PHOTOSYNTHETICA 37 (1): 53-59, 1999

Developmental and photosynthetic characteristics of a photoautotrophic *Chrysanthemum* culture

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Abstract

Chrysanthemum plantlets were cultivated *in vitro* on media with 2.0, 0.3, or 0 % sucrose, or photoautotrophically without an organic carbon source but with supplementation of the culture vessel atmosphere with 2 % CO_2. The photoautotrophically cultivated plantlets showed a better growth and multiplication, higher contents of chlorophyll (Chl) and carotenoids, higher Chl *a/b* ratio, net photosynthetic rate and ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase activities than plantlets grown on the medium with sucrose.

Additional key words: carotenoids; chlorophyll; *in vitro* CO_2 supply; net photosynthetic rate; phosphoenolpyruvate carboxylase; photosynthetic pigments; ribulose-1,5-bisphosphate carboxylase; suncaps.

Introduction

From a photosynthetic point of view, *in vitro* active cultures represent a step forward in the field of plant cultures. The theoretical and practical applications of these culture types are multiple (Kozai *et al.* 1992, Widholm 1992, 1995, Hüsemann 1995, Pospíšilová *et al.* 1997). To regard the specific environmental conditions, classical *in vitro* cultures are heterotrophic or photomixotrophic. However, at present, the capacity of plantlets to photosynthesise *in vitro* has been proved in several species: sweetgum, tobacco, rose, potato, strawberry (*e.g.*, Pospíšilová *et al.* 1987, Tichá *et al.* 1988, Kozai *et al.* 1992, Desjardins *et al.* 1995).

The achievement of photosynthetically active aseptic cultures is important for the obtaining of an increased efficiency in plantlets acclimatisation (Fujiwara *et al.* 1988, Kozai *et al.* 1995, Van Huylenbroeck and De Riek 1995, Van Huylenbroeck and Deberg 1996).

Received 11 March 1999, *accepted* 25 August 1999.

Abbreviations: Car – carotenoids; Chl – chlorophyll; FM – fresh mass; P_G – gross photosynthesis; P_N – net photosynthetic oxygen release rate; PEPC – phosphoenolpyruvate carboxylase; R_D – dark respiration rate; RuBPC – ribulose-1,5-bisphosphate carboxylase.

Acknowledgements: The authors are grateful to Prof. Nicoletta Rascio for her valuable advice and help. This work was supported by a grant from the University of Padova.

The photosynthetically micropropagated plantlets generally require the supplementation of culture vessel atmosphere with CO_2 (*e.g.*, Kozai 1991, Debergh *et al.* 1992, Solárová and Pospíšilová 1997). The closure systems of the classical *in vitro* culture vessels are as tight as possible, in order to prevent infections favoured by the presence of sucrose in the culture medium. This is why this gas disequilibrium is created, which inhibits photosynthesis. The use of suncaps for the closure of vessels allows gas exchange with the exterior atmosphere enriched in CO_2, but prevents from infecting.

The present work aimed to induce photoautrophy in *Chrysanthemum* plantlets cultivated *in vitro* and to study them from the point of view of some developmental and physiological parameters.

Materials and methods

Plants and conditions of growth: The *Chrysanthemum morifolium* Ramat. apical cuttings were taken from plantlets developed *in vitro* and maintained for 22 d under 16 h photoperiod. The irradiance was 250 μmol m^{-2} s^{-1}. The Murashige and Skoog (1962) culture medium contained 1 g m^{-3} α-naphtaleneacetic acid, 0.5 g m^{-3} kinetin, and 7 kg m^{-3} agar. Three experimental variants were without CO_2 supplementation of the culture vessel atmosphere, with 2.0, 0.3, or 0 % sucrose in the culture medium. The first variant represented the control, *i.e.*, classical *in vitro* culture conditions. The fourth variant consisted of a culture medium without sucrose but with supplying of the culture vessel atmosphere with 2 % CO_2. High CO_2 partial pressures were established using a system similar to Tichá (1996). These culture vessels were closed with suncaps. The vessels of the three other variants were closed hermetically with polyethylene film. The development of plantlets, *i.e.*, their growth, multiplication, organogenesis and rhizogenesis, was studied.

Total pigment analysis: Total Chl and Car from leaf tissues were analysed in a double array spectrophotometer (model *124, Perkin Elmer*, Norwalk, CT, USA) after extraction with N,N-dimethylformamide. The extinction coefficients proposed by Wellburn (1994) were used to determine the pigment concentrations.

Leaf/environment oxygen exchanges (oxygen release or uptake) were measured with an oxygen electrode (*YSI* model *53, Yellow Spring Instruments*, Ohio, USA) on small pieces of leaf tissues according to Ishii *et al.* (1977).

RuBPC (ribulose-1-5-bisphosphate carboxylase) activity was determined according to Di Marco and Tricoli (1983). The spectrophotometric method is based on the reduction of D-3-phosphoglycerate formed in the carboxylase reaction. Changes in absorbance were measured at 340 nm.

PEPC (phosphoenolpyruvate carboxylase) activity was determined spectrophotometrically according to Usuda *et al.* (1984). The method measures a change in absorbance at 340 nm as a result of reduction of oxaloacetate to malate.

Results

The photoautotrophically cultivated *Chrysanthemum* plantlets grew and developed better than those cultivated on a 2.0, 0.3, or 0 % sucrose medium without CO_2 (Table 1). The reduction of sucrose concentration from 2.0 to 0.3 % caused a 2.5-fold reduction in the stem growth and a 2-fold reduction in the number of the newly formed leaves (all values were compared to the control ones). The plantlets grown photoautrophically showed a 2.8-fold growth and 1.7-times as many leaves compared to the control. The highest percentage of inocula generating new plantlets appeared in the photoautrophic culture as well. The rhizogenesis occurred significantly in 67 % of the initial inoculate in photoautotrophic conditions.

Table 1. The effect of different sucrose and CO_2 concentrations on the developmental and photosynthetic parameters of *Chrysanthemum* plantlets, 22 d after the inoculation. Car - carotenoids; Chl - chlorophyll; P_G - gross photosynthetic rate; P_N - net photosynthetic rate; PEPC – phosphoenolpyruvate carboxylase; R_D – dark respiration rate; RuBPC – ribulose-1,5-bisphosphate carboxylase. Means from 15 plants, with standard deviations.

Parameter	Sucrose [%]			
	2.0 % $-CO_2$	0.3 % $-CO_2$	0 % $-CO_2$	0 % $+CO_2$
Plant growth [mm]	8.00±1.56	3.16±2.64	0.91±1.16	22.5±3.5
Leaf number	4.8±1.2	2.5±1.0	0.6±0.5	8.2±1.2
Root number per inoculum	0	0	0.08	0.67
Regenerated plantlets per inoculum	0.33	0.17	0	0.67
Chl *a+b* [mg kg^{-1}(FM)]	1069	531	143	1519
Chl *a/b*	2.05	2.21	1.75	2.76
Car [mg kg^{-1}(FM)]	240±10	123±12	39±25	301±13
P_N [μmol(O_2) kg^{-1}(FM) s^{-1}]	2.55±0.55	6.67±0.83	2.17±0.75	8.95±0.61
P_G [μmol(O_2) kg^{-1}(FM) s^{-1}]	4.91	8.34	3.06	11.03
R_D [μmol(O_2) kg^{-1}(FM) s^{-1}]	2.66±0.20	1.67±0.27	0.89±0.32	2.08±0.16
P_N/R_D	0.91	4.21	2.43	4.72
RuBPC [μmol(CO_2) kg^{-1}(FM) s^{-1}]	0.48±0.05	0.42±0.04	0.08±0.04	1.61±0.03
PEPC [% of control]	100.0	180.6	70.9	145.2

Similar influence of the different experimental variants occurred in the contents of Chl *a+b* and Car (Table 1). By reducing the sucrose concentration from 2.0 to 0.3 %, the pigment contents decreased two-times, and, in photoautotrophic cultures, they increased 1.4-times. The Chl *a/b* ratio was 2.76 in the leaves of photoautotrophic plantlets and 2.05 in those developed on the 2.0 % sucrose medium.

Reduction in sucrose concentration from 2.0 to 0.3 % increased the net photosynthetic rate (P_N) by 2.6-times, and the presence of CO_2 and the absence of carbon organic source from the medium caused a 3.5-fold increase in this parameter (Table 1). Dark respiration rate (R_D) was lower in all variants compared to the control one. In plantlets developed on the medium with 0.3 % sucrose and in those developed under

photoautrophic conditions, the gross photosynthetic rate (P_G) was 1.7- and 2.2-times higher than in the control ones.

The RuBPC activity under photoautrophic conditions was 3.3-times higher than that of the control, and the PEPC activity was 1.8-times higher in the case of sucrose reduction from 2.0 to 0.3 % and 1.4-times higher in photoautotrophic conditions (Table 1).

Discussion

As found in other studies (Cristea *et al.* 1999), the photoautotrophically cultivated *Chrysanthemum* plantlets show a different ultrastructure than those cultivated on 2.0, 0.3, or 0 % sucrose medium without CO_2.

The best growth, multiplication, and photosynthetic activity occur in photoautotrophically cultivated plantlets, *i.e.*, in the culture medium without sucrose and with CO_2 supply, as confirmed by Desjardins *et al.* (1995), Cristea *et al.* (1996), and Tichá (1996). We showed that *Chrysanthemum* plantlets cultivated in vessels normally closed with polyethylene film, which does not allow gas exchange, on a medium without sucrose did not grow and develop. Therefore CO_2 supply allows, in the absence of a carbon organic source, a development similar to that of plants under natural conditions. This photoautotrophic development is superior, from the point of view of all studied parameters, to the other variants, including classical cultures *in vitro*.

The decrease in sucrose concentration without the CO_2 supplementation inhibited the growth and development of the plantlets and caused a reduction of 50 % of Chl content. P_N is enhanced under *in vitro* photoautotrophic conditions, as found by Langford and Wainwright (1987) for rose plants and by Fujiwara *et al.* (1992) for potato cultures, and as demonstrated by our results. However, Fujiwara *et al.* (1992) found a higher growth of the plantlets at a sucrose concentration of 1.5 % than of 2.5 %. We found that the decrease of sucrose concentration in the culture medium from 2.0 to 0.3 % did not allow a good plantlets growth even if the amount of photosynthetic O_2 production was increased. Shimada *et al.* (1988) and Tanaka *et al.* (1991a,b) studied the development of *Chrysanthemum* plantlets grown in vessels with an atmosphere supplemented with 0.04 % CO_2 on media with 3 or 0 % sucrose, where the variable factor was the oxygen concentration of 5, 10, 15, or 21 %. P_N, RuBPC activity and content were significantly higher in plantlets grown on the medium without sucrose than on the 3 % sucrose medium, but the Chl and soluble protein contents were not higher. We found a good development of plantlets with a significant increase in CO_2 concentration even on the medium without sucrose.

The RuBPC activity is higher in photoautotrophically developed plantlets that in control plantlets, as demonstrated for other species by Goldstein and Widholm (1990) or Tanaka *et al.* (1991b). Hdider and Desjardins (1994a), by adding sucrose to the medium of strawberry plantlets initially grown on a medium without sucrose, induced a decrease in P_N. However, by measuring the catalytic turnover rate of the enzyme and the activation state of RuBPC, they suggested that the P_N inhibition was a consequence of the reduction in RuBPC efficiency due to deactivation. According

to Desjardins *et al.* (1995), under uncontrolled environmental conditions the CO_2 exhaustion in the culture vessels or the feedback inhibition of the Calvin cycle by exogenous sucrose lead to RuBPC deactivation.

Hdider and Desjardins (1994b) have confirmed that the presence of sucrose stimulated the CO_2 fixation mediated by PEPC. They correlated the presence of sucrose in the culture medium with the enhancement of the respiratory activity in *in vitro* plantlets. In our experiments the R_D was directly proportional to the sucrose amount in the culture medium. Unlike in the control, where the photosynthetic/respiratory O_2 ratio was almost 1, in photoautotrophic plantlets this ratio significantly increased.

In a closed space with equilibrium between the two O_2 types, a development on photosynthetic basis is not possible, therefore these cultures are photomixotrophic or photoheterotrophic. Moreover, because the photosynthetic O_2 released by the plantlets developed without the CO_2 supply on media containing 2 or 0 % sucrose was similar, a weak contribution of photosynthesis at the development of classical *in vitro* cultures was evident.

Hence we conclude that *Chrysanthemum* plantlets cultivated *in vitro* on media without an organic carbon source but with a supply of CO_2 have a photoautotrophic development with all the studied parameters superior to those of the plantlets developed in other experimental conditions.

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