may not be necessary. Recent work in our laboratory has shown that cotton cells with a Chl level of <30 $\mu g\ g^{-1}$ fresh weight will grow photoautotrophically using the system described in the present paper (C. Blair, personal communication).

These rapidly growing soybean cultures will be studied further to attempt to determine why such high CO_2 levels are needed for growth and why O_2 evolution cannot be measured under culture conditions. Tsuzuki *et al.* (11) have postulated that low levels of carbonic anhydrase might account for the high [CO_2] requirement but at the pH of our growth medium (4.5 to 5.0) CO_2 is essentially the only species present with little or no HCO_3^-. These cells should be useful in attempts to select photosynthetic herbicide resistance as well as other useful traits.

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