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Growth and Photosynthetic Characteristics of *Solanum tuberosum* Plantlets Cultivated *in Vitro* in Different Conditions of Aeration, Sucrose Supply, and CO_2 Enrichment

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ABSTRACT

Growth characteristics, oxygen exchange, and carbohydrate and chlorophyll contents were determined 30 days after subculturing of single node-derived plantlets of *Solanum tuberosum* cv Haig cultivated *in vitro*. Cultivation conditions were: (a) photomixotrophy in closed vessel, (b) photomixotrophy in closed vessel on medium supplemented with silver thiosulfate, (c) photomixotrophy in aerated vessel, (d) photoautotrophy in air, (e) photoautotrophy in CO_2-enriched air. In photomixotrophic conditions, aeration of the vessel enhanced sucrose utilization and had a positive effect on plantlet growth. In photoautotrophic conditions, growth of the plantlets was slow in air and was strongly enhanced by CO_2 enrichment of the atmosphere. Starch to sucrose ratios were higher in plants grown photoautotrophically than in plants grown with sucrose in the medium. Oxygen exchange characteristics on a chlorophyll basis were similar between the plantlets when measured under moderate light, and resembled those of greenhouse plant leaves. In high light, however, plantlets grown photoautotrophically in a CO_2-enriched atmosphere had higher oxygen exchange rates. We concluded from these results that potato plantlets *in vitro* in conditions (c), (d), and (e) developed C3-plant photosynthetic characteristics, which were in photoautotrophically grown plantlets comparable to those of field-grown plants.

The confinement of the atmosphere during cultivation of plantlets *in vitro* in closed vessels can lead to dramatic modifications of the gaseous composition. Volatile products such as ethylene can accumulate and inhibit plant growth and development of photosynthetic abilities (5). In photosynthetically active cultures grown photomixotrophically under a light/dark regime in closed vessels, CO_2 accumulates in the dark and is consumed in the light. Thus, in the long term, CO_2 can reach very high concentrations when photosynthetic CO_2 fixation does not balance the respiratory losses, and depletion of O_2 can occur. Or, depending on the photosynthetic activity of the plantlets, CO_2 concentration can decline to the compensation point during the light period (11): in this case, the carbon gain from CO_2 fixation is limited by sucrose utilization activity and can be very small. Depletion or excess of CO_2 may also cause deleterious effects on plantlets *in vitro* and could be a cause of growth abnormalities during the *in vitro* stage or during acclimatization.

One can expect that achievement of photoautotrophic plant growth or aeration in photomixotrophic culture systems will improve *in vitro* cultivation efficiency, as well as plantlet behavior (8, 14). Nevertheless, information is scarce on the effects of such culture conditions on plantlet physiology. We therefore investigated how culture conditions, *i.e.* different carbon sources (photomixotrophy and photoautotrophy at two CO_2 levels) and confinement status (photomixotrophy with or without gas exchange with the atmosphere), could affect the growth, photosynthetic characteristics, and carbon metabolism of white potato (*Solanum tuberosum*) plantlets cultivated *in vitro*.

MATERIALS AND METHODS

Plant Material

Stock plantlets of *Solanum tuberosum* cv Haig were routinely subcultured from stem axillary buds on a medium containing Murashige and Skoog (18) salts, 15 g liter^{-1} sucrose, without phytohormones (19). Light was supplied by fluorescent tubes (Grolux and Coolwhite 1/1) at a photon flux density of 60 μE m^{-2} s^{-1} at the vessel periphery. The temperature was 25°C in dark and light periods, and the photoperiod was 18 h light/6 h dark.

Growth Conditions

In the experiments, vessel volume was 500 mL, and each vessel contained 150 mL of medium. Plantlets were cultivated *in vitro* in the same environmental conditions as stock plantlets. The following growth conditions were compared. (a) Closed vessel + sucrose: the vessels were sealed with an airtight cap; the medium contained 15 g Liter^{-1} sucrose. (b) Closed vessel + sucrose + STS[1]: conditions were as in (a) but STS was added to a final concentration of 2 mg liter^{-1} in order to prevent ethylene production (22). (c) Aerated vessel + sucrose: the vessels were sealed with an air-permeable cap in polyurethane foam; the medium contained 15 g liter^{-1} sucrose. (d)

[1] Abbreviations: STS, silver thiosulfate; E, gross photosynthesis; U, O_2 uptake in the light; P, net photosynthesis.

Photoautotrophy + air: the vessels were flushed with hydrated air containing 340 μL liter^{-1} CO_2 at a flow rate of 5 liters h^{-1} through a Millipore 0.2-μm filter; the medium contained no sucrose. (e) Photoautotrophy + CO_2: the vessels were flushed with hydrated air containing 20,000 μL liter^{-1} CO_2 at a flow rate of 5 liters h^{-1} through a Millipore 0.2-μm filter; the medium contained no sucrose.

Sampling

Five replica vessels were used for each growth condition, and each vessel contained five plants. After 30 d of cultivation, one plantlet from each vessel was used for dry weight measurement, another for Chl determination, and the three remaining plantlets for sugar content determination. Fresh weight, height of aerial parts, and leaf number of each plant were measured.

Analysis

Chl *a* and *b* were extracted in 80/20 (v/v) acetone/water mixture and determined according to the method of Lichtenthaler and Wellburn (16).

Carbohydrate contents were determined enzymatically. The plantlets were ground in liquid nitrogen and incubated in 0.5 M NaOH for 30 min at 4°C. The samples were neutralized using 5 N HCl and were centrifuged for 5 min at 6000 rpm; the supernatant was used for glucose, fructose, and sucrose determination. Glucose and fructose concentrations were measured successively in pH 7.6 ethanolamine buffer (1, 2). To determine sucrose content, an aliquot (500 μL) was incubated with β-fructosidase for 25 min at room temperature in 1 mL of pH 4.6 citrate buffer and then analyzed following the same protocol that was followed for glucose and fructose measurements. Determination of starch was carried out by glucose determination after α-amylase digestion of the extract in pH 4.6 citrate buffer for 30 min at 40°C (3). Enzymes were supplied by Boehringer Mannheim.

Statistical analysis (Student-Newmann-Keuls multiple comparison test) of the results was performed using the computer program VOYONS (26).

Gas Exchange Measurements

O_2 exchange rates of plantlets were determined following the $^{18}O_2$ technique described previously (6): U was calculated from the rate of decrease of the isotopic tracer $^{18}O_2$:

$$U = -\frac{d(^{18}O_2)}{dt} \times \frac{^{16}O_2 + {}^{18}O_2}{^{18}O_2}$$

E was then measured as the rate of $^{16}O_2$ production (coming from cell water photolysis):

$$E = \frac{d(^{16}O_2)}{dt} + \frac{^{16}O_2}{^{16}O_2 + {}^{18}O_2} \times U$$

P was then calculated as the difference between E and U:

$$P = E - U$$

A single focusing magnetic mass spectrometer (model MM-8-80; VG Instruments) was used. The setup described in Dimon *et al.* (6) was modified as follows. The culture vessel was connected to the gas circuit by means of ultra-torr union (Cajon) and immersed in a thermoregulated water bath (25°C). Total volume was 834 mL, and 5 mL $^{18}O_2$ were added before measurements. Light was supplied by four 150-W Mazda "Cool beam" lamps, allowing a PPFD range from 0 to 2000 μE m^{-2} s^{-1}. O_2 exchange rates were measured for periods of 30 min at various CO_2 concentrations and PPFD.

RESULTS AND DISCUSSION

Growth Characteristics

The growth characteristics of potato plantlets grown in the five cultivation conditions were compared. Concerning plant height (Table I), photoautotrophy + CO_2 treatment produced the tallest plantlets. Those grown in the aerated vessel + sucrose condition were smaller but still vigorous. Plantlets grown in closed vessel + sucrose, closed vessel + sucrose + STS, and photoautotrophy + air were small and fragile. These three height classes were statistically discriminated ($P < 0.05$).

The dry weight of the plantlets was affected by culture conditions (Table I) in the same manner as height. The stimulation of growth observed at 20,000 μL liter^{-1} CO_2 was

Table I. *Growth Characteristics of Plantlets in Vitro*

Height and dry weight of *S. tuberosum* plantlets grown under various conditions of culture vessel aeration, sucrose supply, and CO_2 enrichment. Values followed by the same letter are not significantly different (height: $n = 25$, dry weight: $n = 5$, $P < 0.05$). Means ± SE are indicated.

Treatment	Height	Dry Weight
	mm	*mg*
Photoautotrophy + CO_2	81.31 ± 18.53 (a)	52.90 ± 22.56 (a)
Aerated vessel + sucrose	55.00 ± 11.60 (b)	29.30 ± 9.03 (b)
Photoautotrophy + air	35.83 ± 24.13 (c)	9.30 ± 7.17 (c)
Closed vessel + sucrose + STS	32.37 ± 14.77 (c)	7.12 ± 2.41 (c)
Closed vessel + sucrose	31.10 ± 12.33 (c)	5.76 ± 2.68 (c)

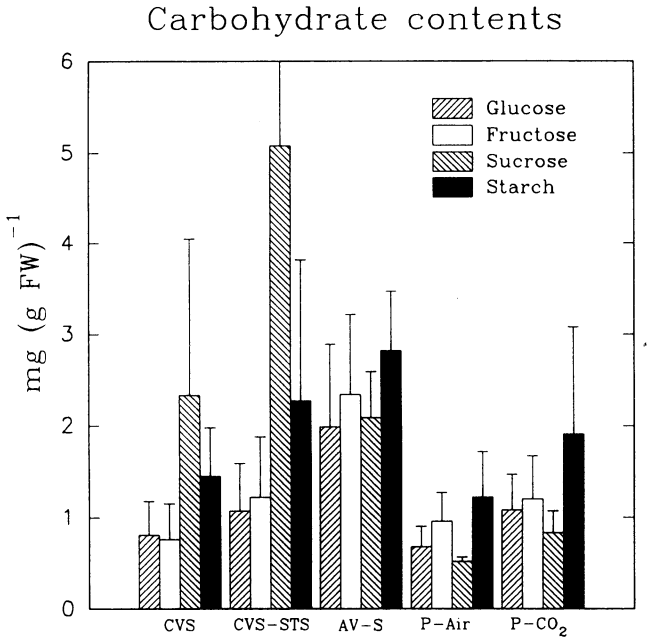

Figure 1. Carbohydrate contents of plantlets of *S. tuberosum* grown *in vitro* under closed vessel + sucrose (CVS), closed vessel + sucrose + STS (CVS-STS), aerated vessel + sucrose (AV-S), photoautotrophy + air (P-Air), and photoautotrophy + CO2 (P-CO_2) conditions. Glucose, fructose, and sucrose contents are expressed in mg·(g fresh weight)$^{-1}$, starch contents are expressed in (mg glucose equivalent)·(g fresh weight)$^{-1}$. Results are shown with SE bars.

considerably stronger than reported by Mousseau (17) or by Kozai and Iwanami (13) at lower enrichment levels (1000 μL liter^{-1}). This strong stimulation is in agreement with recent observations suggesting that photosynthetically over-saturating concentrations of CO_2 could considerably accelerate plantlet growth, at least in the first stages of photoautotrophic cultivation (FX Cote, personal communication).

The number of leaves per plantlet was slightly affected by the different treatments, except for the closed vessel + sucrose treatment (data not shown), where several plants produced numerous but only partially developed leaves, which is most likely a result of ethylene accumulation (10, 22).

Based on the hypothesis that differences between closed vessel + sucrose and closed vessel + sucrose + STS conditions were the consequences of ethylene regime, we found that ethylene affected morphogenesis and weight, but less so than observed by De Proft *et al.* (5) or by Perl *et al.* (22). Indeed, the fact that growth was limited in closed vessels even in the presence of STS in comparison to aerated vessel + sucrose suggested that either STS had a toxic effect or that other confinement constraints had acted. It has been observed that ethanol or acetaldehyde could accumulate in closed vessels (25) and possibly inhibit plant growth. Dry weight of plantlets grown in aerated vessels in our conditions was much higher than dry weight of plantlets grown in closed vessels or photoautotrophically in air. These results support the assumption of Kozai *et al.* (15) that the beneficial effect of vessel aeration on plant growth in mixotrophic conditions is the consequence of a better utilization efficiency of medium sucrose allowed by suppression of confinement constraints, rather than of the atmospheric carbon supply.

Carbohydrates

The contents in glucose and fructose were higher in plantlets grown in the aerated vessel + sucrose treatment than in all (Fig. 1). Sucrose content was much higher in plantlets cultured in photomixotrophic conditions than in plantlets cultured in photoautotrophic conditions. Starch content of the plantlets was higher in the aerated vessel + sucrose condition than in all other treatments. (All differences stated above were statistically significant at $P < 0.05$.)

The high contents of glucose, fructose, and starch in plantlets from aerated vessel + sucrose suggest that their rates of sucrose incorporation and utilization were higher than those of plants grown in closed vessel conditions, although one can assume that photosynthetic activity would result in a preferential production of starch. The starch/sucrose ratio varied between the treatments. Based on this criterion, the growth conditions were divided into three groups, discriminated in a highly significant way ($P < 0.01$). In confined conditions—closed vessel + sucrose and closed vessel + sucrose + STS—all the fixed carbon originated from the medium sucrose; there, a low starch to sucrose ratio was observed (respectively, 0.46 and 0.77). In aerated vessel + sucrose, *i.e.* photomixotrophic treatment, where potential carbon sources were both medium sucrose and atmospheric CO_2 (17), the starch to sucrose ratio of plantlets was higher (1.39). In photoautotrophy + air and photoautotrophy + CO_2, *i.e.* photoautotrophic treatments, where all the carbon assimilated came from atmospheric CO_2, the starch to sucrose ratio was the highest (2.19 and 2.77). It is worth noting that the values of this ratio in the photoautotrophic treatments were comparable to those measured in leaves of greenhouse-grown potato plants (2.1 when measured at noon in mature leaves; our unpublished data).

Chl

The lowest Chl content based on fresh weight was observed in closed vessel + sucrose plantlets (Table II). STS addition, or vessel aeration, led to higher Chl contents. The Chl *a*/*b* ratio was significantly lower in photoautotrophy + CO_2 treatment than in all.

Chl contents of *in vitro* plantlets (except in closed vessel +

Table II. *Chl Content*

Total Chl content and Chl *a* to Chl *b* ratio of *S. tuberosum* plantlets grown under various conditions of culture vessel aeration, sucrose supply, and CO_2 enrichment. Values followed by the same letter are not significantly different ($n = 5$, $P < 0.05$). Means ± SE are indicated.

Treatment	Chl	Chl *a*/*b* Ratio
	mg (g fresh wt)$^{-1}$	
Aerated vessel + sucrose	1.33 ± 0.25 (a)	3.86 ± 0.44 (a)
Photoautotrophy + air	1.01 ± 0.11 (a)	3.95 ± 0.71 (a)
Photoautotrophy + CO_2	0.95 ± 0.23 (a)	3.15 ± 0.32 (b)
Closed vessel + sucrose + STS	0.92 ± 0.35 (ab)	3.73 ± 0.88 (a)
Closed vessel + sucrose	0.70 ± 0.21 (b)	4.04 ± 0.85 (a)

sucrose treatment) and Chl *a* to Chl *b* ratios were in the same range as those determined in greenhouse plants (our unpublished data) or in leaves sampled in agricultural conditions (20).

O_2 Exchange Characteristics

To determine gas exchange of plantlets (when total plant weight in the vessel was about 1 g), vessels from aerated vessel + sucrose, photoautotrophy + air, and photoautotrophy + CO_2 treatments were sealed and connected to the gas exchange measurement device (see "Materials and Methods").

To determine the responses to variations in light and CO_2, O_2 exchange was measured at 230 and 1000 μE m^{-2} s^{-1} PPFD: (a) at the CO_2 compensation point, (b) in air containing 340 μL $liter^{-1}$ CO_2 (atmospheric concentration), and (c) at saturating CO_2 concentration (about 10,000 μL $liter^{-1}$). Average values from three gas exchange measurements under each condition are shown in Figure 2.

When CO_2 concentration increased, net photosynthesis (Fig. 2A) was stimulated, whereas O_2 uptake in the light (Fig. 2B) was inhibited. The same effect was observed under all growth conditions. Such a response is characteristic of C3 plants (4, 9) and can readily be explained by the kinetic properties of Rubisco. It is remarkable that the rates of O_2 exchange expressed on a Chl basis were in the same range, at atmospheric CO_2 concentration, as those of potato leaves sampled in the greenhouse (our unpublished data).

Whatever the growth conditions of plantlets, high light levels stimulated gross photosynthesis (Fig. 2C), O_2 uptake in the light, as well as net photosynthesis. The stimulation was the highest for photoautotrophy + CO_2 plantlets. This would suggest that their photochemistry is better adapted to high light levels (Chl *a* to Chl *b* ratio is different, suggesting differences in light harvesting complex and PSII densities) or they are less limited by sink capacity by growing faster than plantlets from the two other conditions, or both.

In all plantlets, at saturating CO_2 concentration, which is known to inhibit photorespiration, O_2 uptake in the light was stimulated by light increase. The appreciable rate of O_2 fixation at saturating CO_2 may include both photoinduced O_2 fixation (Mehler-type reactions) and respiration. Based on the finding that O_2 uptake in the light at saturating CO_2 was similar to the O_2 uptake measured during a dark period after the illumination (0.23 μmol O_2 min^{-1} [mg Chl]$^{-1}$ in aerated vessel + sucrose plantlets, 0.38 in photoautotrophy + air, and 0.51 in photoautotrophy + CO_2), one can argue that most of the measured O_2 uptake was due to mitochondrial respiration. Assuming this, the fact that U at saturating CO_2 increased proportionally with net photosynthesis when light increased can be explained by an increased availability of substrates for respiration. *In vitro* potato plantlets seemed to maintain a respiratory activity in the light that was linked to the rate of carbon fixation. These results could be an argument in the controversy about the persistence of mitochondrial respiration in the light in higher plants, as observed in algae (21).

E declined when the CO_2 concentration approached the CO_2 compensation point. Possible reasons for this decline are: a deficiency in terminal electron acceptors, because oxygenase activity of Rubisco is too low to completely balance CO_2

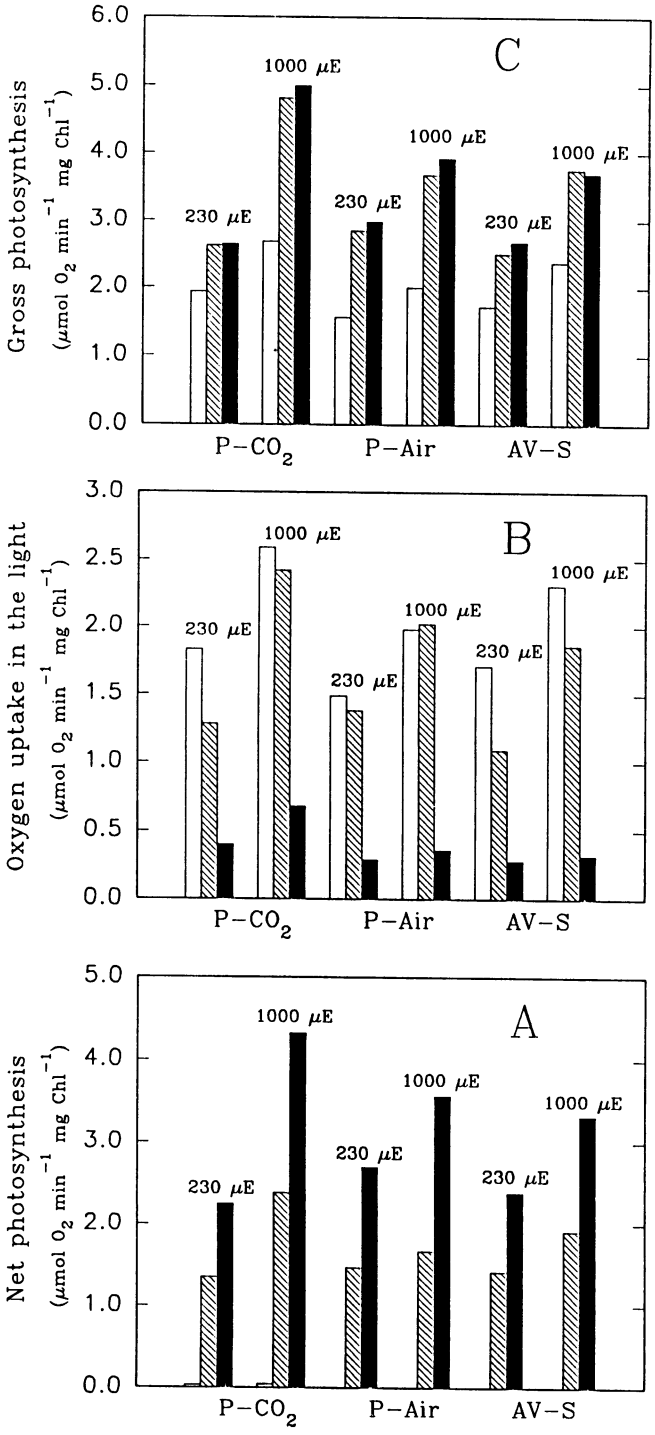

Figure 2. P, U and E of plantlets grown *in vitro* under aerated vessel + sucrose (AV-S), photoautotrophy + air (P-Air), and photoautotrophy + CO_2 (P-CO_2) conditions, measured at 230 μE m^{-2} s^{-1} and at 1000 μE m^{-2} s^{-1} PPFD, at three CO_2 concentrations: the CO_2 compensation point (□), 340 μL $liter^{-1}$ (▨), and 10,000 μL $liter^{-1}$ (■).

Table III. *U/P Ratios*

U/P ratios of *S. tuberosum* plantlets grown *in vitro* on aerated vessel + sucrose, or photoautotrophically in air with or without CO_2 enrichment, measured at 340 μL liter^{-1} and at 10,000 μL liter^{-1} CO_2 concentration, under 230 μE m^{-2} s^{-1} and 1,000 μE m^{-2} s^{-1} PPFD.

CO_2 Concentration	PPFD	Treatment		
		Photoautotrophy + CO_2	Photoautotrophy + air	Aerated vessel + sucrose
	$\mu E\ m^{-2}\ s^{-1}$		*U/P ratio*	
340 μL liter^{-1}	230	0.95	0.94	0.77
	1,000	0.92	1.02	0.89
10,000 μL liter^{-1}	230	0.14	0.11	0.12
	1,000	0.15	0.11	0.10

depletion, and a low activation state of Rubisco due to the lack of CO_2 (27). The observation that, in low light, relative depression of E at the compensation point was less than in high light was again in agreement with results obtained with whole plants (4) and justifies the conclusion that *in vitro* plants seem to fix atmospheric CO_2 by the C3 pathway irrespective of the growth condition.

The result that U/P ratios (Table III) under a given light regime at 340 μL liter^{-1} CO_2 were similar in the three types of plantlets can be attributed to similarity in CO_2 to O_2 ratio at the Rubisco catalytic site (28). The observation that U/P was constant over the light range, whereas P increased, indicated that leaf diffusive resistance to CO_2 changed in response to light so that internal CO_2 concentration should be maintained; this result is not in agreement with the general assumption that stomata of plantlets *in vitro* may be poorly functional (24).

The CO_2 compensation point (Table IV) was the highest for plantlets grown under high CO_2. Its level in C3 plants depends on the kinetics of Rubisco and on the rate of nonphotosynthetic CO_2 exchange (7). Using clonal material, we can assume that Rubisco kinetic properties were similar. This result is another indication that higher respiratory gas exchange occurs in photoautotrophy + CO_2 plantlets. High light increased the CO_2 compensation point of all plants, perhaps because of increased leaf temperature, which could stimulate respiration and lower CO_2 specificity of Rubisco (12).

We observed that O_2 exchange rates decreased during long exposure to high light in plantlets grown in photomixotrophic conditions, but not in photoautotrophic plantlets. One possible explanation is that photomixotrophic plantlets have a low capability of dissipation of excess energy.

Table IV. *CO_2 Compensation Point (μL liter^{-1})*

CO_2 compensation point of *S. tuberosum* plantlets grown *in vitro* on aerated vessel + sucrose, or photoautotrophically in air with or without CO_2 enrichment, measured at two PPFD conditions: 230 and 1000 μE m^{-2} s^{-1}. Values followed by the same letter are not significantly different ($n = 5$, $P < 0.05$).

Treatment	PPFD (μE m^{-2} s^{-1})	
	230	1000
	CO_2 compensation point	
Photoautotrophy + CO_2	65 (a)	52 (b)
Photoautotrophy + air	56 (b)	49 (c)
Aerated vessel + sucrose	52 (b)	45 (c)

CONCLUSION

Development of photosynthetic function appeared to be fully achieved in photoautotrophic and aerated photomixotrophic treatments. This contradicts the results of Pospísilová *et al.* (23) obtained on potato plantlets grown *in vitro*.

As observed in other studies (13, 17), allowance of gas exchange and photosynthetic CO_2 fixation proved to be beneficial to potato growth and vigor *in vitro*. Photoautotrophy, however, brought better growth than photomixotrophy only when air was CO_2-enriched. The strong enhancement of growth by CO_2 enrichment on photoautotrophic plantlets *in vitro* could explain the ability of such plants to better cope with high rates of photosynthesis in high light.

Starch synthesis occurred in all conditions. The starch to sucrose ratio was strongly affected by the relative contribution of photosynthetic CO_2 fixation to growth, *i.e.* the balance between heterotrophic and autotrophic carbon metabolism, and, in photomixotrophic conditions, by the rate of sucrose utilization.

The characteristics of photoautotrophic plantlets resembled those of field grown plants, and therefore make them usable as model systems to study plant behavior in controlled conditions or to initiate the precocious selection of plant varieties on photosynthesis linked criteria.

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