The Effects of Carbon Dioxide Enrichment, Natural Ventilation, and Light Intensity on Growth, Photosynthesis, and Transpiration of Cauliflower Plantlets Cultured in vitro Photoautotrophically and Photomixotrophically

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ABSTRACT. The effects of natural ventilation and CO₂ enrichment during the rooting stage on the growth and the rates of photosynthesis and transpiration of in vitro cauliflower (*Brassica oleracea* L.) plantlets were investigated. In vitro plantlets were established in airtight or ventilated vessels with or without CO₂ supplied ($\approx 1200 \mu\text{g}\cdot\text{L}^{-1}$) through gas permeable films attached to the vessel's cap for 15 days before transplanting ex vitro. Leaves generated in vitro in ventilated vessels had a higher photosynthetic rate than those produced in airtight vessels, which lead to greater leaf expansion and shoot and root dry matter accumulation during in vitro culture and acclimatization. Enhanced photosynthesis in leaves of ventilated plantlets was positively correlated with chlorophyll content. Increasing photosynthetically active radiation from 70 to 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ enhanced the growth of in vitro plantlets under ventilated conditions but it depressed photosynthesis of the leaves grown photomixotrophically with sugar and CO₂ enrichment which might be due to the feedback inhibition caused by marked accumulations of sucrose and starch. Higher CO₂ levels during in vitro culture enhanced photosynthesis under photoautotrophic conditions, but inhibited it under photomixotrophic conditions. Fifteen days after transplanting ex vitro, high photosynthetic ability and stomatal resistance to transpiratory water loss of ventilated plantlets in vitro had important contributions to rooting and acclimatization. Our findings show that the ventilated culture is effective for accelerating photoautotrophic growth of plantlets by increasing photosynthesis, suggesting that, especially for plantlets growing in vitro without sugar, CO₂ enrichment may be necessary to enhance photosynthetic ability.

Micropropagated plantlets face inevitable changes in the environment from aseptic and heterotrophic conditions to dry and autotrophic conditions. The most serious problem is low survival of the micropropagated plantlets after transfer to ex vitro conditions. Survival during acclimatization ex vitro can be achieved by strict control of the environment (Kozai et al., 1995) or by use of antitranspirants (Sutter and Hutzell, 1984; Voyiatzis and McGranahan, 1994).

Easy acclimatization depends primarily on the rate of root formation and establishment of autotrophic growth. Leaves of in vitro cultured plantlets are characterized by a thin cuticle (Grout, 1975), stomatal malfunction (Sallanon et al., 1993; Sutter, 1988), and low photosynthetic rate (Grout, 1988), which result in reduced growth (Preece and Sutter, 1991). The photosynthetic capability and stomatal regulation of transpiration of in vitro generated leaves are important factors influencing the successful acclimatization of in vitro plantlets. Low photosynthetic rate of in vitro plantlets is caused by low atmospheric CO₂ levels in culture vessels (Mousseau 1986; Sallanon et al., 1995), low irradiance (Kozai et al., 1990; Sallanon et al., 1995), and high sugar content of the medium (Capellades et al., 1990; Hdider and Desjardins, 1994). Several methods have been tried to promote autotrophic growth in vitro to improve acclimatization: increasing the aeration of the vessel

(Kubota and Kozai, 1992), increasing the light intensity (Lees, 1994), changing the lighting cycle (Hayashi et al., 1995), providing sideward lighting (Kozai et al., 1992), and supplying CO₂ into culture vessels (Figueira and Janick, 1994; Kirdmanee et al., 1995).

Cauliflower plantlets transferred to the acclimatization stage have been shown to have too low a photosynthetic rate to achieve normal growth (Abe et al., 1995; Grout and Aston, 1978). Recently, we studied in vitro preacclimatization of photomixotrophic cauliflower plantlets in natural ventilation at a high ($130 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) photosynthetically active radiation (PAR) and showed an enhanced rate of photosynthesis before transferring ex vitro (Abe et al., 1995). Although CO₂ enrichment and supplemental lighting at the rooting stage of micropropagation stimulated growth and photosynthesis of in vitro plantlets, there was little information relating physiological aspects to which enhanced ability of photosynthesis might be attributed. Also, there is limited information relating these physiological aspects to low photosynthesis of the micropropagated plantlets (Desjardins, 1995; Hdider and Desjardins, 1994). However, they did describe how in vitro culture conditions, especially sucrose in the medium affected plantlets sink–source relationships, activity of carboxylating enzymes, and the developmental state of the photosynthetic apparatus.

Objectives of the present study were to investigate the effects of sucrose, ventilation, CO₂ enrichment and light intensity on growth, photosynthesis, and transpiration of cauliflower plantlets during the preacclimatization before transplanting ex vitro. In addition, chlorophyll and carbohydrate (sucrose and starch) content in leaves were measured to evaluate the effects of the different culture conditions on the development of photosynthetic capability.

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Materials and Methods

PLANT MATERIAL AND CULTURE CONDITIONS. Five mm long stem tips with cotyledons were aseptically dissected from cauliflower seedlings (*Brassica oleracea* L. 'Snow New-Dia') grown on 0.1 g·L⁻¹ commercial nutrient medium (6.5N-6P-19K, Hyponex Japan, Osaka, Japan) with 20 g·L⁻¹ sucrose and 8 g·L⁻¹ agar (Nacalai tesque, Kyoto, Japan). Each stem tip was transplanted to 25 mL of one-half strength hormone-free MS medium (Murashige and Skoog, 1962) solidified with 8 g·L⁻¹ agar. The medium pH was adjusted to pH 5.7 before autoclaving. Half of cultures were grown autotrophically on sucrose-free medium and another half of them were grown heterotrophically on medium with 20 g·L⁻¹ sucrose. Cultures were maintained in airtight glass vessels (200 mL capacity, 105 mm high, 50 mm in diameter) in a temperature-controlled cabinet (100 cm wide, 60 cm deep, 60 cm high) at 25 °C and a 16-h photoperiod provided by cool white fluorescent lamps supplying 55 μmol·m⁻²·s⁻¹ PAR at culture level.

After 3 weeks of culture, plantlets with three new leaves and roots were assigned to experimental treatments. For half of the vessels, airtight caps were aseptically replaced with ventilated caps that allowed gas exchange with the surrounding environment through two gas permeable Teflon membranes (Milli-Seal, Millipore, Tokyo, Japan, 10 mm in diameter and 0.5 μm pore size) covering two holes on each of the caps. Carbon dioxide enrichment was initiated by flushing pure CO₂ into the cabinet in which all cultures were placed through a low-density polyethylene CO₂ diffusive tube (Sativa Research Institute, Kobe, Japan) connected to a pressure regulator and a compressed supply of pure CO₂. Two concentrations of CO₂ were supplied, 1200 μg·L⁻¹ for CO₂ enrichment and 360 μg·L⁻¹ for natural ventilation during the 15 d of treatments at low (70 μmol·m⁻²·s⁻¹) and high (200 μmol·m⁻²·s⁻¹) PAR with a 16-h photoperiod. CO₂ concentration in the cabinet was continually monitored with an infrared gas analyzer (ASSA-1600 system, Horiba, Kyoto, Japan).

GROWTH AND GAS EXCHANGE MEASUREMENTS. Growth characteristics, including shoot and root dry mass and total leaf area, were measured at the start (day 0) and at the end (day 15) of treatments and 15 d following ex vitro transplanting. Acclimatization was done in the same temperature and humidity (40% RH) controlled cabinet, in which all cultures had been placed, at 130 μmol·m⁻²·s⁻¹ PAR without any covering, except that plantlets were given a 24-h dark treatment for the first day of acclimatization. As an ex vitro grown control, seedlings, which had the same number of leaves as in vitro plantlets, were grown on vermiculite irrigated with 1 g·L⁻¹ of the same commercial nutrient solution as mentioned above under the same conditions as for acclimatization.

The rates of photosynthesis of newly-expanded leaves were measured at end of the treatment using a leaf disc chamber equipped with a gas phase oxygen electrode (LD2, Hansatech, Norfolk, England), maintained at 25 °C. The carbon dioxide concentration inside the chamber was ≈50,000 μL·L⁻¹, which was a sufficiently high concentration to overcome the stomatal limitation as a diffusion barrier against CO₂ uptake because of the typically thin cuticle layers on the thinner epidermis of in vitro generated leaves. The light-saturated rates of photosynthesis were recorded following 5 min preillumination at 1200 μmol·m⁻²·s⁻¹ PAR. The rates of transpiration and stomatal diffusive resistance of the leaves were measured with a portable steady-state diffusion porometer (LI-1600, LI-COR, Lincoln, Nebr.)

CHLOROPHYLL, SUCROSE, AND STARCH CONTENTS. After measuring photosynthesis, leaf segments were analyzed for chlorophyll, sucrose and starch. Chlorophyll was extracted by grinding

fresh leaves in precooled 80% acetone and was quantified spectrophotometrically according to the method of Arnon (1949).

For quantification of sucrose and starch, leaves were frozen in liquid N₂ immediately after measurement of photosynthesis and ground quickly with a precooled mortar and pestle in 2 mL of 80% (v/v) ethanol in water. The extraction was completed by incubating the ground leaves at 40 °C for 18 h. Extracts were centrifuged at 14,000 g_n for 10 min and the supernatant was used for sucrose determination and the pellet for starch determination. One milliliter of the supernatant was mixed with equal volumes of chloroform and distilled water, to remove pigments and the mixed supernatant was centrifuged at 2,500 g_n for 5 min. One milliliter of the noncolored supernatant was added to 1 mL of 1 N NaOH and incubated at 80 °C for 1 h in a shaking water bath to degrade free fructose. After cooling to room temperature, the sample was mixed with 3 mL of 12.2 N HCl and 1 mL of 0.05 % (w/v) resorcinol then incubated at 80 °C for 10 min followed by rapid cooling to room temperature. Absorbance at 420 nm was used for samples and sucrose standards to estimate the concentration. For starch determination, the pellet was dried at 55 °C, then 0.7 mL distilled water was added, and samples were capped and boiled for 1 h. Samples were cooled and 1 mL of 0.2 M acetate buffer (pH 4.5) containing 5 units of amyloglucosidase (Sigma A7420; Sigma Chemical Co., St. Louis) was added. The mixture was incubated in a shaking waterbath at 55 °C for 24 h, samples were centrifuged at 14,000 g_n for 10 min. Glucose concentration in the supernatant was determined by the mutarotase-glucose-oxidase assay procedure (Glucose CII-test; Waco Pure Chemical Industries Ltd., Osaka, Japan).

STATISTICAL ANALYSIS. Data were evaluated by analysis of variance using the Statistical Analytical Systems (SAS, Institute, Cary, N.C.) with a three factorial design (photomixotrophic or photoautotrophic culture, two levels of PAR, and three types of the vessel ventilation). For each experiment we measured five to ten replications per treatment, and each experiment was conducted at least twice.

Results

GROWTH PARAMETERS. The growth of in vitro plantlets was promoted under photomixotrophic conditions compared with photoautotrophic conditions and largely improved by ventilation and CO₂ enrichment in the culture vessels for 15 days (Table 1). For the plantlets grown on the medium with sucrose and CO₂ enrichment at high PAR, the increased shoot and root dry mass and total leaf area was 380%, 390%, and 85%, respectively, compared with photomixotrophic plantlets in airtight conditions. Shoot and root dry mass and total leaf area of photomixotrophic plantlets at high PAR with CO₂ enrichment were ≈68%, 56%, and 34%, respectively, of ex vitro grown seedlings. The interaction between gaseous environment and sucrose was significant for shoot and root dry mass, so the rates of increase by ventilation or CO₂ enrichment were higher in the plantlets cultured under autotrophic conditions than under heterotrophic conditions, but these plantlets were smaller than photomixotrophic plantlets. The plantlets in airtight vessels lacking sucrose showed almost no growth and eventually died after transplanting ex vitro, demonstrating the requirement for a carbon source for the establishment of photoautotrophic in vitro plantlets grown under airtight conditions. Also, PAR enhanced dry matter accumulation under ventilated and CO₂-enriched conditions. For the plantlets cultured on the sucrose-free medium, CO₂ enrichment decreased growth compared to ventilated conditions at low PAR but high PAR overcame that decrease, indicating a significant CO₂ × PAR interaction for leaf area or dry mass of shoots and roots.

During 15 d ex vitro acclimatization (Table 2), shoot and root dry weight and total leaf area increased ≈ 2 -fold in the plantlets which had been ventilated or CO₂-enriched before transplanting, compared to plantlets grown on medium with sucrose in airtight vessels. Leaf area and shoot dry mass showed a significant CO₂ $\times$ sucrose interaction, so CO₂-enrichment under autotrophic conditions increased shoot growth during acclimatization, but under heterotrophic conditions it decreased growth after transplanting ex vitro. The percentage of plantlets that survived through acclimatization was 100% except the plantlets that had been grown on sucrose-free medium under airtight conditions and showed almost no growth with loss of leaf area due to abscission during 15 d acclimatization.

PHOTOSYNTHESIS. The rate of photosynthesis of in vitro generated leaves was significantly improved by ventilating the vessels and by adding sucrose into medium (Table 1). The potential activity was the highest in the leaves from plantlets grown on the naturally ventilated medium with sucrose. The OER was 21 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (or $\approx 85\%$ of the ex vitro control leaves; 25 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). When the plantlets were grown in the CO₂-enriched environment, enhanced photosynthesis was observed in photoautotrophic conditions but not in photomixotrophic conditions. Carbon dioxide enrichment in combination with the medium containing sucrose significantly depressed the potential activity of photosynthesis of in vitro leaves developed at high PAR. Increasing PAR during preacclimatization in vitro had little effect on the rate of photosynthesis regardless of ventilated or CO₂-enriched conditions but had a negative effect on photosynthesis under airtight conditions

lacking sucrose and under CO₂-enriched conditions with sucrose added to the medium.

CHLOROPHYLL AND CARBOHYDRATE CONTENT. Seedlings contained 0.64 g·m⁻² chlorophyll in leaves. Chlorophyll content of the pre-acclimatized leaves was enhanced in plantlets grown in the ventilated or CO₂-enriched and heterotrophic culture environment and was $\approx 90\%$ of seedling leaves (Table 1). There was a slight increase in chlorophyll content of leaves formed at high PAR compared with it at low PAR except the leaves formed under airtight conditions lacking sucrose and ventilated plantlets on sucrose-containing medium. No significant difference in chlorophyll content was observed between leaves formed with or without CO₂ during culture. A significant positive correlation between photosynthesis and chlorophyll content (Fig. 1) was observed in the leaves of plantlets grown photoautotrophically ($r = 0.82^{**}$, $p = 0.01$). A significant positive correlation was also observed in leaves of photomixotrophic plantlets when CO₂-enriched samples were excluded ($r = 0.76^{**}$, $p = 0.01$) (Fig. 1).

We also analyzed the content of sucrose and starch in the preacclimatized leaves to investigate the physiological basis of those accumulation balances which might affect photosynthesis (Table 1). Both carbohydrates accumulated to a lesser extent in the airtight-grown leaves than in the ex vitro grown leaves but increased significantly in the CO₂-enriched conditions. The increase in both carbohydrates were further stimulated by increasing PAR and by adding sucrose to the medium during the preacclimatization culture period. The synthesis of starch was preferentially stimulated compared with the synthesis of sucrose, resulting in a sucrose

Table 1. Growth parameters (total leaf area, dry mass of shoots and roots) of cauliflower plantlets, oxygen evolution rate (OER), and contents of chlorophyll, sucrose, and starch of the leaves developed during preacclimatization in vitro at two levels of irradiance: 70 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, low photosynthetically active radiation (PAR), and 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, high PAR.

Culture conditions (preacclimatization)	Irradiance level	Leaf area (cm ²)	Shoot dry mass (mg)	Root dry mass (mg)	OER ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	Chlorophyll (g·m ⁻²)	Sucrose (g·m ⁻²)	Starch (g·m ⁻²)
Photomixotrophic (2% sucrose)								
Airtight	Low	18.5	63.8	8.9	10.6	0.36	0.45	0.40
	High	15.0	62.2	7.3	12.3	0.41	0.56	0.32
Ventilated ^z	Low	35.9	156.2	20.2	21.6	0.54	---	---
	High	31.8	171.8	38.0	20.6	0.54	---	---
CO ₂ enriched	Low	27.1	167.0	27.0	11.5	0.59	0.84	2.04
	High	27.8	299.1	36.0	6.1	0.62	1.82	16.36
Photoautotrophic								
Airtight	Low	6.8	13.0	0.9	5.6	0.39	0.09	0.26
	High	6.4	11.3	0.7	-1.3	0.23	0.10	0.30
Ventilated	Low	31.7	68.6	11.0	12.4	0.44	---	---
	High	31.2	100.6	17.3	14.1	0.51	---	---
CO ₂ enriched	Low	15.9	35.9	3.9	14.6	0.50	0.44	0.61
	High	25.3	128.3	18.0	18.0	0.52	0.92	9.03
Seedlings grown on vermiculite ^x		82.9	438.8	64.5	24.8	0.64	3.06	2.84
ANOVA								
Sucrose (S)		***	***	***	***	***	***	***
Gaseous environment (GE)		***	***	***	***	***	***	***
PAR		NS	***	***	NS	NS	***	***
S $\times$ GE		NS	***	***	***	NS	***	***
S $\times$ PAR		*	NS	NS	NS	NS	***	***
GE $\times$ PAR		**	***	***	NS	NS	NS	***
S $\times$ GE $\times$ PAR		NS	**	*	***	*	NS	**

^zGas exchange treatments were done through gas-permeable Teflon membranes covering holes on the vessel's cap (see Materials and Methods).

^yNot determined.

^xAs an ex vitro-grown control, seedlings that had five leaves (same as in vitro plantlets) were grown under the same conditions as for acclimatization.

NS, *, **, *** Nonsignificant or significant at $P = 0.05$, 0.01, or 0.001, respectively.

Table 2. Growth parameters (total leaf area, dry mass of shoots and roots) of cauliflower plantlets after 15 d acclimatization ex vitro.

Culture conditions (preacclimatization)	Irradiance	Leaf area (cm ²)	Shoot dry mass (mg)	Root dry mass (mg)
Photomixotrophic (2% sucrose)				
Airtight	Low	33.2	186.0	31.8
	High	28.5	178.7	31.6
Ventilated ^z	Low	68.2	421.8	82.3
	High	58.3	379.7	74.7
CO ₂ enriched	Low	58.4	302.0	74.7
	High	43.5	272.6	56.9
Photoautotrophic				
Airtight	Low	3.5	18.8	4.4
	High	3.0	16.4	3.4
Ventilated	Low	37.1	157.6	72.6
	High	46.7	212.6	76.3
CO ₂ enriched	Low	71.9	307.4	52.4
	High	72.2	369.0	62.0
ANOVA				
Sucrose (S)		**	***	***
Gaseous environment (GE)		***	***	***
PAR		NS	NS	NS
S × GE		***	***	NS
S × PAR		*	*	NS
GE × PAR		NS	NS	NS
S × GE × PAR		NS	NS	NS

NS, **, ***, **** Nonsignificant or significant at $P = 0.05, 0.01, \text{ or } 0.001$, respectively.

: starch ratio of 0.1 : 1 in leaves grown at high PAR. Leaves from ex vitro grown seedlings had a sucrose to starch ratio of 1.1.

TRANSPIRATION AND STOMATAL DIFFUSIVE RESISTANCE. At the time that preacclimatized in vitro leaves grown under photoautotrophic conditions were transferred to ex vitro conditions, the transpiration rate was 4.0 and 3.7 times higher in the naturally ventilated and the CO₂-enriched leaves, respectively, than in leaves of seedlings grown on vermiculite (Fig. 2). Fifteen days after transplanting to ex vitro conditions the rates decreased but were still higher than that of the seedlings. Addition of sucrose to the medium significantly depressed the rate of transpiration of in vitro and ex vitro-grown plantlets. Fifteen days after transplanting to ex vitro conditions the rate of transpiration in plantlets, which had been grown with or without CO₂ enrichment under in vitro photomixotrophic culture conditions, the rates decreased to a value close to that of the seedlings that coincided with significant increases in stomatal resistance. PAR had no effect on transpiration or stomatal resistance across the other treatments.

Discussion

Natural ventilation during the rooting stage in vitro was very advantageous to the subsequent growth of cauliflower plantlets ex vitro. Leaf expansion and dry matter accumulation occurred in the 15 d during the in vitro preacclimatization on medium lacking sucrose, suggesting that promotion of photoautotrophic growth could stimulate rooting ex vitro. This enhanced effect on growth during preacclimatization in vitro was more remarkable at high PAR and under photoautotrophic conditions than at low PAR and under photomixotrophic conditions. Similar results were also observed during the preacclimatization of potato (Kubota and Kozai, 1992) and *Eucalyptus* (Kirdmanee et al., 1995).

Photosynthesis is the energy and carbon source for active growth under photoautotrophic conditions. Carbon fixation by

leaves developed in vitro is usually low and insufficient to sustain autotrophic growth after transfer to soil (Preece and Sutter, 1991). Light saturation for photosynthesis by most in vitro plantlets is relatively low; PAR below 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and is susceptible to photoinhibition at high PAR (Lee et al., 1985; Serret et al., 1996). Increasing PAR during the rooting stage of in vitro plantlets improved photosynthetic activity of *Cymbidium* (Kozai et al., 1990) and *Hosta* and *Daphne* (Lees, 1994). In the present study with cauliflower plantlets, increasing PAR from 70 to 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 15 d did not significantly stimulate photosynthesis. Similar results have been reported with *Juglans regia* (Sallanon et al., 1995),

Fig. 1. Relationship between oxygen evolution rate (OER) and chlorophyll content of the in vitro leaves grown under different conditions: airtight ($\blacktriangle$, $\triangle$), ventilated ($\bullet$, $\circ$, 360 $\mu\text{g}\cdot\text{L}^{-1}$ CO₂), and CO₂ enrichment ($\blacksquare$, $\square$, 1200 $\mu\text{g}\cdot\text{L}^{-1}$ CO₂) with or without 2% sucrose in the medium at different levels of photosynthetically active radiation (closed symbols, 70; open symbols, 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Seedlings as ex vitro control were shown as $\star$. **Significant correlation at $p = 0.01$ calculated from data excluding samples from the CO₂-enriched treatment (circled by dots) for plantlets grown photomixotrophically.

Clematis (Lees, 1994), and *Gardenia* (Serret et al., 1996). The in vitro generated leaves of cauliflower plantlets grown under typical culture conditions, which were airtight at low PAR and included sucrose in the medium, had about half the photosynthetic potential of vermiculite-grown seedlings. However, the in vitro leaves grown at high PAR under airtight conditions on sucrose-free medium had negative photosynthesis, which might have resulted from photoinhibition as reported in *Clematis* plantlets cultured at high PAR (Lees, 1994) and in *Gardenia* (Serret et al., 1996).

In general, micropropagated plantlets grow photomixotrophically on a medium with sugar, so they need not photosynthesize actively in the culture vessels. In agreement with Lees (1994), however, the development of photosynthetic capability during the rooting stage seems to be a limiting factor for easy acclimatization after transfer to soil. Sucrose in the medium has great influences on both development and rate of photosynthesis (Hdider and Desjardins, 1994; Lees et al., 1991). In tissue-cultured plantlets of *Rosa* (Capellades et al., 1991), the presence of sucrose in the medium under CO₂-enriched conditions led to considerable accumulation of starch in the leaves like the cauliflower plantlets in the present study. Some physiological details of photosynthesis in micropropagated plantlets were described by Desjardins (1995)

who hypothesized that sugar in the culture medium was probably the most important factor for depressing photosynthesis in vitro by leading to the down-regulation or feedback inhibition of ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco). He explained that this feedback inhibition of the Calvin cycle by sucrose accumulation was attributed to an increase in ribulose 1,5-bisphosphate concentration affected by a low level of inorganic phosphate and active starch formation in the chloroplasts. In vitro cauliflower leaves which were grown with sucrose under CO₂-enriched conditions at high PAR had significantly reduced photosynthesis. High CO₂ and high PAR depress photosynthesis in *J. regia* plantlets due to low activity of rubisco (Sallanon et al., 1995). There were positive effects of elevated CO₂ on photosynthesis and acclimatization of *Eucalyptus* during in vitro culture (Kirdmanee et al., 1995), dry matter gain for *Chrysanthemum* (Cuello et al., 1992), and photosynthesis for cacao (Figueira and Janick, 1994) and *Homalomena* (Matysiak and Nowak, 1994). The effect of CO₂ enrichment was clearly more effective during photoautotrophic growth than in photomixotrophic growth (Tichá, 1996). These promotive effects were enhanced by increased PAR for most of these species. The simultaneous increase in both light intensity and CO₂ concentration during in vitro culture generally improve in vitro photosynthetic activity (Kozai et al., 1995). Our results also showed both positive effects of increasing PAR and CO₂ enrichment on photosynthesis of the cauliflower plantlets grown on the sucrose-free medium.

An increase in chlorophyll in the leaves formed in vitro under ventilated conditions might cause improved photosynthesis because of a positive correlation between chlorophyll content and photosynthesis. In cauliflower plantlets, therefore, chlorophyll content was considered to be an important factor in determining photosynthetic ability in agreement with Abe et al. (1995) and Grout and Aston (1978), so the photosynthetic activity will become high in the leaves containing more chlorophyll. These findings suggest that the low photosynthetic rate of in vitro plantlets is partly due to decreases in Hill activity (Grout and Aston, 1978) and quantum efficiency (Lees, 1994) in leaves containing insufficient chlorophyll for active photosynthesis, which can be improved by removing plantlets from airtight growth conditions.

In vitro produced leaves have no structured epicuticular wax and nonfunctional stomata, and fail to control excessive water loss from the plantlets after transfer to ex vitro conditions (Sallanon et al., 1993). Cauliflower plantlets with poorly developed epicuticular wax apparently cannot survive under the dry conditions outside vessels, as reported by Grout and Aston (1978), who also reported that in cauliflower plantlets stomatal malfunction was a major cause of the low survival due to an excessive loss of water from plantlets at transfer from in vitro to ex vitro conditions. During 15 d of acclimatization, stomatal regulation was evident as indicated by decreasing transpiration and increasing stomatal resistance of the ventilated and the CO₂-enriched plantlets.

The results presented in this study indicate that ventilated culture led to an increase chlorophyll content and enhancement of photosynthesis of leaves formed in vitro, which improved growth of plantlets in vitro and during subsequent acclimatization after transplanting ex vitro, thus leading to greater success of acclimatization. Furthermore, CO₂ enrichment had additional positive effects on the growth and photosynthesis of plantlets grown under photoautotrophic conditions, but had negative effects on photosynthesis of plantlets grown under photomixotrophic conditions.

Fig. 2. Transpiration rate and stomatal diffusive resistance (R_s) of leaves from plantlets grown under different conditions: ventilated (360 $\mu\text{g}\cdot\text{L}^{-1}$ CO₂) and CO₂ enriched (1200 $\mu\text{g}\cdot\text{L}^{-1}$ CO₂) with (+S) or without (-S) 2% sucrose in the medium at different levels of photosynthetically active radiation (solid bars 70 and shaded bars 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Measurements were done at the time of transplant to ex vitro conditions and at 15 d post-transplant. Leaves grown under airtight conditions could not be measured due to a quick wilting with excessive transpiration. Vertical bars indicate $\pm$ SE of 10 measurements. The black bar indicates seedlings as the ex vitro control.

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