PHOTOAUTOTROPHISM IN CULTURED PLANT CELLS

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CONTENTS

Abbreviations

BA; 6-benzyladenine
CAM; crassulacean acid metabolism
Chl; chlorophyll
DCIP; 2,6-dichlorophenol indophenol
DCMU; 3-(3,4-dichlorophenyl)-1,1-dimethylurea
2,4-D; 2,4-dichlorophenoxy acetic acid
D.W.; dry weight
F.W.; fresh weight
GSH; glutathione
NAA; α-naphthalene acetic acid
PEP; phosphoenol pyruvate
PEPCase; phosphoenol pyruvate carboxylase
PGA; phosphoglyceric acid
RuBP; ribulose-1,5-bisphosphate carboxylase
vvm, aeration volume/medium volume/min

INTRODUCTION

Plant tissue culture is a practical tool for the basic and applied sciences. Its culture methods produce results that have many advantages:

(1) Some cultured plant cells have totipotency; a whole plant can be regenerated from a single cell or from protoplasts (80,81).

(2) Differentiation, growth and metabolism in cultured cells can be regulated with environmental factors such as growth substances (77), nutrients, pH, temperature, light, etc.

(3) Cultured cells grow more rapidly than intact plants and produce large amounts of usable cells (64).

(4) Cultured cells can be produced under controlled environmental conditions independent of climatic changes or soil conditions, and they are free of microbes and insects.

These merits make plant tissue culture not only a useful experimental tool for biologists, but a significant tool for use in agriculture and industry as new methods to propagate clones, to establish pathogen free

plants, to modify genetic potential, and as new producers of natural products such as alkaloids, steroids, pigments, vitamins, etc.

There has been a dramatically increasing demand for the production of natural products by cultured cells because of the drastic decrease in plant resourced due to human disturbance of the natural environment, ruthless exploitation, increasing labor costs, and technical and economic difficulties in cultivating wild plants (84). It is, however, very difficult to obtain cultured cells which produce useful metabolites; redifferentiated tissue often produces secondary products (14,31,39).

Photoautotrophism is a distinctive characteristic of plant metabolism, It is a good subject for the investigation of functional differentiation under controlled conditions, and has the economic advantage of using solar energy directly. Thus, the photoautotrophism of cultured cells should provide a good model for us to use for increasing the productivity of plant cells. In this study, I have investigated the regulation of the functional differentiation of chloroplasts in cultured cells separated from their tissues and have cultivated these cells photoautotrophically.

From the time of Harberlandt (1902), there have

been many studies on chloroplast functions in cultured tissues and cells that used chemical and physico-chemical regulation for photoautotrophic culture. The effects of plant growth substances (especially auxins) on chloroplast development have been the most fully investigated.

Auxins In cell cultures, the addition of an auxin is essential to induce cell division and to maintain cell growth (97). But the addition of auxin to a medium often inhibits the greening of cultured cells (3,18,30, 83,96). For example, *Cucumis* callus needs auxin (NAA 1 ppm) for greening, but too much auxin (NAA 2 ppm) decreases the chlorophyll content (23). Of the various auxins, 2,4-D in particular inhibits chloroplast development both in intact plants (75) and in cultured cells (6,18,29,83). Yamada et al. (102) reported that NAA was the auxin most promotive for the greening and development of photosynthetic activity in cultured tobacco cells.

Cytokinins Cytokinins prevent senescence during the ageing of mature leaves, and they stimulate greening in etiolated seedlings. Cytokinins also are effective for the greening of cultured cells, even though some cells do not need the addition of a cytokinin for growth (30,46, 60,74,79).

Sugars The addition of organic carbon sources to a medium is needed for the culture of green cells; although some cultures have developed chloroplasts without it (46,89), growth is regulated by the amount of sugar present (4,17,18,24,100). The addition of sugars to the medium, especially sucrose which is the product of photosynthesis, inhibits photosynthetic activity and suppresses the development of chloroplasts (19-21,30,42, 69). In fact a decrease in the sugar concentration of a medium is known to increase the chlorophyll content of cells even though growth is reduced (4,17,18,24). Starch, a substance which is difficult for cultured cells to use, is a better promoter of greening than sucrose which is easily used by cells (40). The inhibitory effect of sucrose on greening has been observed in cultured cells which had lost their free space invertase activity (19,20).

Light Weak illumination is sufficient for cultured cells to develop chloroplasts. Venketeswaran (90) reported that strong illumination (1350-2000 lux) during the early stage of greening decolored cells, but that weak illumination (600 lux) promoted greening. Illumination stimulates the growth of cultured green cells (7,60,67, 100), and may stimulate the enzyme activity for glycolysis and respiration (25).

The quality of light effects the growth and greening of cultured green cells (7,8,34). Chloroplast development is induced by blue light, whereas red light reduces both the chlorophyll content and growth (7,8).

Inorganic elements Conventianal heterotrophic culture media are useful for cells grown under illumination. The Murashige-Skoog medium is usually the best for the greening and growth of cultured cells (7,89). Modified White's medium (7) and R-2 medium (66), in which the nitrogen concentration had been increased, were more effective for greening than the same media without modification

Gas phase Ethylene is produced and accumulated in cultures of green spinach cells (16). It inhibits both the greening and growth of these cells. Auxin stimulates the production of ethylene. CO_2 enrichment reduces the inhibitory effect of ethylene on both greening and growth. A low, partial pressure of oxygen (5% O_2), which does not inhibit growth, stimulates greening.

I have reviewed above the conclusions of many researchers about the results of their studies of what stimulates or regulates greening. Many have tried to stimulate the photosynthetic function by regulation and

to culture cells photoautotrophically. Few attempts have been successful, because the chlorophyll contents of cultured cells and their photosynthetic activities were too low to maintain photoautotrophic growth, and the improvement of the photosynthetic function was too slight.

What was needed was to establish a line of cultured cells that maintained a high chlorophyll content and high photosynthetic activity during photoautotrophic culture. I noticed that there is a difference in greening among cultured cells. Cells, even when derived from the same segments, showed heterogeneous greening. In addition, certain species of plant cells seemed to have a greater potential for developing chloroplasts in cultures; cultured cells derived from some plant species (*Cytisus, Phellodendron*) were greener than others (*Atropa, Hyoscyamus*).

I started my investigation with the induction of callus and selected those cells that had a high chlorophyll content. The easily detectable depth of color provided a good index for chloroplast development during the establishment of cultured chlorophyllous cells (Chapter I). Then I investigated the chemical and physiological requirements of greening and cultivated my high-chlorophyll cells photoautotrophically (Chapter II). Next,

I analyzed the photosynthetic activity of these photomixotrophically cultured green cells and investigated the relationship of photoautotrophism and photosynthetic activity in cultured cells (Chapter III). A more efficient method for selecting photoautotrophic cells was developed, which was based on their photosynthetic activity (Chapter VI), then I improved the culture conditions for photoautotrophic culture (Chapter V). I next investigated the characteristics of photosynthetic carbon metabolism in photoautotrophically cultured tobacco and scotch broom (*Cytisus*) cells in detail (Chapters VI & VII). For cultured cells to be useful in industrial processes, they must be cultured on a large scale; thus, I investigated the photoautotrophic cultivation of green tobacco cells in a jar-fermenter (Chapter VIII).

From the results of my several investigations, I am able to discuss what is essential for the establishment of cultured cells with a high, characteristic potential for photosynthesis and the production of secondary metabolites.

CHAPTER I

SELECTION OF CULTURED GREEN CELLS (100)

INTRODUCTION

Chloroplasts can develop (51,74,79) and maintain photosynthetic activity (6,27,54,60,86,103) in cultured tissue. Light plays an important role in growth (7,60), but most chlorophyllous cells cannot grow without sugar. Many investigators have tried to cultivate chlorophyllous tissues photoautotrophically, with some success for short periods (6,27) or with a low growth rate (11,12). The lack of vigorous photoautotrophic growth has been attributed to the low chlorophyll content and low photosynthetic activity of these chlorophyllous cells.

There are many studies on the physico-chemical conditions and nutritional requirements for the growth and greening of chlorophyllous cells. They include research on auxins (6,36,83), cytokinins (46,60,79), sugars (19,46,49,60,69), inorganic nutrients (89,93), light intensity (7,49,89), temperature (49), and the gas phase (12,16,89). But, although the number of studies have increased, there have been only a few reports of successful photoautotrophic cultures (6,9,11,12,36,85). Therefore, I first tried to obtain highly developed

chlorophyllous cells in order to establish photoautotrophic cultures.

MATERIALS AND METHODS

Plant materials Almost every part of the plant has been used as the material for callus induction; but seeds have been particularly common and easy materials to use. The following is the procedure used for the sterilization of seeds and other plant tissues.

(1) The plant material first was rinsed in a tap water.

(2) The sample then was transfered to 70% (v/v) ethanol in a beaker and shaken briefly.

(3) The alcohol was discarded and a 0.2% (v/v) benzalkonium chloride solution, a cationic surface active agent, was added and left in the beaker for 5-10 min.

(4) The detergent solution was discarded and 1-2% sodium hypochloride (as available chlorine) was added. This was left in the beaker for 5-10 min.

(5) The plant tissue then was rinsed in sterile water 3 or 4 times.

(6) This rinsed tissue was cut into small sections (0.5 cm) which was used to induce callus.

Culture conditions Fifteen species of plant calluses (Table 1) originally were induced in 1975 on the Linsmaier-

Skoog basal medium (52) with 10 μM NAA and 1 μM BA in the light. Tobacco callus (*Nicotiana tabacum* var Samsun) was received from Dr. M.Ito of Nagoya Univ. in 1975. His highly chlorophyllous tobacco cells, originally derived from the pith, were subcultured at 14-day intervals on liquid Linsmaier-Skoog basal medium with 10 μM NAA, 1 μM kinetin and twice the concentration of vitamins in the original solution. All cells were cultured at 26±1°C under continuous light from fluorescent lamps at an intensity of about 2000 lux. Other tobacco cells (cv. Bright Yellow) had been cultured with 2,4-D, IBA and NAA in the dark from 1968, 1971 and 1973, respectively (99,102).

RESULTS

Many calluses could be induced from the fifteen plant species (Table 1), but the greening and growth rate of these calluses differed greatly. *Cytisus*, *Nicotiana tabacum* var Samsun and *Phellodendron* cells had the highest chlorophyll content and grew well. Other calluses had low chlorophyll contents, and some cells were colorless. The greenest portions of the *Cytisus*, *Nicotiana* and *Phellodendron* calluses were selected and recultured on fresh medium at 21-day intervals to

Table 1 *Characteristics of calluses induced in the light*

Plant species	tissue used	growth*	color of induced callus	Chl content (μg/g F.W.)
Atropa belladonna	seed	+++	green	10-20
Beta vurgaris	leaf	++	white, red, green	10
Camellia sineasis var Assamica	leaf	+	pale green	0-5
Carica papaya	seed	+++	white, green	5-10
Chelidonium majus	stem	+	dark green	5
Coptis japonica	stem & leaf	+	yellow	
Cytisus scoparius Link	seed	+++	green	80-200
Datura metal L.	seed	+++	green	5-10
Datura tatula L.	seed	+++	green	5-10
Hyoscyamus niger L.	stem & leaf	+++	white, green	10
Lavandula vera D.C.	flower stalk	++	green	40-80
Nicotiana tabacum var. Samsun	pith	+++	green	70-120
Phellodendron amurense	seed	+++	green	60-150
Rheum paumatum	tuber	+	white	
Stevia vebaudiana	stem	++	yellow	
Chenopodium rubrum (36)	hypocotyl	+++	green	72
Daucus carota (19)	root	+++	green	64
Spinacia oleracea (16)	seedling	+++	green	73

On the average a green leaf contains 500-2000 μg/g fresh weight.

* +, grow slowly; ++, grow well; +++, grow rapidly

improve the yield of chlorophyll.

DISCUSSION

To establish photoautotrophic cultures, I first had to obtain cells with high photosynthetic activity. As stated in the introduction, there have been many attempts to enhance photosynthetic activity by metabolic regulation. These attempts to enhance photosynthetic activity have been based on the belief that all cells are homogeneous and that all have totipotency. My experiment, however, started with the idea that all cultured cells are not the same; thus, they may function differently. Therefore, I selected the most highly chlorophyllous cultured cells of a callus because a high concentration of chlorophyll appears to be the basic indicator for high photosynthetic activity.

It was difficult to obtain highly chlorophyllous cells; however, newly derived callus, illuminated from the beginning on a medium of simple composition, produced four highly chlorophyllous calluses (*Cytisus*, *Lavandula*, *Nicotiana* and *Phellodendron*). When calluses which had been subcultured in the dark for long periods were transferred to light, they turned only slightly green or showed no green color. Thus, it is important to culture cells

under illumination from the start of callus induction, this ensures the easy selection of highly chlorophyllous cells.

The constituents of the medium also are important for the selection-culture of chlorophyllous cells. We usually use 2,4-D as the auxin because it is necessary for callus induction and propagation, but 2,4-D inhibits chlorophyll synthesis in intact leaves and callus (6,75, 83). IAA, a natural auxin, decomposes easily under illumination. NAA is more effective than IBA and 2,4-D for promoting active chlorophyll synthesis among auxins (102). Cytokinins, which regulate cytodifferentiation with auxin, are known to promote greening in nongreen callus (46,79); hence, I added BA or kinetin as the cytokinin. A hormonal combination of 10 μM NAA and 1 μM BA was best, from my experience, for use with the Linsmaier-Skoog basal medium, whose effectiveness for the growth and greening of callus has been shown by Vasil and Hildebrandt (89). This combination of basal medium and hormone is not necessarily the best for all types of chlorophyllous callus induction and propagation, although, so far, it has been the most useful.

The chlorophyll content of my new, highly chlorophyllous cells ranged from 70 to 200 μg/g of their fresh

weight. This puts these cells in the highest previously reported class of cultured chlorophyllous cells.

SUMMARY

Chlorophyllous calluses were induced from fifteen species of plants in the light. *Cytisus scoparius* cells, *Nicotiana tabacum* cells and *Phellodendron amurense* cells had relatively high chlorophyll contents (70-200 µg/g fresh weight). The greenest cells observable were selected throughout each callus induction and cell subculture.

CHAPTER II

PHOTOAUTOTROPHIC CULTURE OF CULTURED CHLOROPHYLLOUS CELLS[100]

INTRODUCTION

Many investigators have tried to culture chlorophyllous tissues photoautotrophically with some success for short periods or with a low growth rate (6,9,11,12, 27,36,85). The lack of vigorous photoautotrophic growth has been partially attributed to the low chlorophyll contents and the low photosynthetic activity of these chlorophyllous cells, as well as to deficiencies in the culture conditions for chloroplast development and photosynthesis.

I could establish highly chlorophyllous tobacco, *Cytisus* and *Phellodendron* cells (70-200 ug/g fresh weight) after selection of the greenest cells after callus induction and each subculture. In this experiment, I examined the effect of light intensity, one of the most important factors in photosynthesis, on the growth and greening of green cells. I then estimated photosynthetic activities during growth by comparing growth in the dark and under strong illumination, or by inhibiting photosynthesis with DCMU or 2-chloro-4,6-bis(ethylamino)-s-triazine (Simazine). Finally, I cultured these

green cells photoautotrophically and investigated the effects of aeration with CO_2 enriched air and light intensity on photoautotrophic growth.

MATERIALS AND METHODS

Plant and subculture cell lines Five different types of cells were used. *Cytisus scoparius* Link and *Phellodendron amurense* Rupr. calluses originally had been induced in 1975 from seeds on Linsmaier-Skoog basic medium (52) with 10 μM NAA and 1 μM BA in the light. The greenest cells were selected and cultured from the calluses induced. The greenest cells in each culture were then recultured at 21-day intervals in the same medium. The other three cell types used came from the tobacco cell strain and were derived from the pith. Highly chlorophyllous tobacco cells (*Nicotiana tabacum* var Samsun, TN-I cells) received from Dr. M. Ito of Nagoya University in 1975 were subcultured at 14-day intervals on Linsmaier-Skoog basal liquid medium with 10 μM NAA, 1 μM kinetin and twice the concentration of vitamins used in the original solution. The other two types of tobacco cells (T5 and TN-II strains) were derived from *Nicotiana tabacum* cv. Bright Yellow. The T5 (99) strain had been subcultured on Linsmaier-Skoog medium with 1 μM 2,4-D only. TN-II cells were

produced with 10 μM NAA in the Linsmaier-Skoog basal medium, after which they were subcultured with 2 μM NAA. The T5 and TN-II strains, which had been subcultured at 3-week intervals in the dark for 5 years and for half a year, respectively, were transferred to illumination in 1973 and continuously subcultured under light. Stock culture media were supplemented with 3% sucrose, and all media, except for the TN-I stock culture, were solidified with 1.0% agar. All cells were cultured at 26±1°C under light. Continuous light was provided by fluorescent lamps at an intensity of about 2000 lux.

Cultured cells for experiments Throughout the experiments, I made two replications of 25 ml of the solid medium in 100 ml Erlenmeyer flasks twice. The inocula were taken from cultured stock cells when the TN-I cells were 14 days old, and the other strains were 21 days old. These cells were in the early stationary stage of the growth curve. TN-I cells were collected from the liquid culture medium by filtration and were washed with sterilized water. The other cultured cells, after careful removal of the agar medium, were broken into small pieces (about 1mm in dia.) and transplanted. For photomixotrophic culture, the TN-I cells were continuously cultured for 2 weeks and the other cells for 3 weeks.

For photoautotrophic culture, all the cell types were incubated for 4 weeks, after which they were analyzed.

Light intensity In the light intensity experiment, the intensity was varied from 0 to 14,000 lux by shading the source of illumination with black gauze or by using a dark incubator.

Inhibitors of photosynthesis I used the photosynthesis inhibitor DCMU or 2-chloro-4,6-bis(ethylamino)-s-triazine (Simazine) in a solution of 10% methanol at a final concentration of 100 μM to inhibit the photosynthetic growth of chlorophyllous cells.

CO_2 aeration The aeration system was constructed as follows. Air containing various amounts of CO_2 was bubbled through distilled water and led into the culture flasks at flow rates of about 5 ml/min. The air inlet and outlet were protected by cotton wool filters. The CO_2 enriched airs (CO_2 concentration 0.2, 1.0 and 5.0%) were supplied by Seitetsu Kagaku Co., Ltd.

Growth and chlorophyll contents Fresh weights were measured after removing excess water by blotting the cells on paper. The dry weights of the samples were measured after 2 days in an oven at 60°C. The chlorophyll contents were determined spectrophotometrically in an 80% v/v acetone extract. Chlorophyll was extracted by

the method of Sunderland (83), and its concentration was calculated with the equations derived by Arnon (2).

I consider that dry weight is a better growth index, because the water contents of fresh cultured cells frequently vary. The chlorophyll contents are expressed as the total chlorophyll content of a culture flask divided by the fresh weight. Chlorophyll synthesis has given as the change in the amount of chlorophyll content per culture flask, because changes in chlorophyll content do not necessarily coincide with cell growth.

RESULTS

Effect of light intensity and sucrose concentration on the growth of chlorophyllous cell cultures

Highly chlorophyllous tobacco TN-I cells (chlorophyll 70-120 μg/g fresh weight) were cultured in a medium containing various concentrations of sucrose (0, 0.5, 1.0 and 3.0%) under different light intensities. Fig. 1 shows their growth and greening. An increase in intensity from 600 to 6000 lux stimulated chlorophyll synthesis (changes in the amounts of chlorophyll from the original value per flask) at all concentrations of sucrose (Fig. 1-a). The optimum light intensity was between 6000 lux and 14,000 lux for chlorophyll synthesis. A 0.5% sucrose

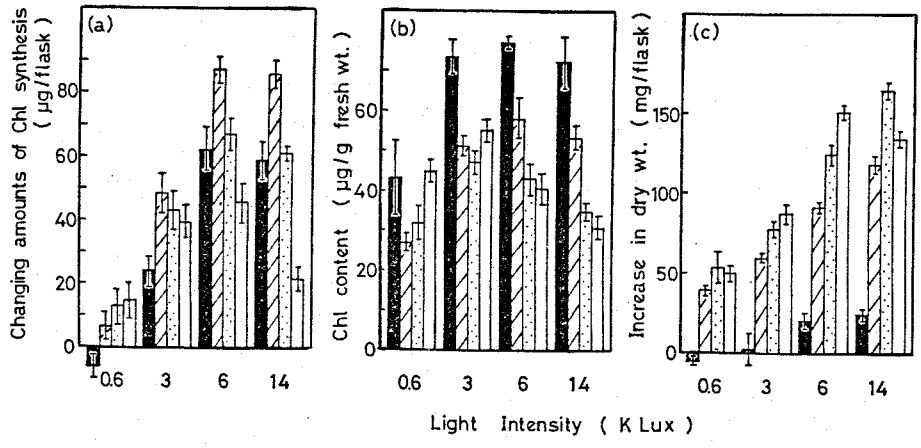


Fig. 1. *Effect of light intensity on the growth and greening of TN-I tobacco cells in a medium with various sucrose concentrations* Each flask planted with 35 mg tissue as dry weight (25 μg as total chlorophyll amount) was cultured for 2 weeks. The sucrose concentration in the medium was : ■ : 0%, ▨ : 0.5%, ⁘ : 1.0%, and □ : 3.0%. The bars represent the standard error of the mean values.

concentration produced the greatest chlorophyll synthesis. A very small addition of sucrose to the medium stimulated synthesis, but a high sucrose concentration inhibited the rate of increase when the light intensity was increased. The relation between greening and sucrose concentration became clear when the chlorophyll content per fresh weight was used as the index of greening (Fig. 1-b).

An increase in light intensity increased the chlorophyll a:b ratio from 3.0 to 4.0 in all cells except those in the sucrose free medium.

Light intensity also greatly affected the growth of TN-I cells. A high intensity enhanced the increase in the dry weights of TN-I cells (Fig. 1-c). A high sucrose concentration supported a high growth rate, but growth was nearly the same for all the sucrose concentrations under weak illumination (600 lux).

The general effect of light intensity on growth was examined with highly chlorophyllous, cultured *Cytisus* and *Phellodendron* cells (chlorophyll 114 μg/g and 68 μg/g fresh weight, respectively) and with low chlorophyllous, cultured tobacco cells; T5 and TN-II (chlorophyll 17 μg/g and 36 μg/g fresh weight, respectively)(Fig. 2). An increase in light intensity stimulated the growth of *Cytisus* and *Phellodendron* cells, whereas the growth of low chlorophyllous tobacco cells was only slightly stimulated by an increase in intensity.

The effect of photosynthesis inhibitors on the growth of cultured chlorophyllous cells

DCMU greatly inhibited the growth of TN-I tobacco cells under 14,000 lux (Table 2). DCMU also inhibited the growth of *Cytisus* and *Phellodendron* cells under

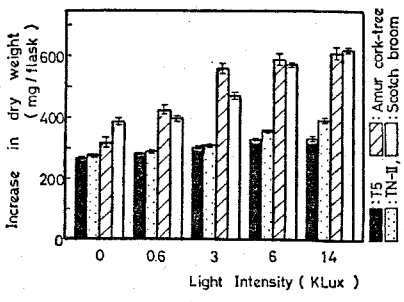


Fig. 2

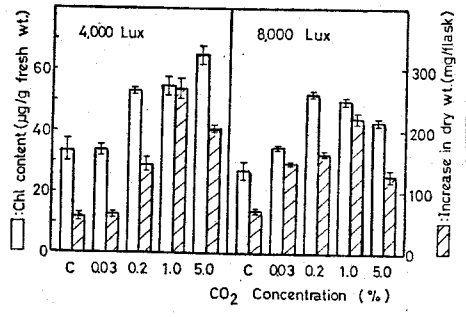


Fig. 3

Fig. 2. *Effect of light intensity on the growth of high and low chlorophyllous cultured cells. Cytisus, Phellodendron,* T5 and TN-II tobacco cells were planted as 70 mg, 52 mg, 30 mg and 39 mg dry weight, respectively. Each culture was harvested after 3 weeks of incubation. The sucrose concentration in the medium was 3%. Bars represent standard errors of the mean values.

Fig. 3. *Growth and greening in a photoautotrophic culture of TN-I tobacco cells aerated with CO_2-enriched air under weak (4000 lux) or strong (8000 lux) illuminaion.* Each flask was planted with 54 mg (dry weight) of cells (105 ug total chlorophyll) and cultured with no aeration or with aeration with air containing various CO_2 concentrations. C denotes the culture with no aeration. Bars represent standard errors of the mean values.

Table 2 *Effect of a photosynthesis inhibitor (DCMU) on growth of cultured chlorophyllous cells*

Treatment	Tobacco TN-I	*Cytisus*		*Phellodendron*	
	Increase in dry weight (mg/flask)	Increase in dry weight (mg/flask)	%[c]	Increase in dry weight (mg/flask)	%[c]
Light					
Control[a]	254 (8.3)	647 (10.2)	216	632 (11.4)	188
DCMU[b]	50 (2.4)	439 (7.9)	137	288 (6.0)	85
Dark					
Control[a]	No growth	321 (5.9)	100	337 (6.9)	100
DCMU[b]	- (-)	300 (5.4)	93	336 (7.3)	100

Chlorophyllous TN-I, *Cytisus and Phellodendron* cells were planted as 35 mg, 65 mg and 60 mg dry weight, respectively. TN-I cells were cultured for 2 weeks; the other cells for 3 weeks. Light cultures were made at 14,000 lux. Parentheses show the ratio of the harvest per inoculum in weight.

[a] The control medium contained 0.4% MeOH added to the basal medium.

[b] The DCMU medium contained 100 μM DCMU added to the control medium.

[c] % is the growth percentage per control culture in the dark.

illumination. These inhibitions by DCMU were not observed in the completely dark culture of *Cytisus* and *Phellodendron* cells, in which the increase in dry weight for these two cell lines was the same. TN-I cells ceased to grow and died during dark culture.

The same experiment was carried out with Simazine. Similar results were obtained.

Photoautotrophic culture of chlorophyllous cells

The results clearly show that the growth of highly chlorophyllous cultured cells depends to a great extent on photosynthesis under illumination. Hence, I enriched the CO_2 concentration of the aeration culture and investigated the photoautotrophic growth of chlorophyllous cells and their greening in relation to light intensity. TN-I tobacco cells in a sucrose-free medium grew vigorously (the increase in dry weight was 4- to 6-fold) when aerated with CO_2-enriched air under illumination at 4000 and 8000 lux (Fig. 3). Aeration with 1.0% CO_2 in air supported maximum photoautotrophic growth in which the increase in dry weight reached 270 mg in 25 ml of the medium. This value was nearly the same as that for the growth of T5 and TN-II tobacco cells in a dark culture with 3% sucrose (Fig. 2). Chlorophyll synthesis also was stimulated by CO_2 enrichment. Aeration with 1.0% CO_2 in air supported maximum synthesis. No aeration, or aeration with ordinary air, also enhanced photoautotrophic growth, but growth and greening were lower than with CO_2-enrichment, and cells did not grow in subsequent subcultures.

In the TN-I cell culture, the increase in light intensity from 4000 to 8000 lux had little effect on growth or on the chlorophyll content (Fig. 3). At 8000 lux

the callus was apt to be harder, whereas at 4000 lux it was friable and sometimes showed slightly more growth.

CO_2 enrichment maintained the vigorous autotrophic growth of TN-I cells, and these could be subcultured photoautotrophically for more than one and a half years (Table 3). In the 7th subculture, a mechanical accident occurred and some cells were damaged. I was able, however, to select some of the greenest undamaged cells for the 8th subculture, thus maintaining the cell line.

The photoautotrophic growth of the other chlorophyllous cells aerated with CO_2-enriched air was examined at 4000 lux (Figs. 4 and 5). The chlorophyllous *Cytisus* cell culture showed photoautotrophic growth and maintained a high chlorophyll content, but in a subsequent culture at 4000 lux, growth declined (Fig. 4). When I increased the illumination from 4000 to 8000 lux, growth was stimulated and chlorophyll synthesis increased. Growth was about two-thirds that in the dark culture with 3% sucrose, and there was a high chlorophyll content (230 μg/g fresh weight).

A similar photoautotrophic culture of *Phellodendron* cells was examined (Fig. 5). The cells showed only slight growth, even with aeration with CO_2-enriched air, at both 4000 and 8000 lux (Fig. 5). *Phellodendron* cells died

Table 3 *Growth and chlorophyll contents of chlorophyllous TN-I tobacco cells in photoautotrophic culture*

Subculture No.	Subculture Duration (week)	Increase in dry weight (mg/flask)	Growth rate[a] (dry weight)	Chlorophyll contents (μg/g fresh weight)
1st	4	214	5.1	51.6
2nd	4	108	2.8	61.7
3rd	4	98	3.8	63.3
4th	4	98	3.7	53.7
5th	4	114	3.2	81.9
6th	4	76	3.0	92.2
7th[b]	5	34	2.0	—
8th	7	170	8.7	—
9th	7	147	5.7	71.3
10th	7	156	8.7	89.3
11th	6	83	3.1	99.1
12th	8	158	7.0	62.5
13th	6	146	7.5	108.9
14th	6	138	4.9	84.3

Each culture was maintained by aerating it with CO_2-enriched air (1% CO_2). In the 1st and 2nd cultures, the light intensity was 4000 lux and in subsequent subcultures it was increased to 8000 lux. Experimental data are the means of three to five replicates.

[a] The growth rate is the ratio of the harvest per inoculum in weight

[b] A mechanical accident occurred in the incubator.

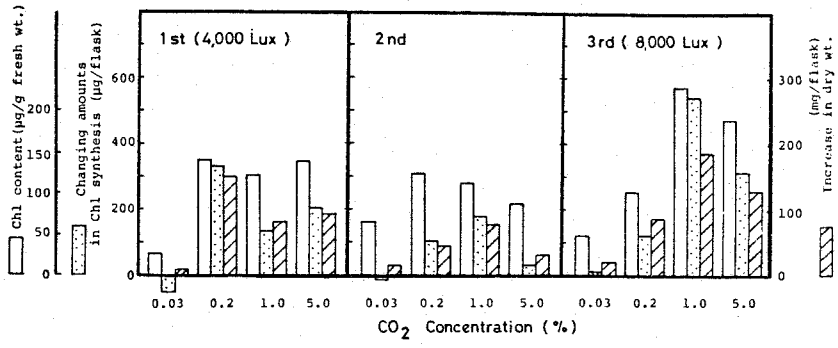


Fig. 4. *Growth and greening in photoautotrophic cultures of chlorophyllous Cytisus cells aerated with CO_2-enriched air.* Each flask was planted with about 40 mg (dry weight) of cells. In the 1st and 2nd cultures the light intensity was 4000 lux and in the 3rd it was increased to 8000 lux.

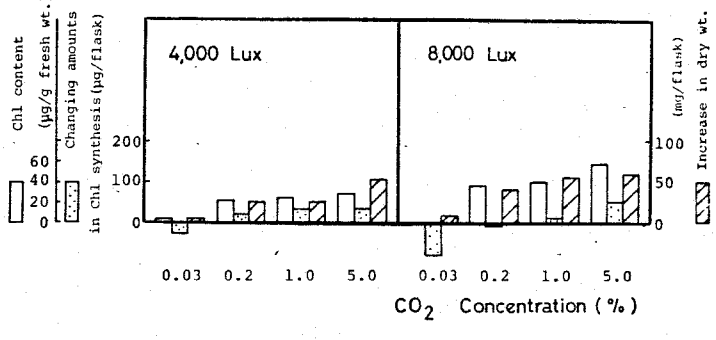


Fig. 5. *Growth and greening in photoautotrophic cultures of chlorophyllous Phellodendron cells aerated with CO_2-enriched air.* Each flask was planted with about 40 mg (dry weight) of cells. During the 2nd culture the *Phellodendron* cells died at both 4000 and 8000 lux.

during the 2nd culture at both 4000 and 8000 lux.

DISCUSSION

These experiments show a simple method of photoautotrophic culture. When highly chlorophyllous cells are obtained, adequate illumination (about 10,000 lux) and aeration with CO_2-enriched air (CO_2 concentration, about 1%) assure photoautotrophic growth. Previously it had been difficult to obtain highly chlorophyllous cells; however, newly derived callus illuminated from the beginning on a medium of simple composition, produced highly chlorophyllous calluses (Chapter I). The chlorophyll content in these highly chlorophyllous cells ranged from 70 to 200 μg/g for fresh weight. This puts them in the highest previously reported class of cultured chlorophyllous cells. A light intensity of about 10,000 lux and aeration with 1% CO_2 in air supported vigorous photoautotrophic growth of these cells, which was nearly the same as growth with 3% sucrose in the dark. These conditions are similar to the conditions for photoautotrophic culture reported previously (6,9, 27,36). Thus, the selection of highly chlorophyllous callus is the most important condition for establishing an photoautotrophic culture. *Phellodendron* cells can

not grow in photoautotrophic cultures, although they are highly chlorophyllous. Therefore, it is also important to investigate the physiological nature of cultured cells (Chapter III) and to improve the conditions for culture (Chapter V).

I obtained information on the effect of light intensity, the sucrose concentration and the CO_2 concentration on the growth and greening of chlorophyllous cells. Some researchers (49,89) have reported that the effect of light intensity differs with the callus species, e.g. carrot cells require a high light intensity (about 15,000 lux) for growth, whereas endive cells need only a low intensity (about 1500 lux). My results clearly show that an increase in light intensity stimulates an increase in the dry weight of highly chlorophyllous cells, whereas stimulation of an increase in fresh weight depends on the cell strain. In the TN-I cell culture, the increase in fresh weight was stimulated by light as was the increase in dry weight, but in the *Phellodendron*, *Cytisus* and T5 tobacco cell cultures no stimulation of the increase in fresh weight was observed. In the TN-II cell culture, an increase in light intensity decreased the fresh weight. This indicates that light has two effects; one the stimulation of an increase in dry

weight and the other stimulation of an increase in the percentage of dry weight which often means the increased hardness of tissue.

The growth of chlorophyllous cultured cells under strong illumination is about twice that of cells cultured in the dark. This stimulation by light was dependent on the chlorophyll content of the cell, and disappeared when photosynthesis inhibitors were added. Thus, chlorophyllous cells under photomixotrophic conditions and illumination can, by effectively using photosynthesis, refix the CO_2 derived from the degradation of carbohydrates in the cells. In fact, in a low sucrose medium (0% and 0.5%), aeration with CO_2-deficient air decreased growth more than did no aeration. Aeration with ordinary air stimulated growth more than did no aeration, but in a 1% sucrose medium there was no effect on growth. This means that cultured cells in a medium containing a high concentration of sucrose contain CO_2 which can be re-used for photosynthesis.

Sucrose is known to inhibit chlorophyll synthesis (19,60,69). Under strong illumination a large addition of sucrose inhibited chlorophyll synthesis, although a small addition (about 0.5%) stimulated it. Under illumination weaker than the light compensation point (about

600 lux) a large addition of sucrose promoted chlorophyll synthesis. All syntheses need energy and a substrate. Under strong illumination a low sucrose concentration in the medium is enough to synthesize chlorophyll because it is supported by photosynthesis. But, under weak illumination high concentrations of sucrose are needed. We also are able to study greening apart from photosynthesis when we culture chlorophyllous cells with a high concentration of sucrose under weak illumination.

CO_2 is essential for plant life, but the CO_2 concentration in air (0.03%) is usually not the optimum one for high photosynthesis. Air enriched with 0.1% CO_2 greatly promotes photosynthesis, whereas an enrichment of more than 1% inhibits it throughout the intact plant. In cell cultures the optimum CO_2 concentration for photoautotrophic growth is 1%, even at 8000 lux.

SUMMARY

Photosynthesis in chlorophyllous *Cytisus*, *Phellodendron* and TN-I tobacco cells in photomixotrophic cultures was investigated. When cultured under various light intensities, the growth of chlorophyllous cells was stimulated by increases in the intensity of the light given. This stimulation depended on the chlorophyll contents of the cells. It disappeared when photosynthesis inhibitors (DCMU or 2-chloro-4,6-bis(ethylamino)-s-triazine) were added. These phenomena indicate that photosynthesis accounted for a third to a half of the cell growth produced under strong illumination.

These photomixotrophic cultures then were developed as photoautotrophic cultures. When their chlorophyllous cells were cultured with aeration using CO_2-enriched air under light, the *Cytisus* and tobacco cells grew photoautotrophically. Nearly the same amount of growth as produced with 3% sucrose in the dark was observed in a photoautotrophic culture provided with air containing 1% CO_2. These green tobacco cells now have been subcultured photoautotrophically for more than one and a half years.

CHAPTER III

PHOTOAUTOTROPHY AND THE PHOTOSYNTHETIC POTENTIAL OF CHLOROPHYLLOUS CELLS IN PHOTOMIXOTROPHIC CULTURES [72]

INTRODUCTION

The photoautotrophic culture of cultured chlorophyllous cells had been very difficult, because of their low chlorophyll contents (6,11,12,18,27,54,82,85,103) and low photosynthetic capacity (17). Recently, however, I succeeded in producing photoautotrophic cell cultures of two plant species, tobacco (*Nicotiana tabacum* var. Samsun) and scotch broom (*Cytisus scoparius*), by selecting highly chlorophyllous cells from newly induced calluses (Chapter I & II).

In contrast, chlorophyllous amur cork-tree (*Phellodendron amurense*) cells could not grow photoautotrophically, even though their chlorophyll content was as high as that in tobacco and that in scotch broom cells. Growth was stimulated by an increase in light intensity, and this stimulation was reduced by a photosynthesis inhibitor.

Thus, chlorophyll content is not a suitable indicator for photoautotrophism, and direct measurement of the photosynthetic potential of chlorophyllous cells is

necessary. A number of researchers have used determinations of the photosynthetic activity of cultured chlorophyllous cells to investigate the regulation of photosynthetic activity in mixotrophic cultures (6,27) and in photoautotrophic cultures (10,36) and to compare the activity of CO_2 fixation and subsequent metabolism in photoautotrophic and mixotrophic cells (12,54) or in leaf cells (9,103). However, very few studies of photosynthetic potential in relation to the establishment of photoautotrophy have been reported (11). And, there are no reports on the activity of the component reactions (the Hill reaction, electron transport in photosystem I and RuBPCase) in photosynthesis.

I first traced the oxygen exchange by chlorophyllous cells with an oxygen electrode; then I determined the activity of the component photosynthetic reaction, including photosystems I and II and RuBPCase. Photoautotrophy and photosynthesis in photomixotrophic cultures also are discussed in this chapter.

MATERIAL AND METHODS

Callus culture Photomixotrophic stock cultures of tobacco (*Nicotiana tabacum* var. Samsun), scotch broom (*Cytisus scoparius* Link) and amur cork-tree (*Phellodendron*

amurense Rupr.) were maintained on medium containing 3% sucrose as described in Chapter II. When cells were in the early stationary stage after 3 weeks (scotch broom and amur cork-tree) or 2 weeks (tobacco) of incubation, they were harvested and their photosynthetic potential was determined. For photoautotrophic cultures, photomixotrophic cultured stock cells were cultured on a sucrose-free medium that was aerated with air containing 1% CO_2 for 4 weeks.

Measurements of growth The fresh and dry weights of the cells and their chlorophyll contents were determined as described in Chapter II.

Measurements of oxygen exchange by cultured cells Oxygen exchange was measured at 20°C with a Hansatech oxygen electrode after suspending 0.1 g of the cells (fresh weight) in 1 ml of 50 mM phosphate buffer (pH 7.8). To measure photosynthetic oxygen evolution, I illuminated the cell suspensions with a projector through a 10 cm water layer at a light intensity of about 100,000 lux, in the presence of bicarbonate at a concentration of 5 mM for tobacco and scotch broom cells and 2 mM for amur cork-tree cells. Respiratory oxygen uptake was measured in the dark under aerobic conditions.

Isolation of chloroplasts Cooled material (about 10 g)

was homogenized in a Waring blender for 1 min in 20 ml of 0.4 M sucrose solution containing 0.02 M tricine-NaOH (pH 7.8), and 0.01 M NaCl; the homogenate was filtered through four layers of gauze. The filtrate was centrifuged at 200 x*g* for 1 min, then its supernatant was further centrifuged at 1500 x*g* for 10 min. The sediments were suspended in 10 mM NaCl for 10 min, after which the chloroplast fragments were sedimented at 10,000 x*g* for 15 min. These fragments were resuspended in 10 mM NaCl. All procedures were carried out at 0-5°C.

Assay of photosystem I and of the Hill reaction in isolated chloroplasts The methyl viologen-mediated oxygen uptake in photosystem I activity (22) was measured with DCIP/ascorbate as the electron donor in the presence of DCMU. The mixture (1 ml) used to measure photosystem I contained (μmoles): tris-HCl (pH 7.2) 50; ascorbate 1.0; methyl viologen 0.1; DCIP 0.05; NaN_3 1; DCMU 0.01 and chloroplasts containing 5 μg of chlorophyll. The Hill reaction was measured from the oxygen evolved with ferricyanide as the electron acceptor. The reaction mixture contained (μmoles): tricine-NaOH (pH 7.8) 20; NaCl 10; potassium ferricyanide 1; NH_4Cl 1; and chloroplasts containing 5 μg of chlorophyll in a total volume of 1 ml. Measurements were made after saturation of the

reaction mixture with air (photosystem I) or argon (Hill reaction) at 20°C under 100,000 lux.

RuBPCase activity Cooled chlorophyllous cells (5g) were ground for 1 min in a glass homogenizer with 10 ml of ice-cold medium containing 200 mM tris-HCl pH 7.8, 10 mM $MgCl_2$ and 20 mM 2-mercaptoethanol. The homogenate was filtered through four layers of gauze; then the filtrate was centrifuged at 20,000 x*g* for 30 min. The supernatant was used as the enzyme preparation. RuBPCase (EC 4.1.1.39) was assayed at 30°C by measuring the incorporation of $^{14}CO_2$ into acid-stable compounds in reaction mixtures (pH 7.9) containing (μmoles): tris 100; D-ribulose-1,5-bisphosphate 0.25; $NaH^{14}CO_3$ (1.6 μCi) 25; $MgCl_2$ 5; EDTA 0.03; GSH 3 and 0.15 ml of enzyme preparation; total volume 0.5 ml (95). The reaction was started by adding D-ribulose-1,5-bisphosphate and was stopped after 3 min by adding 0.2 ml 6 N HCl, then the reaction mixture was evaporated to dryness. The acid-stable ^{14}C-product was dissolved in 0.2 ml of water, and its ^{14}C was measured with a liquid scintillation counter.

RESULT

Oxygen exchange in cultured cells

I traced the respiratory oxygen uptake and photo-

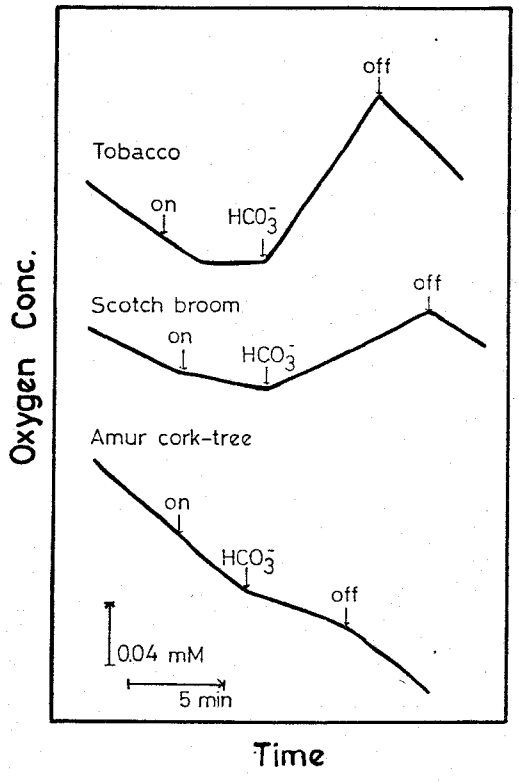


Fig. 6. *Oxygen exchange by cultured chlorophyllous cells* Photosynthetic O_2 evolution and respiratory O_2 uptake were traced with an oxygen electrode. CO_2 was added as $NaHCO_3$ at a concentration of 1 mM. Cultured cells (0.1 g fresh weight) were suspended in 20 mM phosphate buffer, pH 7.8. On: light on, Off: light off

synthetic oxygen evolution of cultured cells (Fig. 6). Chlorophyllous cells in the early stationary stage were used. All cells showed active oxygen uptake in the dark. When suspended cells were illuminated, the rate of oxygen uptake was reduced. The addition of $NaHCO_3$ induced oxygen

evolution in chlorophyllous scotch broom and tobacco cells, but bicarbonate scarcely affected the oxygen exchange in chlorophyllous amur cork-tree cells. In the dark following illumination, cells again took up oxygen.

To better estimate the net photosynthetic potential of these chlorophyllous cells, I had to determine the reaction conditions. The reaction mixture used to measure the maximum net photosynthetic potential contained 50 mM phosphate buffer (pH 7.8) with 5 mM $NaHCO_3$ for tobacco and scotch broom cells or 2 mM $NaHCO_3$ for amur cork-tree cells. Photosynthesis was saturated at these concentrations of $NaHCO_3$ under 100,000 lux.

Measurements were started under anaerobic conditions and carried out under a low oxygen concentration ($<5\%$ O_2), but chlorophyllous cells were cultured under aerobic conditions. Therefore, I checked the effect of the partial pressure of oxygen on photosynthetic potential (Fig. 7). Aerobic conditions inhibited oxygen evolution by chlorophyllous tobacco and scotch broom cells, especially by scotch broom cells, even though these chlorophyllous cells evolved oxygen under this condition. An increased oxygen concentration inhibited oxygen evolution, but this inhibition was reversible. This phenomenon suggests that the photorespiratory system in

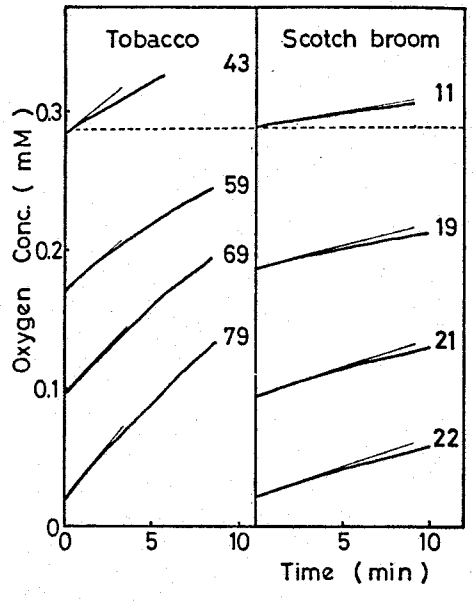


Fig. 7. *Effect of the oxygen concentration on the photosynthetic activity of chlorophyllous cells.* For reaction conditions see "Materials and methods". The O_2 concentration was changed by bubbling argon gas through the mixture. Numbers on the trace lines show the initial rate of O_2 evolution (μmol/mg chlorophyll/hr).

these cells was active under aerobic conditions.

The maximum net photosynthetic potential of chlorophyllous cells, as compared with photoautotrophic growth, is shown in Table 4. Tobacco cells had the highest rate of photosynthetic oxygen evolution, 80 μmol/mg chlorophyll/hr. Scotch broom cells also actively evolved oxygen, but amur cork-tree cells showed only slight

Table 4. *Photosynthetic potential of chlorophyllous cells*

Plant	Mixotrophic cells		Photoautotrophic cells	
	Photosynthetic O_2 evolution		Dry wt. increase	Chl content
	μmol/mg Chl/hr	μmol/g Fr.wt./hr	mg/flask	μg/g Fr.wt.
Tobacco	79,81,105	13.2,14.0,11.6	129	58
Scotch broom	26,26, 59	3.0, 7.0, 4.1	97	133
Amur cork-tree	3, 0, 0	0.3, 0 , 0	-	-

For reaction conditions for measurement of photosynthetic oxygen evolution in photomixotrophic cells, see "Materials and methods". In photoautotrophic culture each flask was inoculated with about 40 mg (dry weight) of tobacco or 50 mg (dry weight) of scotch broom cells, then cultured for 4 weeks. Photoautotrophic growth amounts are the means of four successive photoautotrophic subcultures.

evolution. The rates of oxygen evolution by chlorophyllous cells coincided with the photoautotrophic growth of each cell type.

Changes in photosynthetic activity during the growth cycle

Growth curves for scotch broom and amur cork-tree cells are shown in Fig. 8. Changes in the dry and fresh weights of both scotch broom and amur cork-tree cells produced a normal sigmoid curve (Fig. 8-a); a lag phase of about 0.5 week followed by an exponential phase for a period of 1 week, then a decline to the stationary phase. Synthesis of chlorophyll took place with growth (Fig. 8-b), and the chlorophyll content was almost

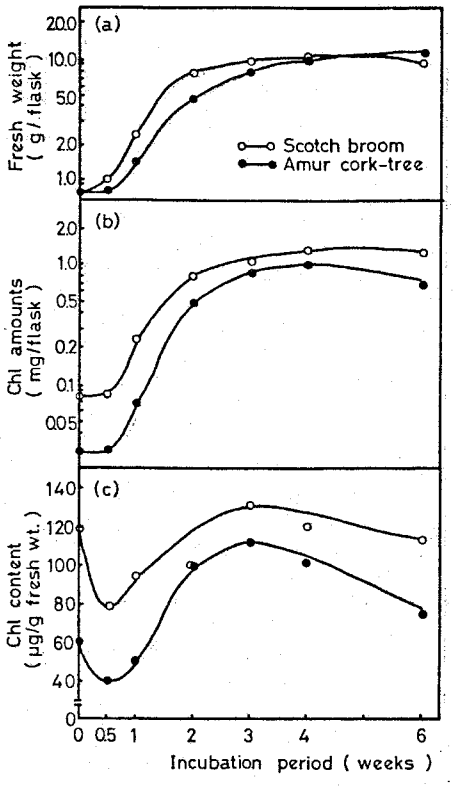


Fig. 8

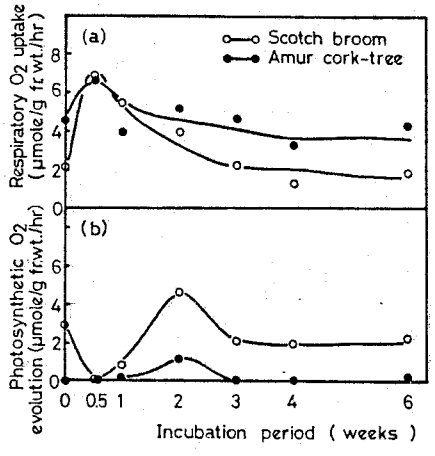


Fig. 9

Fig. 8 *Changes in the increase in fresh weight (a), in the amount (b) and contents (c) of chlorophyll during the culture of scotch broom and amur cork-tree cells.* Flasks were cultured under 4000 lux. Each value is the mean of the contents of three flasks.

Fig. 9. *Changes in respiratory oxygen uptake (a) and HCO_3^--dependent photosynthetic O_2 evolution during the culture of scotch broom and amur cork-tree cells.* For reaction conditions see "Materials and methods".

constant except for the low value in the lag phase (Fig. 8-c). Chlorophyllous scotch broom and amur cork-tree cells showed no HCO_3^--dependent oxygen evolution during the lag and early log phases (Fig. 9). The maximum O_2 evolution activity of scotch broom cells took place late in the log phase, when amur cork-tree cells also evolved photosynthetic oxygen. Scotch broom cells evolved photosynthetic oxygen throughout the stationary phase, but amur cork-tree cells again lost this activity.

Activities of the Hill reaction, photosystem I and RuBPCase in chlorophyllous cells

Measurements of photosynthetic activity during the growth cycle showed that amur cork-tree cells did not lack photosynthetic ability, rather this activity was suppressed strongly by the physiological state of the cells. I then checked the component reaction of photosynthesis (Fig. 10) in chlorophyllous amur cork-tree cells and compared it to that of other chlorophyllous cells.

Activities of the Hill reaction, photosystem I and the RuBPCase of chlorophyllous cells were determined during the early stationary stage, when amur cork-tree cells lost their O_2 evolution activity (Table 5). Each reaction was active in all the chlorophyllous cells.

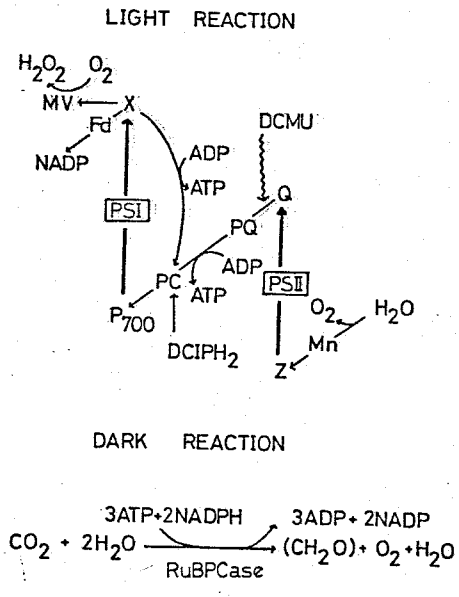


Fig. 10. *Component reaction of photosynthesis*
PS I, photosystem I; PS II, photosystem II; Fd, ferredoxin; MV, methyl viologen; PC, plastcyanin; PQ, plastquinone

Table 5 *Photosynthetic reaction activities of chlorophyllous cells*

Plant	Hill reaction (O_2 evolution) μmoles/mg Chl/hr	Photosystem I (O_2 uptake) μmoles/mg Chl/hr	RuP_2Case activity μmoles/mg Chl/hr
Tobacco	47±15	64±13	54
Scotch broom	77±33	80± 9	53
Amur cork-tree	17± 6	72±14	57

For reaction conditions see **Materials and methods.** Each value is the mean of three individual experiments with standard errors.

Photosystem I and RuBPCase activities in the amur cork-tree cells were as high as those in scotch broon and in tobacco cells, but the Hill reaction activity in the amur cork-tree cells was considerably lower than that in scotch broom and tobacco cells.

DISCUSSION

I confirmed that photosynthetic O_2 evolution is a good index for photoautotrophy. Previously, I had used chlorophyll content as the criterion for selecting photoautotrophic cells, but it is not the best index for photoautotrophy, as seen from the fact that I could not predict the low photoautotrophic growth of chlorophyllous amur cork-tree cells (100). Measurements of photosynthetic O_2 evolution revealed very low photosynthetic activity in amur cork-tree cells. Detailed measurements of photosynthetic activity in chlorophyllous scotch broom and tobacco cells showed that their photosynthetic potentials in photomixotrophic culture coincided with their photoautotrophic growth.

Photosynthetic evolution of oxygen by tobacco cells (80 μmol/mg chlorophyll/hr) was comparable to that of isolated spinach mesophyll cells (1) and was higher than that of the CO_2 fixation rate reported for isolated tobacco leaf cells (41). In previous reports on photoautotrophic culture (6,9,11,36), a similar value for photosynthetic potential was reported. Even though the activity of cultured cells relative to the amount of chlorophyll was comparable to that of the intact plant, the chlorophyll contents of the cultured cells were low.

Thus, their photosynthetic activity per gram of fresh weight is low. Chlorophyllous cells with high photosynthetic activity (per gram of fresh weight) must be selected for vigorous photoautotrophic culture. As a minimum, the activity of the chlorophyllous cells should be more than 4 μmol/g fresh weight/hr.

The rate of photosynthetic potential measured under anaerobic conditions was higher than that under aerobic conditions, even though chlorophyllous tobacco and scotch broom cells evolved photosynthetic oxygen under aerobic conditions. The existence of an active photorespiratory system is obvious in these chlorophyllous cells.

Photosynthetic activity changed during the growth cycle. Synthesis of chlorophyll and growth showed similar sigmoid curves, but the photosynthetic activities differed. Photosynthetic activity disappeared in the lag and early log stages, then reappeared and increased to a maximum in the log stage. In the stationary phase, photosynthetic activity was constant. This was similar to the pattern produced by *Nicotiana tabacum* cv. Xanthi cells in mixotrophic culture (58). Changes in the photosynthetic potential of *Nicotiana tabacum* cv. Xanthi cells coincided with those in respiratory potential. This

strain did not need an exogenous CO_2 supply to evolve oxygen. Maximum peaks in the respiratory activity of scotch broom and amur cork-tree cells appeared earlier than those in photosynthetic activities for O_2 evolution (Fig. 9); thus scotch broom and amur cork-tree cells needed exogenous CO_2. Photosynthetic activity was regulated by the physiological state of the cells.

Measurement of photosynthetic activity during the growth cycle showed that amur cork-tree cells had photosynthetic potential in a specific growth stage. Amur cork-tree cells possessed photosystem I and RuBPCase activities comparable to those of other chlorophyllous cells which could grow photoautotrophically. Only the Hill reaction activity in the amur cork-tree cells was lower than that in the other cells. Oxygen evolution in the chloroplasts of amur cork-tree cells suggested that photosystem II activity was incomplete.

SUMMARY

The relationship between the photoautotrophy of cultured plant cells and their photosynthetic potentials in photomixotrophic cultures was investigated. All chlorophyllous cells took up oxygen in the dark, but

illumination immediately reduced the uptake. Highly chlorophyllous scotch broom (*Cytisus scoparius*) and tobacco (*Nicotiana tabacum* var. Samsun) cells actively evolved photosynthetic oxygen when $NaHCO_3$ was added, but chlorophyllous amur cork-tree (*Phellodendron amurense*) cells showed very little evolution. The measurement of photosynthetic oxygen evolution in chlorophyllous cells revealed a parallel between photosynthetic potential and photoautotrophy. Activities of photosystems I and II and of rebulose-1,5-bisphosphate carboxylase showed that the low photosynthetic activity of amur cork-tree cells was due mainly to the low activity of photosystem II.

CHAPTER IV

AN EFFICIENT METHOD FOR SELECTING PHOTOAUTOTROPHIC CELLS FROM CULTURED HETEROGENEOUS CELLS[104]

INTRODUCTION

Cultured cells consists of heterogenous cells (Chapter I); therefore, we must select cells with the particular properties for our purpose in addition to regulating the culture conditions.

I selected highly chlorophyllous cells of tobacco and scotch broom during photomixotrophic culture and succeeded in culturing them photoautotrophically (Chapters I & II, 100). Berlyn and Zelitch (9,10) derived white and green haploid calluses from an anther culture of yellow mutant tobacco. The green callus had rapid net photosynthesis. Husemann and Barz (35,36) continued the selective subculture of *Chenopodium rubrum* cells that had survived under photoautotrophic conditions until they obtained cell suspensions with high growth rates. These studies show that cultured cells do differ in their photosynthetic capacities.

In Chapter I, I used chlorophyll content as the criterion for selecting photoautotrophic cells, but it

is not the best index for photoautotrophy, as seen from the fact that I could not predict the low photoautotrophic growth of chlorophyllous amur cork-tree cells (Chapter II, 100). Measurement of photosynthetic O_2 evolution revealed very low photosynthetic activity in amur cork-tree cells, whereas the photosynthetic potential of tobacco and scotch broom cells in photomixotrophic culture coincided with their photoautotrophic growth (Chapter III, 72). I confirmed that the selection of cells with high photosynthetic potential is essential if successful photoautotrophic cultures are to be established. I here report an efficient method for selecting cells capable of growing photoautotrophically, in which photosynthetic potential is the criterion used to select photoautotrophic cells.

MATERIAL AND METHODS

Callus culture Three species from the family Solanaceae; *Atropa belladonna*, *Datura stramonium* and *Hyoscyamus niger*, were the materials used. Segments of excised leaves from redifferentiated and aseptically grown seedlings were inoculated on sugar free Linsmaier-Skoog's agar medium (52) in petri dishes. The hormones used were 5 μM or 10 μM NAA and 0.05 μM, 0.5 μM or 5 μM BA in combination. The petri dishes were placed in transparent

20-liter glass cabinets. From the beginning of callus induction, the cultures in the glass cabinets were aerated with a mixture of 1% CO_2 in air at a flow rate of 1 liter per minute and illuminated continuously with fluorescent lamps at a light intensity of 3000 to 5000 lux. The temperature inside the cabinets was 27°-29°C. In subcultures after callus induction, cells were cultured under these same photoautotrophic conditions.

Measurements of growth Fresh and dry weights of cells, and chlorophyll contents were determined as described in Chapter II.

Measurements of oxygen exchange by cultured cells Oxygen exchange was measured at 25°C with a Hansatech oxygen electrode after suspending 0.1 g cells (fresh weight) in 1 ml of 50 mM phosphate buffer (pH 7.8) as described in Chapter III. To measure photosynthetic oxygen evolution, cell suspensions that contained bicarbonate were illuminated with a projector through a 10 cm water layer at a light intensity of about 100,000 lux.

RESULTS

After being cultured under the above photoautotrophic conditions for about two weeks, the inoculated

leaf segments began to swell. In all the *Hyoscyamus* and *Datura* cultures, green callus was induced in small areas along the edges of each segment, the other parts of the tissue turned white and died. I continued the photoautotrophic subculture of the greenest cells of these calluses. In the *Atropa* cultures, no callus induction took place, but the segments remained swollen. Some segments of the *Atropa* leaves showed root formation.

Data for photoautotrophic growth, chlorophyll content and photosynthetic O_2 evolution in *Hyoscyamus*, and *Datura* cultures are given in Table 6.

Table 6. *Photoautotrophic properties in Hyoscyamus niger and Datura stramonium cultures*

Materials	% Increase in F.W.	Chl content (μg/g F.W.)	Photosynthetic O_2 evolution (μmol/g F.W./hr)	(μmol/mg Chl/hr)
Cultured *Datura* cells	55	129.6	3.4	26
Cultured *Hyoscyamus* cells	198	31.1	4.3	138
Regenerated *Hyoscyamus* seedlings	—	510.2	25.4	50

Hyoscyamus cultures were subcultured for 5 passages of 3 weeks and *Datura* cultures for 7 passages of 3 weeks. Hormone concentrations were 10 μM NAA and 5 μM BA for *Hyoscyamus* and 5 μM NAA and 0.05 μM BA for *Datura* cells. For culture conditions see "Material and Methods". Seedlings were grown with 2 % sucrose in hormone-free medium under illumination.

The chlorophyll content of *Datura* cells was much higher than that of *Hyoscyamus* cells, but the increase in fresh weight for *Hyoscyamus* cells cultured photoautotrophically was higher than that for *Datura* cells. The photosynthetic activity of *Hyoscyamus* cells also was higher than that of *Datura*. The photosynthetic activity of *Hyoscyamus* cells (based on chlorophyll) was higher than that of regenerated *Hyoscyamus* seedlings.

The cultured *Hyoscyamus* cells were divided into small pieces, each of which was cultured photoautotrophically. The relationship between the photosynthetic activity and photoautotrophic growth of each *Hyoscyamus* sample is shown in Fig. 11. There was an obvious relationship between photosynthetic activity and photoautotrophic growth, but none between photoautotrophic growth and the chlorophyll content of *Hyoscyamus* cells.

DISCUSSION

Several factors are known to affect chlorophyll synthesis and the photosynthetic activity based on chlorophyll content; these are sucrose (19,27,46,60,69,100), carbon dioxide (9,35,100), light intensity (9,100) and plant hormones (46). When culturing cells with sucrose under conditions that are unsuitable for photo-

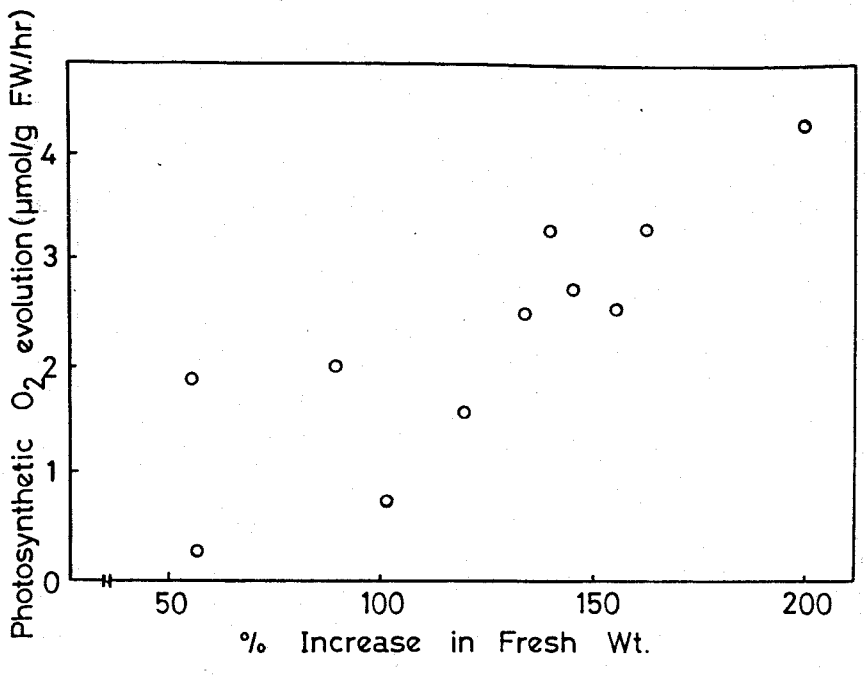


Fig. 11. *Relationship between photosynthetic activity and the photoautotrophic growth of Hyoscyamus cells.* Each culture was plated with 1 to 2 g of cells and harvested after 3 weeks of incubation. Each value is the mean of one to six replications.

synthesis, the cells which have obtained most of their energy from sucrose are allowed to proliferate and to predominate. After several years of subculturing under these conditions, we have found that at times there is a significant loss of cells with high photosynthetic potential. This is one of the problems often encountered when we culture plant cells. We should expect natural selection to take place, sometimes unnoticeably, in any subculture. One way to solve this problem is to select

cells with high photosynthetic potential as soon as we begin to culture them. I was able to set up conditions for induction under which I could select photoautotrophically grown cells at the same time that callus was induced. Furthermore I could change conditions so that the photosynthetic potential of cells would be expressed, as shown in Chapters II & III. The effectiveness of this method is, I believe, attributable to both the timeliness of selection and the selection conditions.

SUMMARY

A new and efficient method was demonstrated for the establishment of photoautotrophic cultures of plant cells. Leaf segments of *Atropa belladonna*, *Datura stramonium* and *Hyoscyamus niger* were inoculated on sugar-free, Linsmaier-Skoog agar medium then aerated with 1% CO_2-enriched air under 3000 to 5000 lux of illumination. Under these regulated conditions I could select photoautotrophic green cells efficiently. These cells subsequently have grown well under photoautotrophic conditions.

CHAPTER V

AN IMPROVED MEDIUM FOR PHOTOAUTOTROPHIC CULTURE

INTRODUCTION

Recently, I and some other researchers have succeeded in culturing plant cells photoautotrophically for long periods (36,44,100). Factors essential for successful photoautotrophic culture were the selection of cell lines with high photosynthetic potentials (Chapter III) and culture conditions favorable for photosynthesis, i.e. adequate light intensity and an enriched CO_2 concentration (Chapter II). The photoautotrophic growth of green tobacco cells was relatively rapid, but the doubling time of photoautotrophically cultured tobacco cells (about 8 days) was longer than that of photomixotrophically cultured tobacco cells (3.5 days). Thus, the culture conditions most favorable for stable and vigorous photoautotrophic growth needed to be investigated in detail.

The basic elements used for photoautotrophic culture can be used for heterotrophic culture if there is an adquate supply of sugar to maintain cell growth and keep photosynthetic activity low. A deficiency in the inorganic elements in the medium causes severe damage to

the metabolism (to the synthesis of chlorophyll and photosynthesis) which usually is seen as chlorosis (93). Plants require nitrogen, potassium and phosphate in relatively large amounts, and a deficiency in any of these elements affects all aspects of the plant's metabolism and growth. Of the minor nutrients, iron, in particular, and manganese and copper are important for photosynthesis. Iron molecules occupy parts of the catalytic sites for many important oxidation-teduction enzymes, and the element is essential for the formation of chlorophyll. Manganese is important in the photosynthetic reaction by which electrons are derived from water and oxygen is liberated. Copper functions exclusively as a catalyst in plants and is present in plastocyanin.

The development and metabolism of plants also are affected or controlled by plant growth substances. Auxin inhibits chlorophyll synthesis and chloroplast development in photomixotrophic cultures (18,30,83,96), but its presence is necessary for the growth of cultured cells. Cytokinins are effective for the greening of photomixotrophically cultured cells (18,30,46,60), but their effect on photoautotrophic cultures is unknown. Thus, I have investigated the effects of nutritional conditions on photoautotrophic growth and have established

an improved medium for the photoautotrophic culture of scotch broom (*Cytisus scoparius* Link) cells.

MATERIALS AND METHODS

Callus culture Stock cells of scotch broom (*Cytisus scoparious* Link) were cultured photomixotrophically on Linsmaier-Skoog (L.S.) basal medium (52), of the same mineral composition as the Murashige-Skoog medium (56), under continuous illumination (3000 lux) at 26°C as described in Chapter II (100). The stock culture medium contained 10 μM NAA, 1 μM BA and 3% sucrose. For photoautotrophic culture, cells were inoculated on basal medium without sucrose and were aerated with 1% CO_2 enriched air under 8000 lux illumination during the 6 week culture period.

Culture conditions For the mineral nutrient experiments, the concentrations of the sodium or chloride salt of the major elements (N,P,K) were halved, doubled or quadrupled to maintain a constant concentration of the other essential elements. The concentrations of the minor elements (Fe, Mn or Cu) were increased 3-fold, 10-fold, and 30-fold.

Chemical analysis Concentrations of the inorganic elements (N,P,K,Ca,Mg,Mn,Fe,Zn) in the cells were assayed as follows: N was measured by the Kjeldahl method after

digestion with sulfuric acid containing salicylic acid; phosphate and the other inorgainc elements were measured after ashing the cells in a muffle at 500°C (33) and treatment with 1 N HCl. Phosphate was measured spectrophotometrically as phosphomolybdate. Potassium was measured by flame photometry, and the other elements were measured by atomic absorption spectrophotometry.

Growth measurement The dry weight increase and chlorophyll content were measured as described in Chapter II.

RESULTS

Effects of the concentrations of inorganic elements in the culture medium on photoautotrophic growth

I first investigated the effects of the concentrations of nitrogen, phosphate and potassium in the medium on the photoautotrophic growth of scotch broom cells (Fig. 12). A 4-fold increase in the phosphate concentration promoted photoautotrophic growth, but increases in the nitrogen and potassium concentrations inhibited it. This stimulation of photoautotrophic growth by the 4-fold increase in phosphate was constant (Table 7). A reduced supply of nitrogen or potassium in the basal medium inhibited cell growth and greening in successive subcultures. The

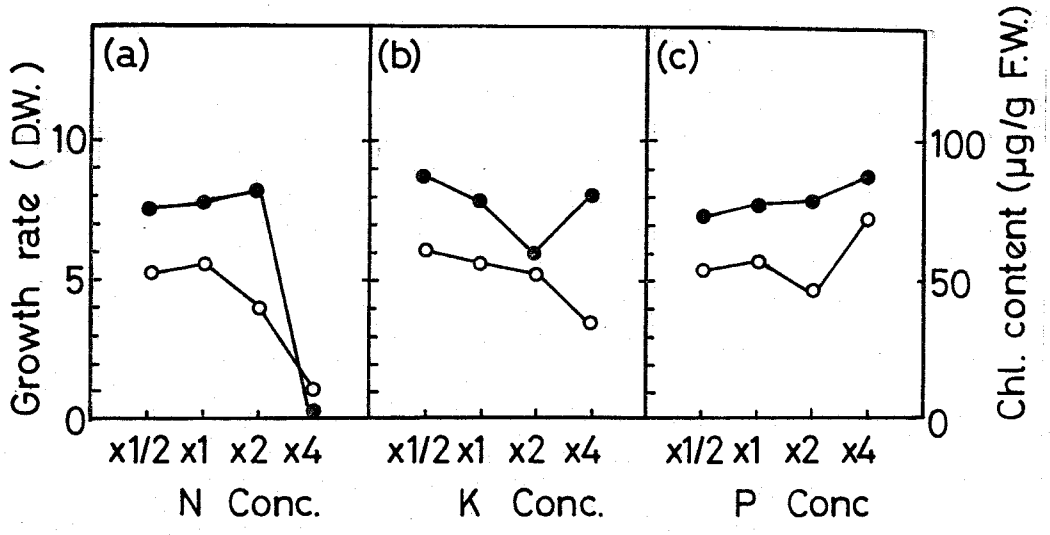


Fig. 12. *Effects of the concentrations of nitrogen (a), potassium (b) and phosphate (c) in the medium used for photoautotrophic growth and for the greening of scotch broom cells.* Cells were cultured for 6 weeks.
○——○ : Growth rate, ●——● : Chlorophyll content

Table 7. *Photoautotrophic growth of scotch broom cells in 2nd culture on modified medium*

	Control	1/2 N	1/2 K	4 P
Increase in D.W.*	3.1	1.1	1.4	7.1
Chlorophyll content (μg/g F.W.)	85	6.1	14	95

Scotch broom cells were cultured on Linsmaier-Skoog (L.S.) medium or modified L.S. medium with half the concentration of nitrogen (1/2 N) or potassium (1/2 K) or a 4-fold increase in phosphate (4 P). Each cell sample was cultured for 6 weeks.

* Ratio of the harvest/inoculum

effects of the concentrations of nitrogen, phosphate, and potassium on chlorophyll synthesis in scotch broom cells was negligible, but the 4-fold increase in nitrogen completely inhibited greening.

The contents of the inorganic elements in the cells matched the contents in the medium (Fig. 13). When the phosphate contents of the cells were halved, doubled or quadrupled, the other elements (N,K,Ca,Mg,Mn,Fe,Zn) had

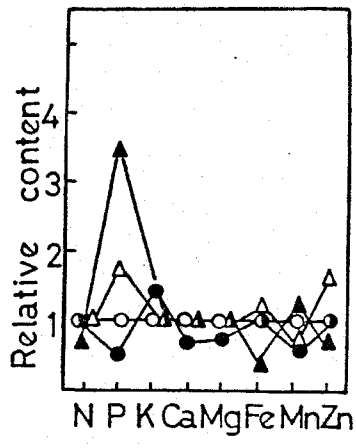


Fig. 13. *The contents of inorganic elements in scotch broom cells cultured on medium in which the phosphate concentration was varied.* The phosphate concentration in the medium was varied as follows; ●—●: x$\frac{1}{2}$, ○—○: x1 (control), △—△: x2, ▲—▲: x4. Cells were harvested after 6 weeks of incubation. The contents of the inorganic elements in control cells cultured on Linsmaier-Skoog medium were (mg/g dry weight); N,79; K,56; P,3.6; Ca,6.7; Mg,2.4; Fe,0.53; Mn,0.23; Zn,0.26

fairly constant concentrations .

Effects of the concentrations of minor elements on photomixotrophic and photoautotrophic cultures also were investigated. An increase in iron severely inhibited cell growth even in the photomixotrophic culture. Increases in copper and manganese had no effect on cell growth in either the photomixotrophic or photoautotrophic culture (Fig. 14).

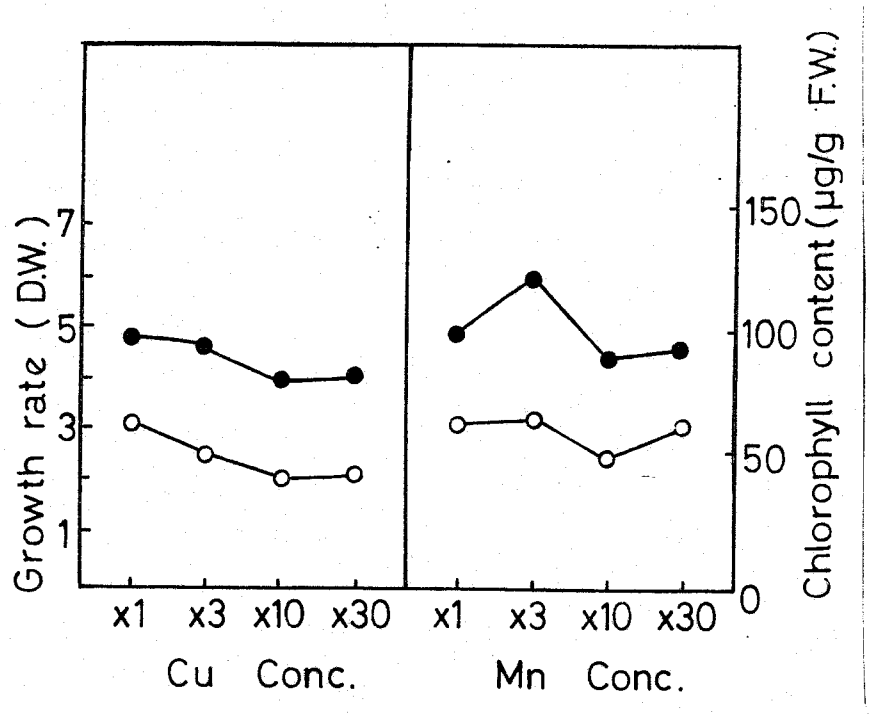


Fig. 14. *Effects of the concentrations of Mn and Cu in the medium used for photoautotrophic growth and for the greening of scotch broom cells.* Cells were cultured for 6 weeks. Symbols are same as in Fig. 12.

Effects of plant growth substances on photomixotrophic and photoautotrophic growth

The concentration of auxin regulated cell growth (Fig. 15 and 16), in both the photomixotrophic and photoautotrophic cultures. A high concentration of NAA (10 μM) promoted photomixotrophic cell growth, especially when the BA concentration was low (Fig. 15). A high BA concentration stimulated growth that had been reduced by lowering the NAA concentration to 0.1 μM, whereas BA had no effect on the culture with a high concentration of NAA

Cells grew better in photoautotrophic culture in media with a low NAA concentration, 1 μM, which was not optimal for photomixotrophic growth. A very low NAA concentration (0.1 μM) also was inhibitory. A high concentration of BA (1 μM) compensated for the inhibitory effect of a high auxin concentration (10 μM) on photoautotrophic growth.

Chlorophyll synthesis was affected by the concentrations of NAA and BA in the photomixotrophic culture (Fig.15). The chlorophyll content of scotch broom cells in photomixotrophic culture was lower when cells were cultured on a medium with high concentrations of NAA and BA. The chlorophyll content found for all the media used for photoautotrophic culture was almost constant (90-100 μg/g F.W.).

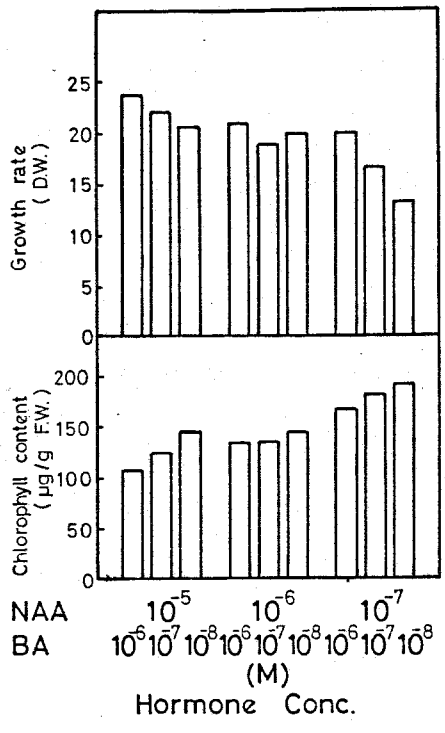


Fig. 15

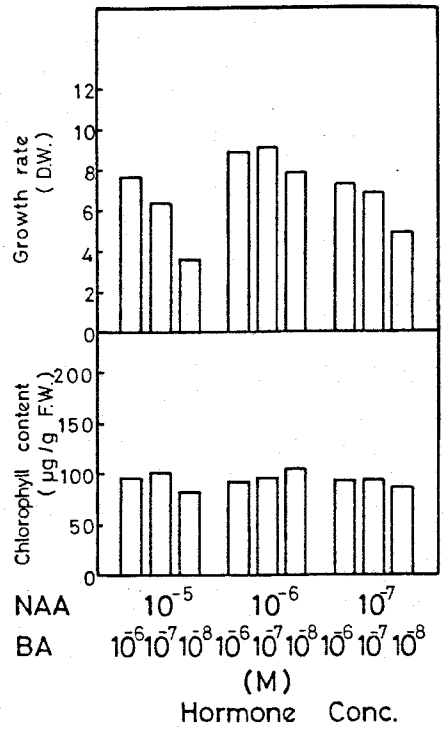


Fig. 16

Fig. 15. *Effects of hormone concentration on the growth and greening of scotch broom cells cultured photomixotrophically.* Cells were cultured for 3 weeks.

Fig. 16. *Effects of hormone concentration on the growth and greening of scotch broom cells cultured photoautotrophically.* Cells were cultured for 6 weeks.

DISCUSSION

I improved the basal medium and produced a vigorous and stable photoautotrophic culture of scotch broom (*Cytisus scoparius* Link) by modifying the Linsmaier-Skoog (L.S.) medium with a supplement of a 4-fold concentration of phosphate, by lowering the auxin concentration (1 μM) and by keeping the cytokinin concentration high (1 μM).

The concentration of inorganic elements in the L.S. basal medium was almost optimal for the photoautotrophic culture of green cells (except for phosphate), even though this medium had been modified for the heterotrophic culture of tobacco cells. Bergmann and Bälz (7), and Vasil and Hildebrandt (89) reported that L.S. basal medium is effective for the greening and growth of different types of chlorophyllous cells in photomixotrophic culture The high concentration of nitrogen in L.S. medium was what was effective for this greening. In fact, nitrogen enrichment of White's (7) or modified R-2 medium (66) increased the chlorophyll content of tobacco and soybean callus, but an increase in nitrogen in L.S. medium inhibited photoautotrophic growth (Fig. 12), as did a deficiency of nitrogen (Table 7). An increase in iron reduced the photomixotrophic growth of scotch broom cells severely, but increases in copper and manganese did not

affect this growth. I concluded that the concentrations of the minor elements in L.S. medium are sufficient for photoautotrophic culture.

The concentration of phosphate in L.S. medium was not optimal for photoautotrophic growth. A 4-fold increase in phosphate stimulated photoautotrophic growth and greening, and this stimulation was constant in successive cultures (Table 8). An increase in the phosphate concentration in the medium results in an increase in the phosphate concentration in the cells. The phosphate concentration in photoautotrophic cells cultured in a medium with a 4-fold concentration of phosphate was about ten times that in photomixotrophic cells. The increases in the other inorganic elements (N,K,Mg, etc.) in the photoautotrophic cells was, at the most, 3 times that in the photomixotrophic cells. This high phosphate content may stimulate greening and photoautotrophic growth. Kato et al. reported that an increase in the phosphate concentration of a medium improved the growth rate of *Marchantia* cells in photomixotrophic culture (45).

The optimum concentrations of auxin and cytokinin in photoautotrophic culture differed from those in photomixotrophic culture. The optimum concentration of

NAA for photoautotrophic growth was lower than that in the photomixotrophic culture (Figs. 15 and 16). Stimulation of photoautotrophic growth when the auxin concentration was lowered also took place in cultured *Chenopodium* cells (36). A high auxin concentration decreased the chlorophyll content of cultured cells in photomixotrophic culture (Fig. 15; 18,30,83,96), but photoautotrophic cells had almost the same chlorophyll contents in media in which the auxin concentration had been varied. Auxin disrupted the structure and the function of the chloroplast; it also inhibited growth in photoautotrophic culture as well as the greening of radish seedlings (57).

A high concentration of BA promoted growth, especially in photoautotrophic culture. A high BA concentration compensated for the inhibitory effect of a high NAA concentration. Cytokinins stimulate the greening of many cultured cells (30,46,60), but in the photoautotrophic culture of scotch broom cells the synthesis of chlorophyll was not stimulated by an increase in the BA concentration. Possibly, cytokinins affect green cell growth directly by stimulating photosynthetic activity (60).

I investigated the conditions needed for vigorous photoautotrophic growth and had partial success. Green

Phellodendron cells, which had a high chlorophyll content, could not grow photoautotrophically under any of the conditions tested. Improvement of culture conditions by enrichment with manganese (Fig. 17) or by lowering the auxin concentration in the medium stimulated the photosynthetic activity of *Phellodendron* cells, but constant photoautotrophic growth was not achieved. Our improved medium stimulated photoautotrophic growth, but the photoautotrophy of cultured cells must depend on some other factor such as the genetic potential of the cells used.

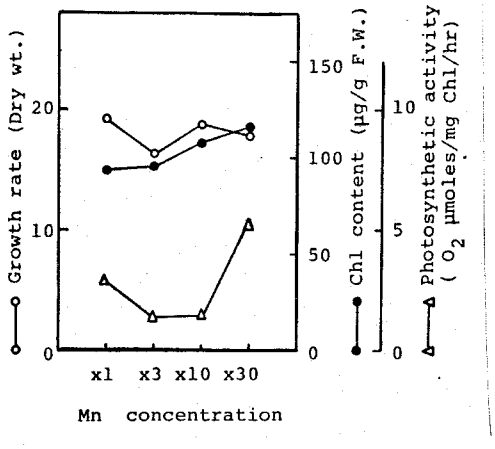


Fig. 17. *Effect of the concentration of manganese in the medium used for photomixotrophic growth, greening and photosynthetic activity of Phellodendron cells.* Cells were cultured for 3 weeks.

SUMMARY

The photoautotrophic culture medium for scotch broom (*Cytisus scoparius* Link) cells was improved, by modifying the Linsmaier-Skoog medium with an addition of a 4-fold concentration of phosphate and by lowering the auxin concentration (NAA 1 μM). Concentrations of all the inorganic elements, except phosphate, in the original basal medium were adequate for the photoautotrophic growth of scotch broom cells. A high concentration of cytokinin compensated for the inhibition of growth caused by a high concentration of auxin.

CHAPTER VI

PHOTOSYNTHETIC CARBON METABOLISM IN PHOTOAUTOTROPHICALLY AND PHOTOMIXOTROPHICALLY CULTURED TOBACCO CELLS

INTRODUCTION

Recently, photoautotrophically cultured cells have been obtained in several laboratories (9,36,44,100). My previous studies (Chapter II, 100,102) show that cells of scotch broom and tobacco cultured photoautotrophically with aeration by CO_2-enriched air under light have nearly the same growth rate as cells cultured heterotrophically with 3% sucrose in the dark.

Photoautotrophically cultured cells get their carbon and energy by photosynthesis as do intact plants. Photomixotrophic cells cultured on a medium with sugar in light, also develop chloroplasts and photosynthetic activities (Chapter III, 27,51,72), but they get their carbon and energy mainly from sugar, e.g. sucrose. Therefore, I investigated the patterns of CO_2 fixation in photoautotrophically cultured cells and in photomixotrophically cultured cells. In addition, I compared the patterns of CO_2 fixation in cultured cells with those in intact C_3, C_4 and CAM plants.

Studies of the products of short term $^{14}CO_2$ fixation in light have been reported for photomixotrophically cultured cells obtained from C_3 (27,51,58-60), C_4 (47, 50,88) and CAM (54) plants. Photomixotrophically cultured cells of carrot (27) and tobacco (58) usually show Calvin-type CO_2 fixation. Nato et al. (58) reported that the $^{14}CO_2$ fixation pathway in light differed at various growth phases; during the exponential phase of growth, the pathway of $^{14}CO_2$ fixation linked to PEPCase being greatly enhanced (59). In the cultured cells from C_4 plants (47,88), the four carbon acids were the most heavily labeled products of $^{14}CO_2$ fixation in the light over a short period. Green callus cultures of *Kalanchoe crenata* showed no crassulacean acid metabolism, but they contained greater quantities of malate, citrate and isocitrate than did colorless callus (54).

There has been little in the literature on the labeling pattern of CO_2 in photoautotrophically cultured cells (12,37). Thus, I undertook this study to determine the labeling pattern of light and dark $^{14}CO_2$ fixation in both photoautotrophically and photomixotrophically cultured tobacco cells.

MATERIALS AND METHODS

Callus culture A photomixotrophic culture of tobacco (*Nicotiana tabacum* var. Samsun) was maintained in light on a solid medium with 3% sucrose as described in Chapter II (100). After 3 weeks of culture, its cells were harvested and used to study $^{14}CO_2$ incorporation. In the photoautotrophic culture, photomixotrophic cells from a stock culture were grown on a sucrose-free medium aerated with air containing 1% CO_2 for 6 weeks under continuous illumination (Chapter III, 72).

$^{14}CO_2$ incorporation experiments (66) Approximately 100 mg of the green photoautotrophically or photomixotrophically cultured cells was broken up and suspended as small fragments in 10 ml glass tubes containing 0.5 ml of 50 mM Na-K phosphate buffer (pH 7.7).

After 5 min of incubation in a water bath at 28°C with 30,000 lux of irradiation provided by two 200 W tungsten lamps, $^{14}CO_2$ incorporation experiments were started by the addition of 40 ul of 23 mM $NaH^{14}CO_3$ (48 μCi/umole) to a final concentration of 1.7 mM. After varying periods of incubation with labeled carbon, the cells were killed quickly with 2 ml of ethanol. After homogenizing them in a glass homogenizer, we used a small sample to measure the total ^{14}C incorporation. After

successive extraction with 80% and 20% ethanol, the alcohol soluble fractions were combined and concentrated under reduced pressure at room temperature. A small sample then was chromatographed with a two dimensional technique using phenol:H_2O:acetic acid (84:16:1) containing 1 mM EDTA as the first, and n-butanol:H_2O:propionic acid (37:25:18) as the second solvent. The products of $^{14}CO_2$ fixation were located by autoradiography and were identified by co-chromatography with known compounds. The radioactivity in each product was counted with a gas flow counter (Aloka, FP type), and the relative percentage was determined. For the dark $^{14}CO_2$ fixation experiments, the glass tubes were covered with aluminum foil to keep out light.

Chlorophyll was determined by the method of Arnon (2).

RESULTS

Time courses of $^{14}CO_2$ fixation

Time courses of light and dark $^{14}CO_2$ fixation by photoautotrophic and photomixotrophic cells are shown in Fig. 18. Both types of cells fixed $^{14}CO_2$ at a steady rate during the first 10 min in both light and darkness. The total light fixation rate of $^{14}CO_2$ by photomixotrophic cells was nearly the same, on the basis of fresh weight,

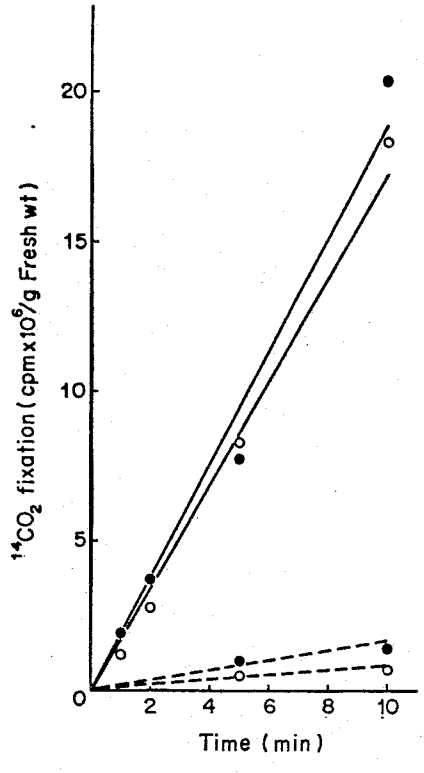


Fig. 18. *Light and dark $^{14}CO_2$ fixation in photomixotrophically and photoautotrophically cultured cells.* $^{14}CO_2$ fixation was measured in the light (——) and dark (----) as in "Materials and methods". Photomixotrophically cultured cells (●). Photoautotrophically cultured cells (○).

as that by photoautotrophic cells (Fig. 18); 7.3 µmol/g fresh weight/hr by photomixotrophic cells and 6.4 µmol/g fresh weight/hr by photoautotrophic cells. In spite of the lower chlorophyll content of the photoautotrophic cells (48 µg/g fresh weight, photomixotrophic cells 77 µg/g fresh weight), they maintained as high a photosynthetic activity as the photomixotrophic cells. That is, photoautotrophic cells had higher photosynthetic activity based on their chlorophyll content than the photomixotrophic cells (Table 9). The rate of CO_2 fixation in light for my photoautotrophic cells,

132 μmol/mg chlorophyll/hr, is similar to the value found for mesophyll cells (77).

The rate of dark fixation of CO_2 also was calculated on a chlorophyll basis and compared with that of light fixation. Photomixotrophic cells possessed a higher rate of dark CO_2 fixation to light CO_2 fixation than the photoautotrophic cells (Table 9).

Table 9 *Activities of light and dark CO_2 fixation in photoautotrophically and photomixotrophically cultured cells*

Condition	Photoautotrophic cells (μmol CO_2/mg chlorophyll/hr)	Photomixotrophic cells (μmol CO_2/mg chlorophyll/hr)
Light	132.0 (100)	94.5 (100)
Dark	4.7 (3.6)	7.3 (7.7)

Figures in parentheses are the percentages of $^{14}CO_2$ fixation in the dark to fixation in the light.

Distribution of ^{14}C among the products of CO_2 fixation

The labeling patterns of the primary products of $^{14}CO_2$ fixation in light in photoautotrophically and photomixotrophically cultured green tobacco cells are shown in Figs. 20 and 21 and those in dark are shown in Fig. 22.

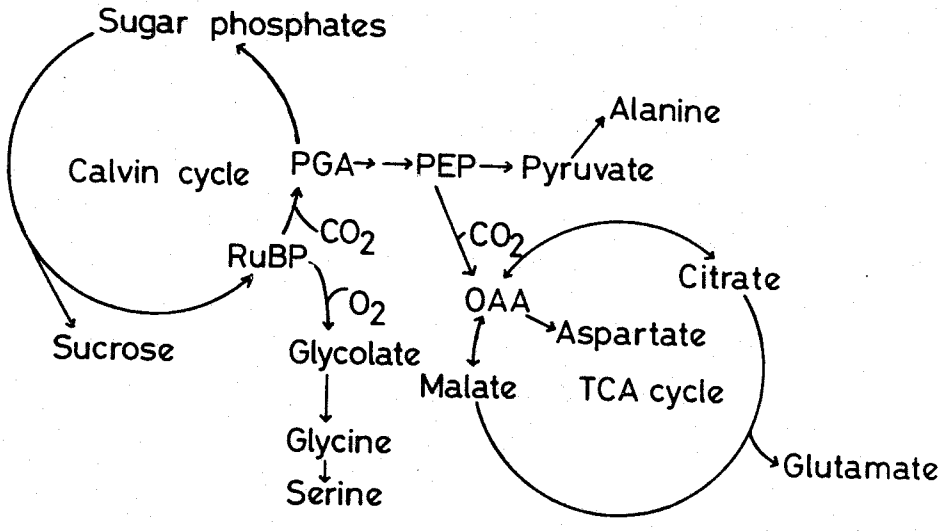


Fig. 19. *CO_2 fixation and the related metabolic pathway*

To evaluate the process of $^{14}CO_2$ fixation, I grouped labeled compounds according to the origin of their carbon chains (Fig. 19). One group consists of C_3 compounds linked to RuBPCase (Calvin cycle) and to its related metabolic pathway, e.g. the glycolate pathway and glycolysis; the other consists of C_4 compounds linked to PEPCase (C_1-C_3 carboxylation) and to its subsequent metabolic pathway, the TCA cycle. Both the activities of RuBPCase and PEPCase accounted for the results of the $^{14}CO_2$ incorporation experiments. The activities of these enzymes will be discussed in Chapter VII (73).

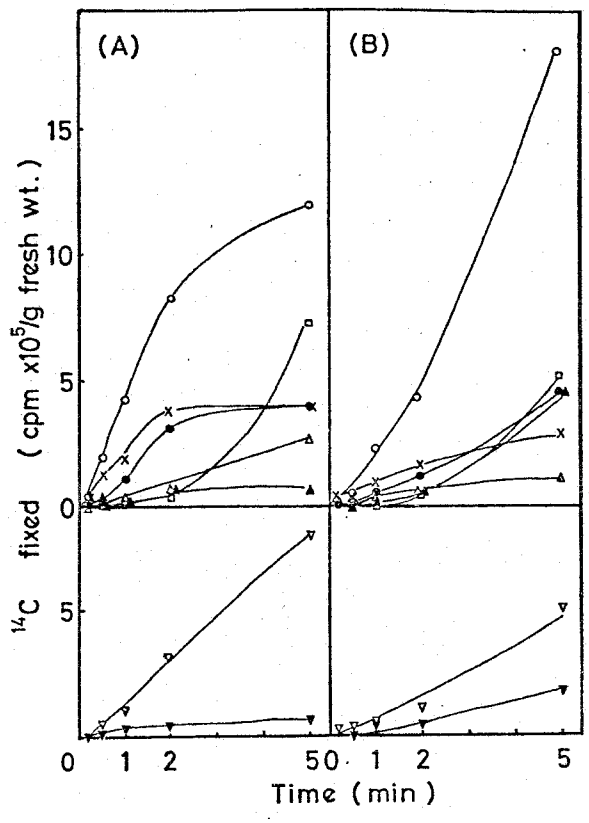


Fig. 20. *Distribution of ^{14}C in C_3 compounds linked to RuBPCase in CO_2 fixation in light in photomixotrophically (A) and photoautotrophically (B) cultured cells.* For reaction conditions see "Materials and methods". Sugar monophosphate (○), sugar diphosphates (●), PGA (×), sucrose (□), glycolate (Δ) and serine/glycine (▲) are grouped as compounds directly linked to the Calvin cycle. Alanine (▽) and PEP (▼) are grouped as metabolized compounds from PGA.

C_1-C_5 carboxylation catalyzed by RuBPCase

The distribution of ^{14}C into C_3 compounds linked to C_1-C_5 carboxylation catalyzed by RuBPCase in CO_2 fixation in light; i.e., sugar phosphates and PGA as

early products, sucrose as the end product, glycolate and serine/glycine as products of the glycolate pathway, and alanine and PEP as the products of metabolism through glycolysis from PGA, is shown in Fig. 20.

The Calvin cycle operated in the *in vivo* CO_2 fixation of both photoautotrophic and photomixotrophic cells. In both types of cells, PGA and the sugar phosphates were labeled rapidly, followed by the labeling of the photosynthetic end product, sucrose. Glycolate and serine/glycine, produced as the other function of RuBPCase (RuBP oxygenase),also were labeled at a steady rate. Alanine and PEP were labeled at a steady rate in both photoautotrophic and photomixotrophic cells.

Photoautotrophic cells incorporated ^{14}C into sugar phosphates at a slightly increasing rate and incorporated more ^{14}C into serine/glycine than into glycolate. But in photomixotrophic cells, the radioactivity in the sugar phosphates became saturated with time and glycolate was labeled more than serine/glycine.

C_1-C_3 carboxylation catalyzed by PEPCase

In addition to C_1-C_5 carboxylation, C_1-C_3 carboxylation took place in both photoautotrophically and photomixotrophically cultured cells. The distribution of ^{14}C in CO_2 fixation in the light into C_4 compounds, i.e.

malate and aspartate as primary products of C_1-C_3 carboxylation; isocitrate/citrate and glutamate as the products of metabolism through the TCA cycle from malate or aspartate, is shown in Fig. 21. The C_4 acids, malate and aspartate, were labeled at a steady rate from zero time, malate being labeled at 43% of the total fixation in photomixotrophic cells, and in photoautotrophic cells at 31% of the total fixation in light during the first 10 seconds. The other highly labeled compounds in light fixation were glutamate in photomixotrophic cells and aspartate in photoautotrophic cells. Photomixotrophically cultured cells incorporated more ^{14}C into their C_4 compounds during CO_2 fixation in light than did photoautotrophic cells.

Dark $^{14}CO_2$ fixation

The distribution of ^{14}C in photoautotrophically and photomixotrophically cultured cells during $^{14}CO_2$ fixation in the dark is shown in Fig. 22. In both types of cells, C_1-C_3 carboxylation was the main reaction during dark fixation. Radioactivity in the C_4 compounds was more than 90% of the total activity during dark fixation.

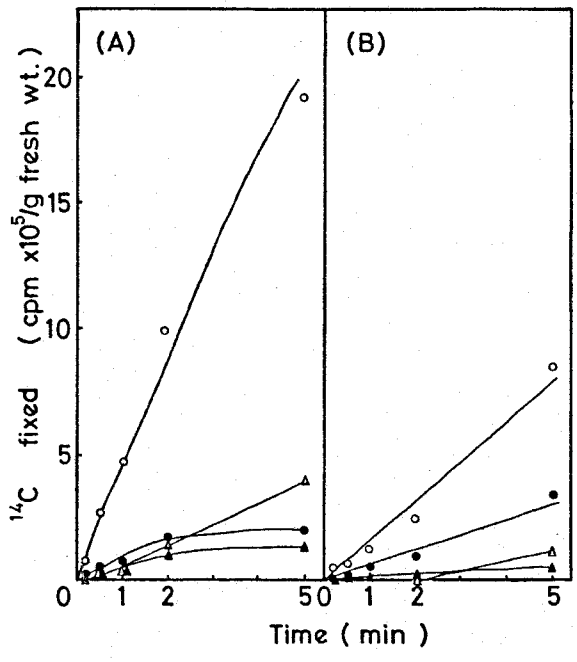


Fig. 21

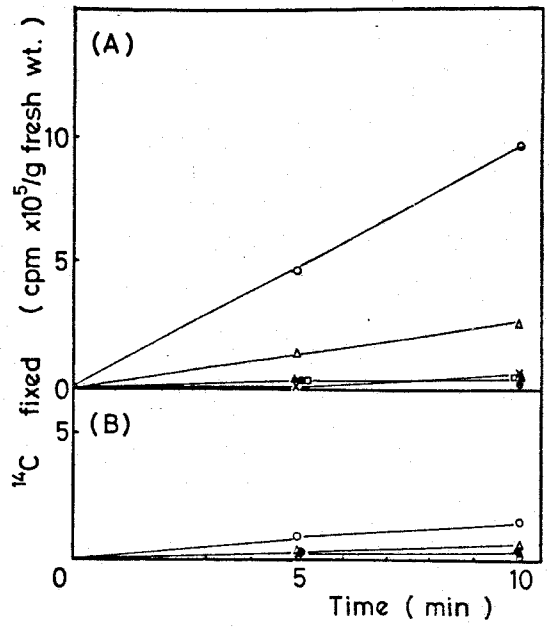


Fig. 22

Fig. 21. *Distribution of ^{14}C in C_4 compounds linked to C_1-C_3 carboxylation in CO_2 fixation in light in photomixotrophically (A) and photoautotrophically (B) cultured cells.* For reaction conditions see "Materials and methods". Malate (○), aspartate (●), glutamate (Δ) and isocitrate/citrate (▲).

Fig. 22. *Distribution of ^{14}C in CO_2 fixation in the dark in photomixotrophically (A) and photoautotrophically (B) cultured cells.* Radioactivity in the minor, labeled compounds, including sugar phosphates and alanine ranged from 7000 cpm/g fresh wt. at 5 min to 31,000 cpm/g fresh wt. at 10 min. Malate (○), aspartate (●), glutamate (Δ), isocitrate/citrate (▲), succinate (□) and glutamine (×).

DISCUSSION

Photomixotrophically and photoautotrophically cultured cells had high rates of $^{14}CO_2$ fixation in light. The rate of light CO_2 fixation was similar to that in mesophyll cells from leaves of *Papaver somniferum* (70). Photoautotrophically cultured green cells had a higher rate of light CO_2 fixation, based on chlorophyll, than did photomixotrophic cells. Moreover, photoautotrophically cultured cells had a lower rate of dark to light CO_2 fixation than did photomixotrophic cells. This means that photoautotrophic cells assimilated carbon more effectively in light than did photomixotrophic cells. Photosynthetic activities of photoautotrophic and photomixotrophic cells were correlated with the fine structure of the chloroplasts of both cell types. Electron microscopic observation showed that photoautotrophic tobacco cells had differentiated chloroplasts with developed grana, whereas photomixotrophic cells were characterized by relatively undifferentiated chloroplasts with few grana (Fig. 23).

From the patterns of the products of $^{14}CO_2$ fixation in light, I concluded that there are two carbon assimilation pathways in photoautotrophically and photomixotrophically cultured green tobacco cells. One is the

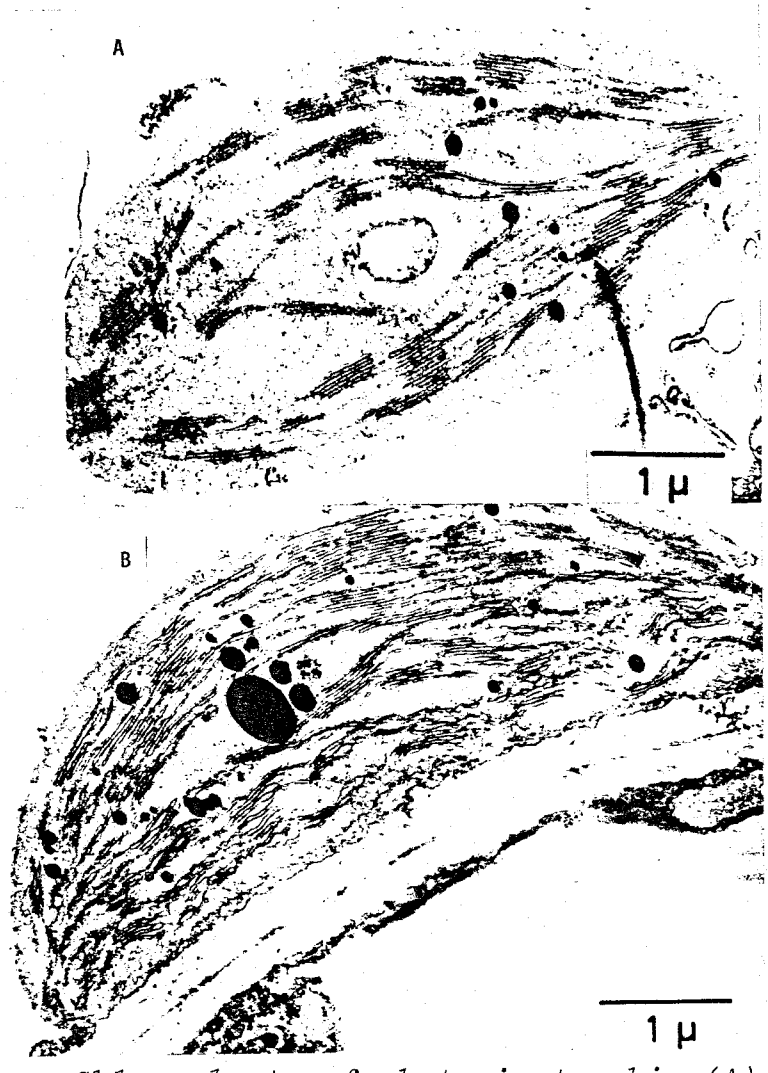


Fig. 23. *Chloroplasts of photomixotrophic (A) and photoautotrophic (B) tobacco cells.* x 25,000

C_1-C_5 carboxylation linked to RuBPCase; the other is C_1-C_3 carboxylation which should be catalyzed by PEPCase, as proposed by Paul et al., from free energy change under physiological conditions (71). The labeling patterns in PGA, the sugar phosphates and the labeling of glyxolate and serine/glycine obviously mean that the Calvin cycle operates in both types of cultured green

cells in light. The fact that malate and aspartate were labeled at a steady rate from zero time suggests that C_1-C_3 carboxylation contributes to CO_2 fixation in light in both types of cultured cells.

The enhancement of malate accumulation (C_1-C_3 carboxylation) as a primary product of $^{14}CO_2$ fixation in light must be a characteristic of CO_2 fixation in light for cultured green tobacco cells. Table 10, calculated from Figs. 20 and 21, shows the total incorporation of radioactivities in C_3 and C_4 compounds

Table 10 *Labeling patterns in C_3 and C_4 compounds in $^{14}CO_2$ fixation in light in photomixotrophic and photo autotrophic cells*

Time (sec)	10	30	60	120	300
	(cpm × 10^5/g fresh wt.)				
Photomixotrophic cells					
Total ethanol soluble fraction	1.69 (100)	8.08 (100)	15.2 (100)	34.3 (100)	66.2 (100)
C_3 compounds	0.76 (45)	4.52 (56)	9.1 (60)	20.2 (59)	39.7 (60)
C_4 compounds	0.93 (55)	3.56 (44)	6.1 (40)	14.1 (41)	26.5 (40)
Photoautotrophic cells					
Total ethanol soluble fraction	1.48 (100)	2.27 (100)	7.14 (100)	13.5 (100)	56.5 (100)
C_3 compounds	0.92 (62)	1.36 (60)	5.36 (75)	10.0 (74)	42.9 (76)
C_4 compounds	0.56 (38)	0.91 (40)	1.78 (25)	3.5 (26)	13.6 (24)

For reaction conditions see **Materials and methods.** Labeled compounds were grouped according to the origin of their carbon chains. C_3 compounds are linked to the Calvin cycle and the related metabolic pathways, the glycolate pathway and glycolysis. C_4 compounds are linked to C_1-C_3 carboxylation and its related metabolic pathway, the TCA cycle.
Figures in parentheses are the percentages of ^{14}C in each compound to the total ethanol soluble fraction.

in CO_2 fixation in light for photoautotrophically and photomixotrophically cultured cells. In comparison with intact plants and *Chlorella*, C_1-C_3 carboxylation was high during CO_2 fixation under light in cultured green tobacco cells.

In the tobacco plant (68) malate was labeled at the rate of 1.5% during the first 10 min of photosynthesis, in the rice plant (61) at 1.7% during the first 3 min, and in *Chlorella* (32) at 3.5% during the first 2 min. In photomixotrophically cultured cells malate was labeled at the rate of 29%, and in photoautotrophically cultured cells at 15% of the total fixation during the 5 min CO_2 fixation in light. In my experiments, the dark CO_2 fixation linked to C_1-C_3 carboxylation could not account for this enhanced C_1-C_3 carboxylation in light. The rate of dark CO_2 fixation to light fixation during 5 min was 3.6% in photoautotrophic cells and 7.7% in photomixotrophic cells (Table 9). The percentage of ^{14}C in C_4 compounds produced by light fixation for 5 min was 24% and 40% of the total $^{14}CO_2$ fixation in photoautotrophic and photomixotrophic cells. In *Chlorella*, blue light stimulated C_1-C_3 carboxylation (65), and the addition of ammonia also stimulated C_1-C_3 carboxylation (PEPCase) (55). In isolated mesophyll cells from the

C_3 plant *Papaver*, a stress response in which C_1-C_3 carboxylation in the light was enhanced just after isolation has been reported (71). The high rate of C_1-C_3 carboxylation is a main process in photosynthesis in C_4 and CAM plants (62).

I conclude that photoautotrophically and photomixotrophically cultured green tobacco cells have the characteristic process of carbon assimilation (high C_1-C_3 carboxylation) during CO_2 fixation in light, but the intact tobacco plant has much higher activity for C_1-C_5 carboxylation than for C_1-C_3 carboxylation during photosynthesis. Further investigations of the patterns of $^{14}CO_2$ fixation in other photoautotrophically cultured cells, and the determination of the activities of RuBPCase, PEPCase and PEP carboxykinase during CO_2 fixation are described in Chapter VII.

SUMMARY

Labeling patterns of light and dark $^{14}CO_2$ fixation in photoautotrophically and photomixotrophically cultured tobacco cells were determined. During short term $^{14}CO_2$ fixation under light, malate (C_1-C_3 carboxylation) was heavily labeled as were phosphoglyceric acid and sugar

phosphates (C_1-C_5 carboxylation). Dark fixation could not account for this high $^{14}CO_2$ incorporation into the C_4 compounds linked to PEPCase. Two carboxylation pathways linked to the RuBPCase and PEPCase were indicated in $^{14}CO_2$ fixation in light in photoautotrophically and photomixotrophically cultured cells.

CHAPTER VII

ACTIVITIES OF CARBOXYLATION ENZYMES AND PRODUCTS OF $^{14}CO_2$ FIXATION IN PHOTOAUTOTROPHICALLY CULTURED CELLS [73]

INTRODUCTION

Cultured green cells from higher plants offer new materials for research in photosynthesis. Photomixotrophic cells cultured with sugars in the light show photosynthetic activity (Chapter III, 27,47,50,54,58,60, 88). Photoautotrophic cells grow without a sugar supply in the light, and their carbon source depends on photosynthesis only (Chapter II, 9,12,36,38,44,63,72,100). Some green cells (photoautotrophic and photomixotrophic cells) have photosynthetic activity that is as high as that of the mesophyll cells in intact plants (Chapter VI, 38,63). Several reports have shown that photomixotrophic cells grown in light fix CO_2 to some extent through the Calvin cycle and that they fix ^{14}C into C_4 compounds ($C_1+C_3=C_4$) in which the percentage distributions of ^{14}C in the organic acids are higher than those in intact C_3 plants (Chapter VI, 38,58,63,88).

In photoautotrophically cultured cells, the Calvin cycle contributes to CO_2 fixation in the light to a

greater extent than it does in photomixotrophic cells, but high labeling of C_4 acids also has been observed (Chapter VI, 38,63). I investigated whether a high $^{14}CO_2$ incorporation into C_4 acids is common in cultured green cells and which carboxylation enzymes may be reponsible.

MATERIALS AND METHODS

Callus culture A photomixotrophic culture of scotch broom (*Cytisus scoparius* Link) was maintained on a solid medium with 3% sucrose as described in Chapter II. After 3 weeks of culture, cells in the stationary phase were harvested and used to study $^{14}CO_2$ incorporation and enzyme activity. In the photoautotrophic culture, photomixotrophic cells from a stock culture were grown to the stationary phase on a sucrose-free medium flushed with air containing 1% CO_2 for 6 weeks under continuous illumination, then they were subcultured three times under the same conditions. The photoautotrophic and photomixotrophic cultures of tobacco (*Nicotiana tabacum* var. Samsun) were maintained as described in Chapter VI.

$^{14}CO_2$ incorporation experiments $^{14}CO_2$ incorporation experiments were carried out as described in Chapter VI. Small fragments of cultured green cells that had been placed in test tubes containing 50 mM Na-K phosphate

buffer (pH 7.7) were incubated for 5 min at 28°C under 30,000 lux of illumination. $^{14}CO_2$ incorporation experiments were started by adding $NaH^{14}CO_3$ (48 μCi/μmole) at a final concentration of 1.7 mM. After various periods of incubation, the cells were killed quickly in ethanol then homogenized, after which samples for analysis were extracted successively with 80% and 20% ethanol The products of $^{14}CO_2$ fixation were located by autoradiography after two dimensional chromatography, and the radioactivity in each product was counted with a liquid scintillation counter. For dark $^{14}CO_2$ fixation experiment, the glass tubes were covered with aluminum foil.

Enzyme preparation Cooled, green cells (about 5 g) were ground with 5 ml of chilled medium containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM ascorbate, 5-10% polyvinylpyrrolidone-30 and 80 mM mercaptoethanol in a glass homogenizer. The homogenate was centrifuged immediately at 20,000 x*g* for 15 min, and its supernatant was used as the enzyme preparation. The above procedures were carried out at 0-4°C.

Enzyme assay In the assays for PEP carboxylase and RuBP carboxylase (68), I followed the changes in absorption at 340 nm (NADH) as described below with a Hitachi spectrophotometer. The total volume of each reaction

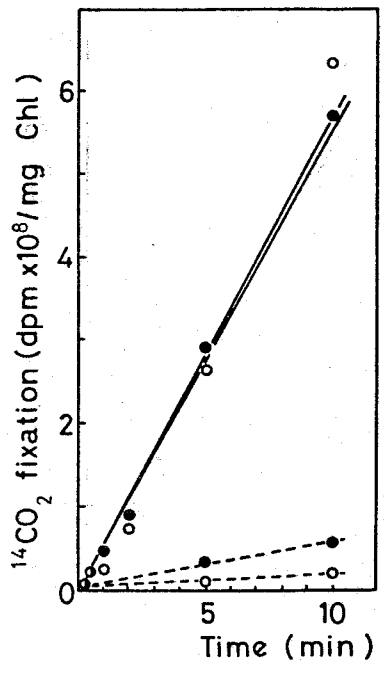
mixture was 3.0 ml, and each reaction was run at 30°C. For the PEP carboxylase assay, the reaction mixture (pH 8.0) contained (in μmoles) Tris, 150; $MgCl_2$, 30; $NaHCO_3$, 30; PEP, 9; NADH, 0.3 and 150 μl of the enzyme preparation. For the RuBP carboxylase assay, the reaction mixture (pH 8.0) contained (in μmoles) Tris, 150; $MgCl_2$, 30; $NaHCO_3$, 60; EDTA, 0.6; ATP, 6; RuBP, 1.2; NADH, 0.3; 1 unit each of 3-phosphoglyceric phosphokinase and glyceraldehyde-3-phosphate dehydrogenase, and 150 μl of the enzyme preparation. For the PEP carboxykinase assay (28), I followed the changes in absorption of oxaloacetate at 280 nm in a reaction mixture (pH 8.0) containing (in μmoles) HEPES, 150; $MnCl_2$, 6; ATP, 1.2; oxaloacetate, 1.8; 1 unit of pyruvate kinase, and 150 μl of the enzyme preparation.

Chlorophyll was determined by the method of Arnon (2) as described in Chapter II.

RESULTS

Activity of $^{14}CO_2$ fixation by green scotch broom cells

Both green, photoautotrophic and photomixotrophic scotch broom cells fixed $^{14}CO_2$ in the light at a much higher rate than in the dark for a period of 10 min (Fig. 24). The rate of $^{14}CO_2$ fixation in the light by



Time course of $^{14}CO_2$ fixation in photomixotrophic (●) and photoautotrophic (○) cells in light (——) and in darkness (- - -).

photoautotrophic cells (64.0 μmol/mg Chl/hr) was nearly the same as that by photomixotrophic cells (62.3 μmol/mg Chl/hr) on the basis of their chlorophyll contents. But, the rate of $^{14}CO_2$ fixation in the dark by photomixotrophic cells (7.0 μmol/mg Chl/hr) was much higher than that by photoautotrophic cells (2.6 μmol/mg Chl/hr).

Labeling patterns in $^{14}CO_2$ fixation products

The labeling patterns of the primary products of $^{14}CO_2$ fixation in the light in photoautotrophically and photomixotrophically cultured, green scotch broom cells are shown in Fig. 25. In both cell types malate, phosphoglyceric acid, sugar monophosphates and sugar diphosphates were labeled as the primary products, and sucrose was labeled as the end product. Glycolate and serine/glycine

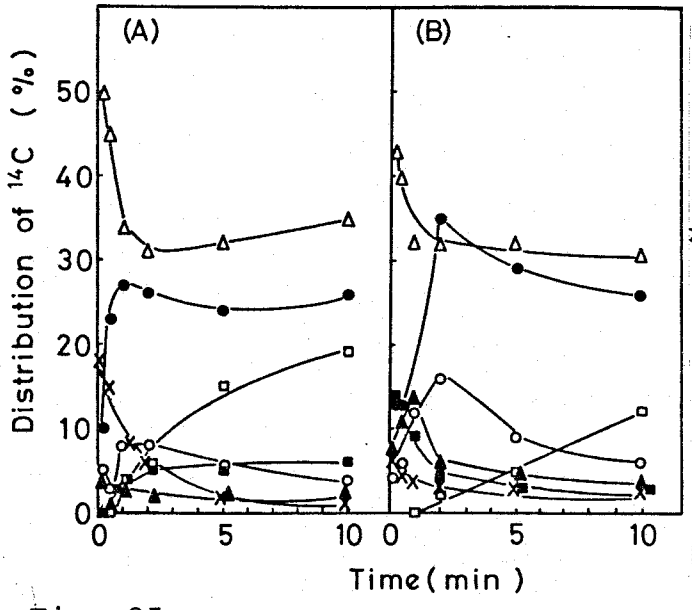


Fig. 25

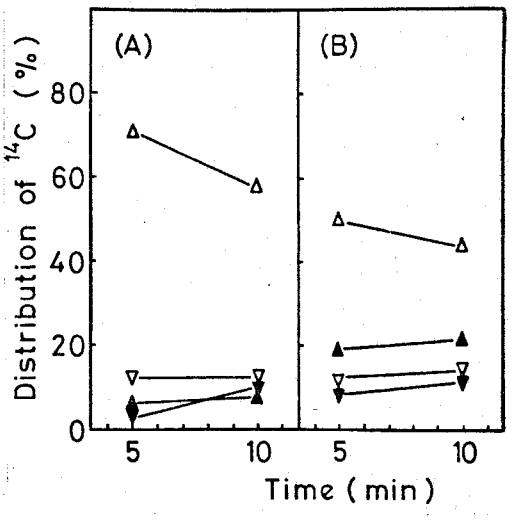


Fig. 26

Fig. 25. *Percentage distribution of ^{14}C in CO_2 fixation by photomixotrophic (A) and photoautotrophic (B) scotch broom cells in light.* Malate (Δ), aspartate (▲), sugar monophosphates (●), sugar diphosphates (○), Phosphoglycerate (×), sucrose (□), alanine (■).

Fig. 26. *Percentage distribution of ^{14}C in CO_2 fixation by photomixotrophic (A) and photoautotrophic (B) scotch broom cells in darkness.* Malate (Δ), aspartate (▲), glutamate (▼), isocitrate/citrate (∇).

were labeled at a steady rate of 1-2% of the total radioactivity. The labeled products of CO_2 fixation in the dark were malate, aspartate, isocitrate/citrate, and glutamate (Fig. 26). The labeling patterns for the products of CO_2 fixation in the light provided evidence

for two types of carboxylation: in one, phosphoglyceric acid was the primary product of CO_2 fixation, in the other, malate was the primary product.

Enzyme activity related to CO_2 fixation

Labeling of C_4 acids possibly might be mediated through PEP carboxylase or PEP carboxykinase if it occurs by the addition of CO_2 to a C_3 precursor ($C_1+C_3=C_4$). Labeling in phosphoglyceric acid and other organic phosphates might take place through RuBP carboxylase. These enzymes were assayed in the green cells of tobacco and scotch broom (Table 11).

In all cells, I detected RuBP carboxylase activity comparable to the Calvin cycle activity calculated from

Table 11 *Enzyme activities related to CO_2 fixation in cultured green cells*

Cells	RuBPCase	PEPCase	PEPCK
	(μmol/mg Chl/h)		
N. tabacum			
Photomixotrophic	36	130	N.D.
Photoautotrophic	39	177	N.D.
C. scoparius			
Photomixotrophic	37	79	N.D.
Photoautotrophic	37	79	N.D.

For reaction conditions see Materials and Methods. RuBPCase, RuBP carboxylase; PEPCase, PEP carboxylase; PEPCK, PEP carboxykinase. N.D., not detected.

$^{14}CO_2$ fixation experiments which accounted for the labeling in phosphoglyceric acid, sugar mono- and di-phosphates and sucrose (Figs. 25 and 26 or Chapter VI). The activities of PEP carboxylase were two or four times higher than the activities of RuBP carboxylase, but no PEP carboxykinase activity was detected in any of the cell extracts.

DISCUSSION

The high distribution of ^{14}C radioactivity in C_4 compounds, the primary products in CO_2 fixation by scotch broom (*Cytisus scoparious* Link) and tobacco (*Nicotiana tabacum* var. Samsun) cells (Chapter VI) in the light, confirmed that cultured green cells have a special carboxylation pathway which is much more active than that in intact C_3 plants. The percentages of ^{14}C in C_4 acids (especially malate) were 44% of the total fixation in the photoautotrophic tobacco cells (Chapter VI) and 41% in photomixotrophic cells of scotch broom during the first 5 min in the light. Results of an enzymological study supported the phenomenon and indicated that this carboxylation reaction is associated with high PEP carboxylase activity. The RuBP carboxylase in the cultured cell extracts used in the present study

has about 1/10 as much activity, on a chlorophyll basis, as that in the leaf extracts of C_3 or C_4 plants. The PEP carboxylase activity of the cultured green cells was about 4 to 8 times higher than that of the leaf extracts of C_3 plants on a chlorophyll basis (78). Thus, in comparison to C_3 leaf tissue, cultured green cells show a large increase in the PEP carboxylase/RuBP carboxylase ratio. This is consistent with the higher percentage labeling found in the C_4 acids of cultured cells as compared to that normally found in C_3 leaf tissue.

Of the photomixotrophic cultures reported in the literature, *Nicotiana* (Chapter VI, 59), *Chenopodium* (38), *Portulaca* (47), *Kalanchoë* (54) and *Amaranthus* (88) also have shown high distributions of ^{14}C in the C_4 compounds produced by CO_2 fixation in the light. The physiological state of cultured cells is reported to regulate the percentage distribution of ^{14}C incorporated in C_4 compounds and the activity of PEP carboxylase during exponential cell growth in tobacco and *Chenopodium* cells (38,59). But, my results show that there was a high incorporation of $^{14}CO_2$ into malate in scotch broom and tobacco cells during stationary cell growth.

The high distribution of ^{14}C in C_4 compounds and the substantial activity of PEP carboxylase are character-

istic of C_4 photosynthesis. Pyruvate, Pi dikinase and a C_4 acid decarboxylase, NAD(P)H-malic enzyme or PEP carboxykinase, also are necessary in C_4 photosynthesis. In preliminary experiments, I detected no activity of pyruvate, Pi dikinase, but I did find high activity of NADPH-malic enzyme (200 μmol/mg Chl/hr). In the absence of pyruvate, Pi dikinase, PEP as a substrate for PEP carboxylase could be generated by glycolysis or from the phosphogylceric acid formed through RuBP carboxylase. Although malic enzyme is often considered to function as a decarboxylase, it possibly could function in carboxylation direction in cultured cells provided relatively high amounts of pyruvate and CO_2 are present.

In photoautotrophic and photomixotrophic cells of *Nicotiana tabacum* var. Samsun, the CO_2 compensation point for photosynthesis is high (87). This may reflect the low concentration of CO_2 in cultured green cells, which is due to the low carbonic anhydrase activity and of high diffusion resistance of cultured cells with large cell volume, and/or high activity of dark respiration in cultured green cells. PEP carboxylase serve to concentrate CO_2 in bundle sheath cells of C_4 plants. Pulse-chase experiments are needed to determine whether the carbon from malate can be donated to the Calvin cycle,

as in C_4 and CAM plants.

Interestingly, C_3 plants show a high incorporation of $^{14}CO_2$ into C_4 acids at a particular physiological stage. In tobacco, high CO_2 fixation and enzyme activity for the C_4 dicarboxylic acid cycle occur in the upper, younger leaf (48). In wheat and the oat, tissues surrounding the grain are capable of relatively high CO_2 fixation by PEP carboxylase (94). For colorless mutants of *Chlorella*, Miyachi et al. reported that illumination with blue light enhanced CO_2 fixation, especially the activity of the C_1-C_3 carboxylation reaction (55). They proposed that the role played by PEP carboxylase is in anaplerotic CO_2 fixation which supplies oxaloacetic acid to the TCA cycle.

In conclusion, my current and previous results (Chapter VI) indicate that photoautotrophically cultured cells fix CO_2 as phosphoglyceric acid through the Calvin cycle and C_4 acids (especially malate) through C_1-C_3 carboxylation. These green cells have substantial RuBP carboxylase and PEP carboxylase activities which could account for the products of CO_2 fixation in the light.

SUMMARY

High activities of $^{14}CO_2$ fixation into malate in the light were observed in photoautotrophically and photomixotrophically cultured cells of the C_3 plants scotch broom (*Cytisus scoparius* Link) and of tobacco (*Nicotiana tabacum* var. Samsun). The percentages of ^{14}C in the C_4 compounds (especially malate) were 44% of the total fixation in photoautotrophic cells and 41% of that in photomixotrophic cells at the end of the first 5 min.

Measurements of the activities of enzymes related to CO_2 fixation indicated that the high labeling of malate in cultured green cells was associated with high PEP carboxylase activity.

CHAPTER VIII

PHOTOAUTOTROPHIC AND PHOTOMIXOTROPHIC CULTURE OF GREEN TOBACCO CELLS IN A JAR-FERMENTER [98]

INTRODUCTION

Recently, high yield production of secondary metabolites from cell cultures has been reported (84,101, 105), and we soon will be able to use cultured cells as industrial materials in a variety of processes. But when cultured cells are used in industry, they must be cultured on a large scale in jar-fermenters or in tank cultures. Successful heterotrophic cultures of *Nicotiana tabacum* cells (43,64) and *Morinda citrifolia* cells (91) have been reported, but most mass cultures of green cells in jar-fermenters under light have not been successful. Usually, secondary metabolites are produced by cultured cells in the dark, but light is required for photosynthesis (Chapter II) and, in some cases, it stimulates secondary metabolism, e.g. essential oil production (13) and flavonoid biosynthesis (26). In addition, photoautotrophically cultured cells can grow without sugar (Chapter II, 36) and their lipid composition is similar to that of mesophyll cells (5,53,76). These cells fix

CO_2, mainly through the Calvin cycle, and have a special carboxylation pathway which is much more active than in intact C_3 plants (Chapters VI and VII). Cultured green cells, especially photoautotrophic cells, differ from heterotrophic cells cultured in the dark.

In this study I assessed the effects of the type of impeller, agitation speed, aeration rate, and gas composition on cell growth when I cultured tobacco cells photomixotrophically and photoautotrophically in jar-fermenters.

MATERIALS AND METHODS

Callus culture Tobacco (*Nicotiana tabacum* var. Samsun) stock cells were maintained photomixotrophically in modified Linsmaier-Skoog (52) basal medium with 10 μM NAA, 1 μM kinetin and 3% sucrose as described in Chapter II. Cells cultured on a rotary shaker (120 rpm) for 2 weeks at 26°C were inoculated in a jar-fermenter at an initial cell concentration of about 10 g fresh weight/liter. Incubation was at 26°C and 0.35 kg/cm^2 of pressure under 8000 lux of illumination.

Jar-fermenter The jar-fermenter used was Model MF-114, New Brunswick Scientific Co., Inc. (vessel volume 14 liters, medium 5 liters). A pH electrode and an NBS

galvanic-type dissolved oxygen elctrode were inserted into the culture vessel, then the pH and the dissolved oxygen concentration were monitored continuously. The fermenter vessel was illuminated by a semi-circular unit containing 6, 15 watt fluorescent bulbs.

Initial volumetric oxygen transfer coefficient

The initial volumetric oxygen transfer coefficient, $k_L a$, of the fermenter was determined by the method of Kato et al. (43). The 14-liter jar-fermenter was filled with 5 liters of deionized water. The dissolved oxygen in the water was removed by the addition of an excess amount of sodium sulfite with a trace amount of $CoCl_2$ as teh catalyst. When the dissolved oxygen concentration had decreased to nearly zero, the jar-fermenter was run at a predetermined agitation speed and rate of aeration. The increase in the dissolved oxygen concentration with time was measured with a galvanic type oxygen electrode to determine the $k_L a$ value at 26°C and 0.35 kg/cm^2 of pressure.

Cell growth Fresh weights of cells were determined as described in Chapter II.

Measurements of oxygen exchange by cultured cells

Oxygen exchange was measured at 20°C with a Hansatech

oxygen electrode as described in Chapter III.
Green tobacco cells (0.1 g fresh weight) were suspended in 1 ml of 50 mM phosphate buffer (pH 7.8). To measure photosynthetic oxygen evolution, I illuminated the cell suspensions with a projector through a 10-cm water layer at a light intensity of about 100,000 lux, in the presence of 5 mM bicarbonate. Respiratory oxygen uptake was measured in the dark under aerobic conditions.

RESULTS

Type of impeller, agitation speed, aeration rate and light intensity

I first assessed the effect of the type of impeller, the agitation speed and the aeration rate on the k_La, the shear stress and the mixing of the culture medium. I used two types of impeller; one was a flat blade turbine, the other was a marine impeller (Fig. 27). The flat blade turbine is the conventional impeller used in biotechnological processes. It mixed the medium well by global radial flow, but at high speed it produced a turbulent region near the impeller and chopped the cells. The marine type impeller mixed the medium gently by convection flow, and this flow washed down the vessel

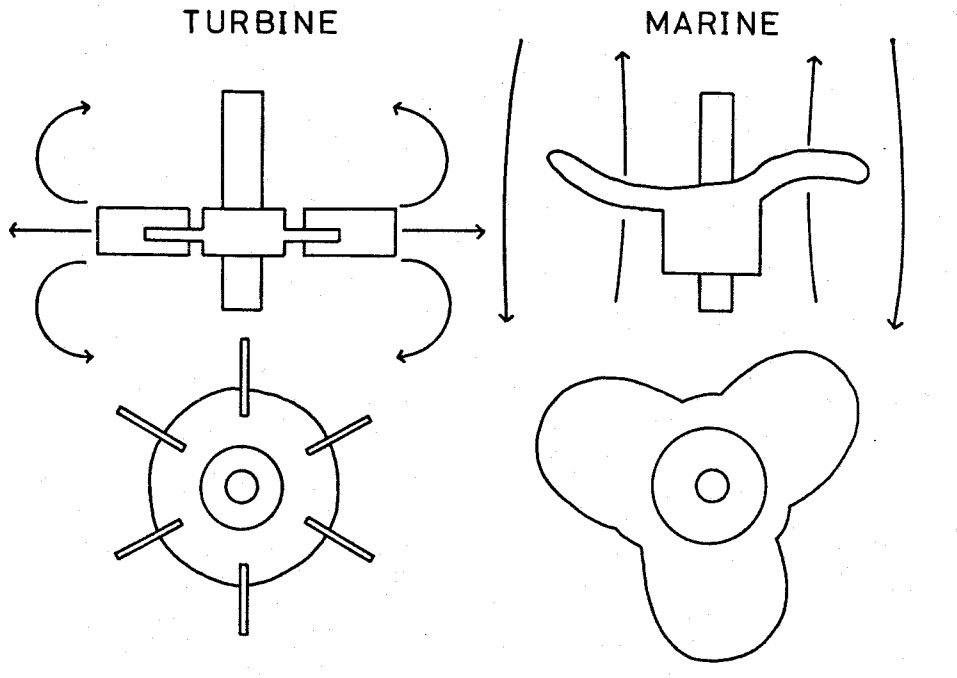


Fig. 27. *Impeller types*

wall and prevented cell growth on the vessel's surface. I judged that the marine impeller is more suitable for plant cell culture in a jar-fermenter than the turbine type.

Cell growth is affected greatly by the rate at which oxygen is supplied. The tobacco cell yield was not affected by the type of fermenter or the method of aeration but it was affected by the $k_L a$. When the supply of oxygen was limited, i.e. the $k_L a$ was set at less than 10 hr^{-1}, there was a linear relationship between the $k_L a$ and cell yield; whereas, final cell growth showed a plateau when the $k_L a$ was above 10 hr^{-1} (43). Green tobacco cells evolve and use photosynthetic oxygen under

illumination. Therefore, I considered that the $k_L a$ at 10 hr^{-1} was sufficient for the growth of photomixotrophic and photoautotrophic cultures in a jar-fermenter, and I determined the agitation speed and aeration rate needed to set the $k_L a$ at 10 hr^{-1}. With the marine type impeller, an agitation speed of 200 rpm and an aeration rate of 1 vvm was necessary to set the $k_L a$ at 10 hr^{-1}.

The growth of green tobacco cells depended on the light intensity but, above 10,000 lux, illumination reduced the green color indicative of cell growth (100). In this jar-fermenter culture, the light intensity was therefore set at about 8000 lux at the surface of the culture vessel.

Photomixotrophic culture in a jar-fermenter

The typical growth pattern of green tobacco cells cultured photomixotrophically in a jar-fermenter under an agitation of 200 rpm with a marine type impeller, an aeration of 1 vvm and an illumination of 8000 lux is shown in Fig. 28. The increase in the amount of green tobacco cells after 17 days of incubation was ten fold, and the maximum doubling time was 3.5 days. The pH in the medium decreased to 5.0 during the lag phase, but began to increase when the cells started to grow. The dissolved oxygen decreased constantly until it reached a

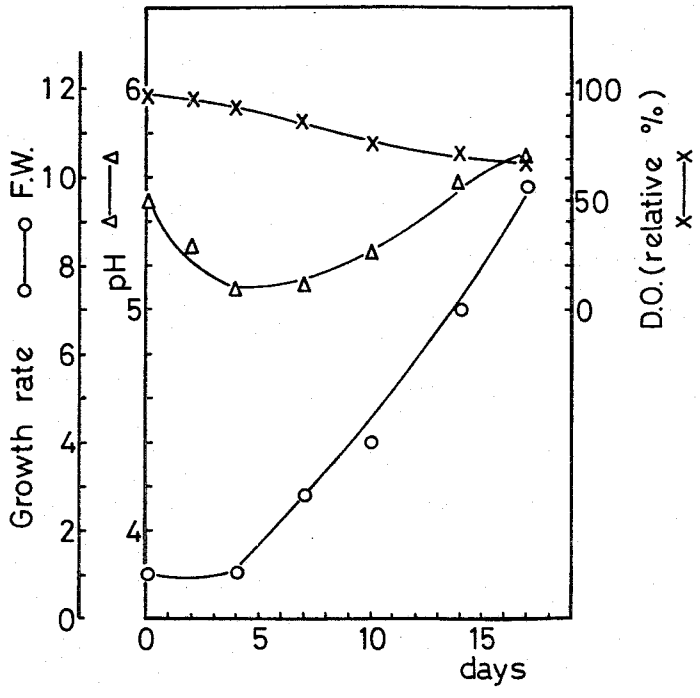


Fig. 28. *Growth kinetics of green tobacco cells cultured photomixotrophically in a jar-fermenter*
About 50 g of green tobacco cells (fresh weight) was inoculated in 5 liters of medium containing 3% sucrose. Cells were cultured at an agitation speed of 200 rpm, an aeration rate of 1 vvm, and an illumination of 8000 lux at 26°C. Increase in fresh weight (O—O), pH (Δ—Δ), Dissolved oxygen concentration (D.O.) (X—X).

final concentration that was 65% of the initial value. Respiration activity increased to 32 μmol O_2 uptake/g fresh weight/hr from 11 μmol O_2 uptake/g fresh weight/hr after 2 days of incubation, then gradually decreased to

15 μmol O_2 uptake/g fresh weight/hr, whereas no apparent photosynthetic oxygen evolution took place during the entire period of culture.

Photoautotrophic culture in a jar-fermenter

Enrichment with CO_2 is essential for photoautotrophic culture in a flask (Chapter II). Photoautotrophic culture in the jar-fermenter was made under the conditions used for photomixotrophic culture, but without sucrose and with 1% CO_2-enriched aeration. The green tobacco cells lost their color, however, and their cell mass gradually decreased.

I believe that the metabolic activity of cells cultured in a jar-fermenter differs from that of cells cultured in a flask. The respiratory activity of tobacco cells (10-30 μmol O_2 uptake/g fresh weight/hr) cultured photomixotrophically in the jar-fermenter was two to five times that (2-10 μmol O_2 uptake/g fresh weight/hr) in a flask, and these cells showed no apparent photosynthetic oxygen evolution, whereas cells cultured in a flask had high apparent photosynthetic activity (2-6 μmol O_2 evolved/g fresh weight/hr). Enhanced cell respiration compensated for, or inhibited, the photosynthetic activity of green tobacco cells in the jar-fermenter, and these cells did not grow photoautotro-

phically.

I consider that oxygen limitation was useful for decreasing cell respiration, for increasing photosynthetic activity, and for culturing cells photoautotrophically. When the aeration rate was lowered to 0.8 vvm, the partial pressure of oxygen lowered to 14% O_2 by the addition of N_2 gas, and the CO_2 concentration enriched to 1%, green tobacco cells grew photoautotrophiclly in the jar-fermenter (Fig. 29). These tobacco cells began to grow after a long lag period (about 7 days), at that time they had begun to evolve photosynthetic oxygen. When cells grew photoautotrophically, the lowered, dissolved oxygen concentration (60% of the saturated value for air) increased gradually and reached 80% of the saturated value for air. The high pH of the medium was maintained during the lag phase of the photoautotrophic culture, but a gradual decrease took place when cells began to grow.

DISCUSSION

Wagner and Vogelmann reported that cultured cells had a high apparent viscosity and sensitivity to shear stress due to the relatively large volume of rigid cells

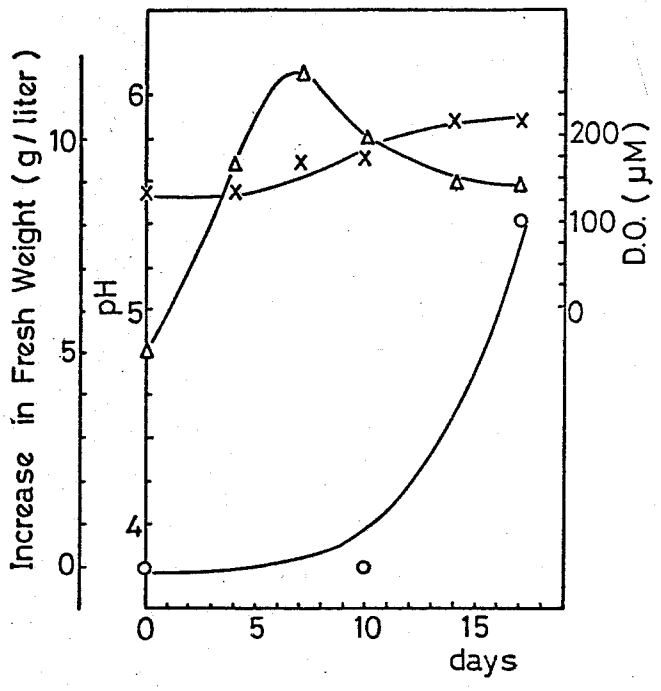


Fig. 29. *Growth kinetics of green tobacco cells cultured photoautotrophically in a jar-fermenter*
About 50 g of green tobacco cells (fresh weight) was inoculated in 5 liters of medium without sucrose. The cells were cultured at 26°C with an agitation speed of 200 rpm, an aeration rate of 0.8 vvm using air with a lowered partial pressure of oxygen (O_2 14%, N_2 85%), an enrichment of 1% CO_2, and an illumination of 8000 lux. Symbols are the same as in Fig. 28.

(91). My mixing by slow agitation with a marine type impeller and aeration led to the successful culture of green tobacco cells in a jar-fermenter. The tobacco cells grew better and more rapidly than in a flask; the maximum doubling time was 3.5 days in the jar-

fermenter and 4.5 days in a flask. No pH drop during the stationary phase, which is a critical indicator of the lysis of cells due to shear stress or to oxygen limitation, was observed in my jar-fermenter culture.

The culture conditions for photoautotrophic growth differed from those for photomixotrophic growth in the jar-fermenter. Photoautotrophic culture of the green tobacco cells in the jar-fermenter did not succeed under the same conditions with the 1% CO_2 enriched aeration used for the photomixotrophic culture because stimulated cell respiration compensated for the net photosynthesis. Green tobacco cells cultured photoautotrophically with ordinary aeration in a jar-fermenter had no apparent photosynthetic activity. I believe that the oxygen limitation decreased cell respiration and increased the apparent photosynthesis. A lowered oxygen supply was produced by reducing the rate of aeration and by using air with a partial pressure of oxygen lowered by the addition of N_2 gas; this enhanced photoautotrophic growth. Usually, cells require large amounts of oxygen to metabolize sugars during dark, heterotrophic culture in a jar-fermenter; whereas, green cells in a photoautotrophic culture can produce their own substrate for growth and their own oxygen by photosynthesis. This

is probably sufficient for their respiration. Recently, Dalton succeeded in cultivating spinach cells photoautotrophically in a continuous culture by controlling the dissolved oxygen concentration (15).

Cells cultured in a jar-fermenter probably differ from those cultured in a flask. The respiratory activity of tobacco cells cultured in a jar-fermenter was two to five times the value for cells grown in a flask, and photosynthetic activity was suppressed in the jar-fermenter. In addition, the characteristics of secondary metabolite production in *Catharanthus* cells cultured in a jar-fermenter differ from those found for cells cultured in a flask (105).

SUMMARY

Green tobacco cells were cultured photomixotrophically and photoautotrophically in a jar-fermenter (working volume, 5 liters). Optimum aeration and agitation, i.e. the type of impeller (marine), agitation speed (200 rpm), aeration rate (1 vvm) and light intensity (8000 lux) were determined in order to get high cell growth. Under these conditions, tobacco cells increased about ten fold after 17 days of photomixotrophic culture.

In the photoautotrophic culture aerated with 1% CO_2 enriched air, the mass of the tobacco cells did not increase because stimulated cell respiration compensated for photosynthetic activity. A low oxygen supply was essential for photoautotrophic culture in a jar-fermenter. The fresh weight of green tobacco cells doubled under photoautotrophy after 17 days of culture with an agitation of 200 rpm; aeration of 0.8 vvm with air containing 1% CO_2, 14% O_2 and 85% N_2; and an illumination of 8000 lux.

CONCLUSION

Photoautotrophism is the most distinctive characteristic of plant metabolism; thus the establishment of photoautotrophism in cultured cells must enhance the productivity of plant cells. I was able to establish the photoautotrophic culture of some species of plant cells. The factors essential for successful photoautotrophic culture were the selection of cell lines with high photosynthetic potentials and culture conditions favorable for photosynthesis, i.e. adequate light intensity and an enriched CO_2 concentration.

I induced my original calluses from fifteen plant species under favorable conditions for greening, i.e. in Linsmaier-Skoog medium containing NAA (10 μM) and cytokinin (BA 1 μM) in the light. The calluses induced consisted of cells that showed different degrees of green, even though they were derived from the same segments. The various plant species had different potentials for the development of chloroplasts in calluses. The greenest cells from each culture were selected for both callus induction and subcultures. Scotch broom (*Cytisus scoparius* Link), the amur cork-tree (*Phellodendron amurense* Rupr.) and tobacco (*Nicotiana tabacum* var. Samsun) cells had

relatively high chlorophyll contents (70-200 μg/g fresh weight).

Because of this, I next investigated photosynthesis in the green amur cork-tree, scotch broom and tobacco cells in photomixotrophic cultures. Samples cultured under various light intensities showed that the growth of green cells was stimulated by increases in light intensity. This stimulation depended on the chlorophyll contents of the cells; it disappeared on the addition of photosynthesis inhibitors. These phenomena indicate that photosynthesis accounts for a third to a half of cell growth under strong illumination.

These photomixotrophic cultures then were developed as photoautotrophic cultures. When these chlorophyllous cultures were aerated with CO_2 enriched air in the light, the scotch broom and tobacco cells grew photoautotrophically for long periods.

Amur cork-tree cells could not grow photoautotrophically, even though their chlorophyll contents were as high as scotch broom and tobacco cells. Measurements of photosynthetic O_2 evolution revealed very low photosynthetic activity in amur cork-tree cells. Detailed measurement of the photosynthetic activity in green scotch broom and tobacco cells shows that the photosyn-

thetic potential of these cells in photomixotrophic cultures was a sign of their ability to undergo photoautotrophic growth.

Photosynthetic activity is a good indicator of photoautotrophism; thus, I could produce a new and efficient method for establishing photoautotrophic cultures of plant cells. When leaf segments of *Atropa*, *Datura* and *Hyoscyamus* were inoculated on sugar-free, Linsmaier-Skoog medium then aerated with 1% CO_2 enriched air under 3000-5000 lux, I could easily select photoautotrophic green cells from their cultures.

I improved the photoautotrophic culture medium by adding a 4-fold concentration of phosphate and lowering the auxin concentration (NAA 1 μM). This increased the photoautotrophic growth of scotch broom cells.

The photoautotrophic cells had more differentiated chloroplasts with developed grana, and assimilated carbon more effectively in light than did the photomixotrophic cells. Thus, I could next determine the labeling patterns for light and dark $^{14}CO_2$ fixation in photoautotrophically and photomixotrophically cultured scotch broom and tobacco cells.

Both photoautotrophically and photomixotrophically cultured green cells mainly fix CO_2 through the Calvin cycle, and they have a special carboxylation pathway

which is much more active than that of intact plant cells. A high degree of $^{14}CO_2$ fixation into malate in the light was observed in both photoautotrophically and photomixotrophically cultured cells of C_3 plants. Dark fixation could not account for this high $^{14}CO_2$ incorporation into C_4 compounds. Measurements of the enzyme activities related to CO_2 fixation indicated that the high labeling of C_4 compounds in cultured green cells was associated with high PEP carboxylase activity. I concluded that photoautotrophic cells are not the same as mesophyll cells, even though the fine structure of their chloroplasts and their lipid composition are similar to those of mesophyll cells.

When cultured cells are used in industrial processes, they must be cultured on a large scale. Optimum aeration and agitation, i.e. the type of impeller (marine), agitation speed (200 rpm), aeration rate (1 vvm) and light intensity (8000 lux) were determined in order to obtain good cell growth in the photomixotrophic cultures. Under these optimum conditions, tobacco cells increased their volume about ten fold after 17 days of photomixotrophic culture, and the maximum doubling time in the jar-fermenter was shorter than that in a flask. For photoautotrophic culture, a low oxygen supply with CO_2 enrichment was essential in the jar-fermenter procedure.

One of the most important subjects scientists need to understand is the mechanism of gene expression and how to control it. I could not regulate the development of chloroplasts in any of my cultured cells, but I could select cells which had a desirable characteristic, photosynthetic potential, and propagate them under controlled conditions. The procedures I have used are not limited to the establishment of photoautotrophic cultures. I believe they can be effectively applied to other fields such as the production of secondary metabolites.

ACKNOWLEDGMENTS

I am grateful to Dr. Eiichi Takahashi, Professor of Kyoto University, for his guidance and encouragement during this work. I also appreciate the continuous guidance and advice of Dr. Yasuyuki Yamada, Associate Professor of Kyoto University, which enabled me to carry out this investigation. Thanks for guidance and advice also go to Dr. Koji Asada, Associate Professor of The Research Institute for Food Science, Kyoto University, Dr. Kojiro Nishida, Professor of Kanazawa University, and Dr. Katsumi Ueda, Professor of Nara Women's University. In addition, I wish to thank the members of the Laboratory of Plant Nutrition, Department of Agricultural Chemistry, Kyoto University, especially Drs. Takeshi Yasuda and Toru Kuboi, Mr. Kazumitsu Imaizumi, Mr. Katsumi Watanabe, Mr. Takashi Hashimoto, Mr. Naruhiko Nakagawa, Mr. Tadashi Tanio, Miss Tomoko Shiomi, Miss Michiko Kato and Miss Yukie Tanaka for their valuable discussions and encouragement and Mrs. P. Yamada for proofreading this manuscript.

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