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Growth of Suspension-cultured *Acer pseudoplatanus* L. Cells in Automatic Culture Units of Large Volume

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ABSTRACT

A phytostat to mass culture higher plant cells in liquid medium is described. This apparatus allowed the culture in batch, turbidostat and chemostat of 20 liters of cells. Automatic control of cell suspension growth was based on culture turbidity. Changes with time of certain cell characteristics, particularly cell respiration and phospholipid content, indicated that the test time to harvest large amounts of sycamore cells (*Acer pseudoplatanus* L.) in good physiological state was about 2 days before the end of the exponential phase of growth, when the cell density reached one million cells per milliliter of culture.

Suspension cultures of isolated plant cells provide a very good material for studying the physiology of the cell. For this purpose, it is important to be able to obtain easily large amounts of cells under rigorously defined culture conditions.

Different methods for cultivating higher plant cells in liquid medium have been described (7, 12, 18, 19), but very little work has been done on semicontinuous or continuous culture systems of large volume (17). The reasons for this are numerous. First, suspension-cultured higher plant cells form aggregates which deposit very quickly on the bottom of the vessel; second, the culture medium must be well oxygenated and be replenished under aseptic conditions; third, it is necessary to measure the cell density continuously for following and controlling the growth of the culture.

It is the aim of this paper to present simple solutions to these problems and to discuss the possibilities of our phytostat.

MATERIALS AND METHODS

Biological Material and Culture Medium. The strain of *Acer pseudoplatanus* L. used in this study was a gift of J. Guern. The basic nutrient medium was prepared according to Lamport (7), as modified by Lescure (10). The culture medium was sterilized by Millipore filtration (pore size, 0.22 μm; diameter, 142 mm). Cell suspensions were maintained in exponential growth by frequent subcultures as recommended by Doree *et al.* (3).

Culture Apparatus. Functional diagrams of the phytostat can be seen Figures 1 and 2.

Each culture unit was started by introducing 19 liters of sterilized medium into a culture vessel (CV), either directly or indirectly from a medium reservoir (MR), through a double peristaltic pump (P). The culture medium was then inoculated in two steps: an aliquot of about 1 liter of suspension-cultured cells was put in an intermediate inoculation vessel (IV) and from there into the culture vessel.

Each culture unit was immersed in a thermo-regulated water bath (WB), which assured an accurate thermal regulation between 0 and 50 C. The culture temperature of the experiments was 25 C.

The culture was stirred by a Teflon-coated magnetic bar (MB) flexibly suspended on a Teflon stalk 1 cm from the bottom of the culture vessel and rotated by a magnetic stirrer (M) placed under the culture vessel. We verified that a rotation rate of 200 rpm was sufficient to maintain cells in homogeneous suspension. This stirring process avoided the cell abrasion which inevitably occurred if the magnetic bar were lying on the bottom of the culture vessel.

The culture medium was supplied with O_2 by bubbling air at a flow rate of 60 l/hr. The air was sterilized by passage through a sintered nickel filter (F) placed at the air inlet (AI). A second filter was placed at the air outlet (AO) to avoid bacterial and fungal contaminations.

Culture aliquots were harvested by clamping the air outlet and opening the culture output (CO).

The inoculation and harvesting systems were adapted from a process perfected in our laboratory for the culture of *Chlorella pyrenoïdosa* (2).

Measurement and Control of Cell Density. Preliminary experiments showed that sycamore cells illuminated at one defined point through their culture vessel reflected an amount of light proportional to their concentration. Therefore an optical measuring system was developed with these elements: (*a*) a white light generator (LG) of 150 w (générateur EF 150, Fort, Paris); (*b*) an optical fiber (OF) for leading the light to a definite point under the bottom of the culture vessel; (*c*) a second optical fiber for transmitting the reflected light to a photoresistance; (*d*) a measuring and monitoring apparatus, the turbidimeter (TU), for amplifying and analyzing the optical signal received by the photoresistance, and for operating automatically the dilution of the culture. This turbidimeter allowed continuous measuring and recording of the culture turbidity in four culture vessels simultaneously. It also controlled the dilution of each culture individually; (*e*) a timer (Ti) to modulate the frequency and the duration of work of the turbidimeter; (*f*) a double peristaltic pump (P), to transfer simultaneously new medium from the medium reservoir (MR) to the culture vessel, and the excess of culture from the fermentor to the outflow reservoir (OR). In turbidostat[1] culture, this pump was operated by the turbidimeter; in chemostat[1] culture, it was directly operated by the timer.

General Methods. Cell counting was done under the microscope with the help of the Nageotte cell. Culture aliquots were macerated in 10% chromic acid solution as described by Butcher and Street (1), and sonicated (20 sec; 20 kcycles; 60 w) with a Sonimass 250 T (Ultrason-Annemasse, Annemasse, France).

Culture aliquots were washed twice with distilled H_2O and strained on a Fiberglass filter (15 sec; pressure of suction, 0.2 bar) to ascertain cell fresh weight. Cell dry weight was measured

[1] According to the definition of Street, ref. 13, p. 334.

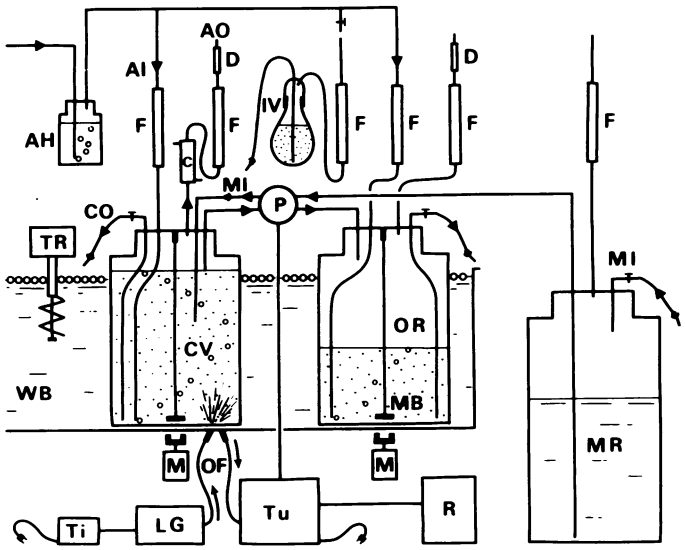

FIG. 1. Phytostat for mass culture of suspended cells. Apparatus for culture in batch, turbidostat, and chemostat with continuous and automatic control and recording of culture turbidity.

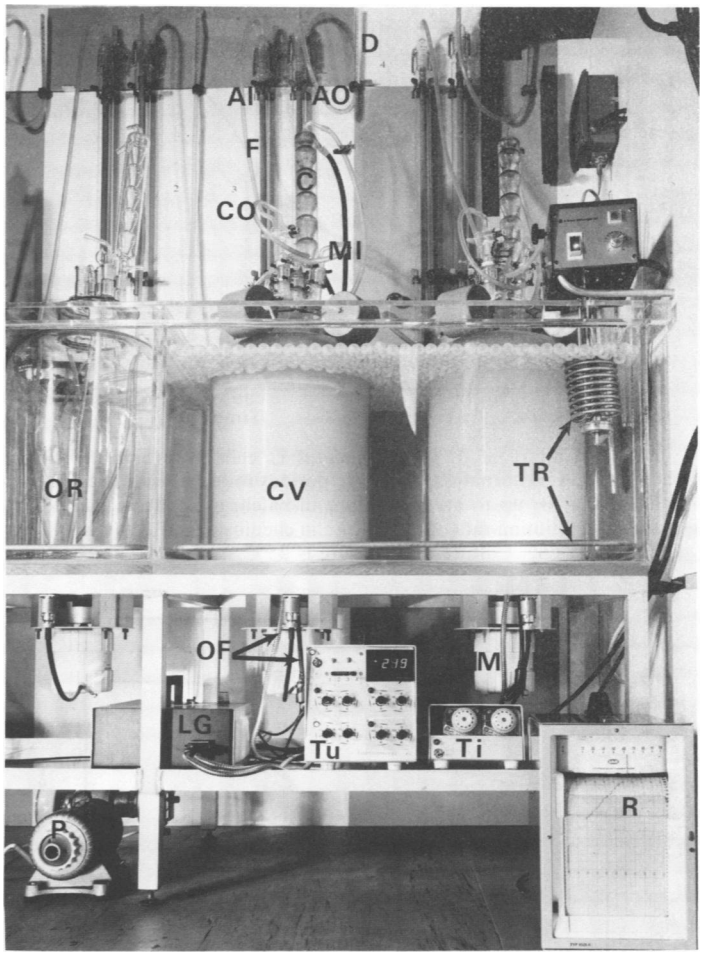

FIG. 2. Functional diagram of the phytostat. AH: air humidifier; AI: air inlet; AO: air outlet; C: humidity condensor; CO: culture output; CV: culture vessel; D: debimeter; F: air filter; IV: inoculation vessel; LG: light generator; M: magnetic stirrer; MB: suspended magnetic bar; MI: medium input; MR: medium reservoir; OF: optical fiber; OR: outflow reservoir; P: pump; R: recorder; Ti: timer; TR: thermic regulation; Tu: turbidimeter; WB: thermo-regulated water bath.

after lyophilization of the preceding samples. Total cell proteins were measured according to the method of Lance (8), after elimination of the soluble N_2 by precipitation and washing of the proteins with 10% trichloracetic acid. Total lipids were extracted according to the method of Folch *et al.* (5). Phospholipids were determined by measurement of the phosphorus (15) after mineralization of the total lipids. Sycamore cell respiration was measured at 25 C in a 3 ml stirred cell, using an O_2 electrode (Beckman, fieldlab O_2 analyzer). The O_2 concentration in air-saturated medium was taken as 240 μM (4).

RESULTS AND DISCUSSION

The apparatus including four culture units has functioned over 16 months, producing 45 cultures free of bacterial or fungal contamination.

CELL NUMBER AND CULTURE TURBIDITY

In our culture conditions, the cell number (Fig. $3A_1$) and the culture turbidity (Fig. 3A) increased exponentially without a lag phase. The cell number doubling time was 48 to 50 hr.

Throughout the exponential phase of growth, cell number and culture turbidity were closely correlated. Therefore, the cell suspension turbidity was used as a reliable indicator to record the growth in the different types of culture: batch culture (Fig. 3A), semicontinuous culture (Fig. 3B), turbidostat (Fig. 4A), and chemostat (Fig. 4B). Similar results have been obtained very recently by turbidimetric measurements using transmitted light (14).

A difference appeared only at the stationary phase of growth. The cell number remained constant (2.6×10^6 cells/ml), whereas the culture turbidity continued to increase slowly (Fig. 3, A and A_1). This rise in culture turbidity—it doubled after 2 weeks of stationary phase—could be explained by the increase of cell volume and by the modification of cell shape which is usually observed at this phase of growth (6). In connection with this observation, it must be pointed out that cell fresh weight increased from 28 to 45 mg/10^6 cells during the stationary phase (Fig. 5) and cell water rate increased from 10.5 to 15 mg H_2O/mg dry weight.

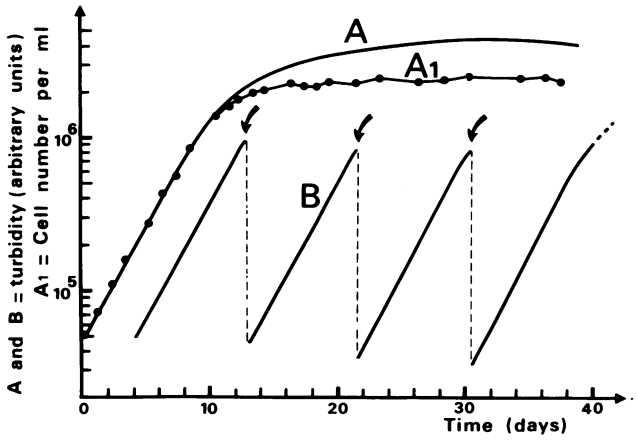

FIG. 3. Use of culture turbidity to monitor growth of batch and semicontinuous *A. pseudoplatanus* L. cells cultures. A and A_1: Time course evolution of cell suspension turbidity (A) and cell number (A_1) in batch cultures; B: semicontinuous cultures. Arrows indicate culture dilutions. Culture temperature is 25 C.

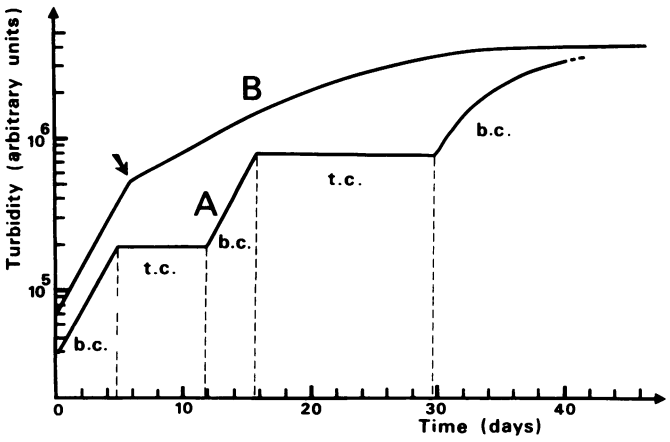

FIG. 4. Culture of *A. pseudoplatanus* L. cells in turbidostat and in chemostat. A: Alternation of culture periods in turbidostat (t.c.) and in batch (b.c.); B: up to arrow, culture in batch; after arrow, culture in chemostat. Dilution rate of the culture in chemostat is 0.1 v (v = culture volume) per day. Culture temperature is 25 C.

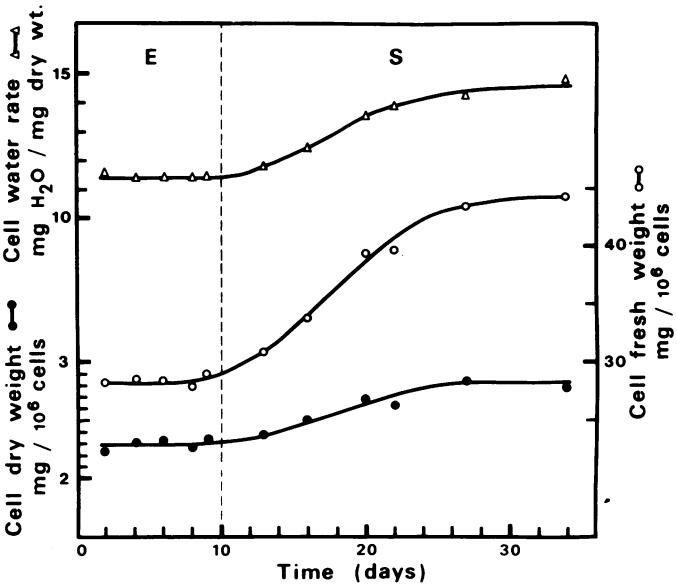

FIG. 5. Changes in *A. pseudoplatanus* L. cells fresh weight, dry weight, and water rate with time in batch culture of 20 liters. Culture temperature is 25 C. Dashed line separates the exponential phase of growth (E) and the stationary phase of growth (S).

PHYSIOLOGICAL STATE OF SYCAMORE CELLS IN BATCH MASS CULTURES

The alterations of sycamore cell protein, total lipid, phospholipid, and also of the cell respiration were studied to determine more accurately the physiological state of the cells at each stage of the exponential and stationary growth phases.

Protein Content and Oxygen Uptake. The average protein content of sycamore cells produced from batch cultures in early exponential phase of growth was 0.31 ± 0.03 mg/10^6 cells (Fig. 6). Just before the end of the exponential phase, the cell protein content decreased to 0.21 ± 0.02 mg/10^6 cells. It then remained constant in stationary phase.

This early diminution of cell protein content, preceding cessation of culture growth, is probably a consequence of the lack of N_2, which is considered a limiting factor of our culture medium (9).

On the other hand, the cell respiration rate (10 ± 1 nmol O_2/

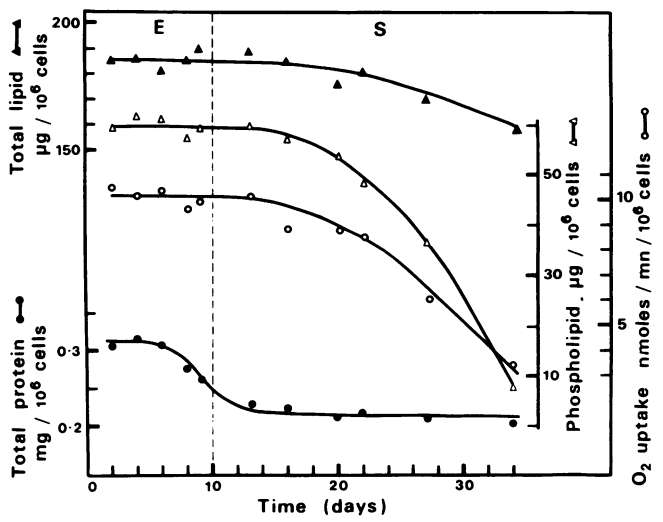

FIG. 6. Changes in cells *A. pseudoplatanus* L. proteins, total lipids, phospholipids, and cell respiration with time in batch cultures of 20 liters. Culture temperature is 25 C. Dashed line separates the exponential phase of growth (E) and the stationary phase of growth (S).

min/10^6 cells) remained constant longer (Fig. 6). After the 1st week in stationary phase, it decreased rapidly. Expressed on the basis of cell proteins, sycamore cells showed a maximum of O_2 uptake at the end of the exponential phase of growth; a value of 45 ± 5 nmol O_2/min/mg proteins was reached.

Lipids and Phospholipids. The total lipids of sycamore cells, 185 ± 15 µg/10^6 cells, was remarkably constant during the first 2 weeks of the stationary phase and decreased slowly thereafter (Fig. 6).

In marked contrast, the phospholipid content decreased rapidly, from 55 ± 5 to less than 10 µg/10^6 cells, between the 2nd and the 3rd weeks of stationary phase.

The phospholipid content gives an accurate reflection of the expansion of the membrane systems inside the cell; it is a good indicator of the physiological state of the living material. The dramatic decrease in the phospholipid content/cell during the stationary phase indicated clearly that after 3 weeks of stationary phase, our sycamore cells were in bad physiological condition.

Accordingly, it appeared that the best time to harvest large amounts of sycamore cells in good physiological state was about 2 days before the end of the exponential phase of growth, when the cell density reached one million cells/ml of culture.

CONCLUSION

The importance of batch, semicontinuous, and continuous cultures of higher plant cells has been pointed out by many authors (11, 13, 16, 17). Our phytostat possesses three main advantages. (*a*) It produces large quantities of cells, up to 1 kg of cells/unit of culture/day. (*b*) The growth of the cell population is monitored automatically and continuously. Thus, it is easy to anticipate the best time of harvesting cultured cells in well defined physiological conditions. (*c*) The culture apparatus is made of simple and readily adaptable components that are available in every laboratory.

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