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CO_2 dynamics and growth in photoautotrophic and photomixotrophic apple cultures

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Abstract

The daily dynamics of CO_2 concentration in the culture vessels and the photoautotrophic or photomixotrophic growth capacity of apple (*Malus pumila* hybrid MM 106 *paradisiaca* × Northern Spy) cultures were studied. The photoautotrophic cultures were grown on a sugar-free growth medium and submitted (0S+CO_2) or not (0S-CO_2) to periodic injections of exogenous CO_2. The photomixotrophic cultures were grown in the presence of 30 g dm^{-3} sucrose, with (30S+CO_2) or without (30S-CO_2) CO_2 enrichment. The photosynthetic photon flux density applied was of 210 ± 5 μmol m^{-2} s^{-1}. In the 0S-CO_2 treatment, CO_2 showed rather uniform and narrow light-dark fluctuations throughout the culturing cycle. In the 30S-CO_2 treatment, the daily ratio between CO_2 produced during the dark period and that uptaken during the following light period, was almost always above 1 with the only exception of a few days (from the 5th to the 9th day) when the amount of photosynthesised CO_2 was equal to or higher than that produced during dark respiration. The 0S+CO_2 cultures needed to be enriched all days with exogenous CO_2 to avoid periods of gas deficiency while in 30S+CO_2 the CO_2 injected the first culturing day was uptaken over 5 d; thereafter, daily injections were necessary. Culture fresh and dry mass, number of newly formed shoots and number of nodes per shoot in 0S+CO_2 treatment did not statistically differ from the values obtained with 30S-CO_2. The highest growth was observed in 30S+CO_2 treatment. The increase in culture fresh mass due to 1 μmol of CO_2 added to the culture vessels was 1.54 and 1.36 mg for 30S and 0S respectively, while in terms of dry mass the increase was about 2.5 times higher in the sugar-enriched treatment. CO_2 enrichment accounted for 77.3 % and 21.2 % of the final fresh mass in 0S+CO_2 and 30S+CO_2, respectively.

Additional key words: CO_2 enrichment, *in vitro* culture, *Malus pumila*, photosynthetic photon flux density, sucrose.

Introduction

Photoautotrophy may be induced in *in vitro* cultures by increasing irradiance and enriching the culture vessel atmosphere with CO_2 from an external source (Kozai *et al.* 1990, Kozai 1991, Fournioux and Bessis 1993, Figueira and Janick 1994). This process has shown to be improved by the removal of sucrose from the growth medium (Capellades *et al.* 1991, Deng and Donelly 1993, Hdider and Desjardins 1994, Navarro *et al.* 1994). Micropropagation procedures based on these findings were thus proposed to test the possibility of using sugar-free growth media while increasing photosynthetic activity to levels suitable for a satisfactory culture growth. The use of sugar-less media was claimed to avoid or reduce contamination and improve the acclimatation ability in micropropagated plantlets submitted to photosynthetically active conditions (Kozai 1988, Kozai and Iwanami 1988, Deng and Donelly 1993, Lucchesini *et al.* 2001). Photoautotrophy also induced important changes on leaf anatomy and ultrastructure (Serret and Trillas 2000), on stomatal density and size (Voleniková and Tichá 2001), and on some other factors such as abscisic acid and chlorophyll content and thylakoid membrane proteins (Hofman *et al.* 2002). Experiments on micropropagation of photoautotrophic cultures have been conducted on a number of species and interesting results have also been obtained with some woody plants (Predieri *et al.* 1991, Rasai *et al.* 1993, Righetti *et al.* 1993, Figueira and Janick 1994). Nevertheless,

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Abbreviations: BA - 6-benzylaminopurine; DKW - nutrient medium according to Driver and Kuniyuki (1984); DM - dry mass; FM - fresh mass; GA_3 - gibberellic acid; IBA - indole-3-butyric acid.

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knowledge today available on the behaviour of photoautotrophic cultures should not yet be considered comprehensive and further research to fully assess their applicability and advantages is still necessary.

The purpose of this work was to furtherly contribute to knowledge on the dynamics of CO_2 and on growth of MM 106 apple rootstock cultures submitted to photoautotrophic and photomixotrophic conditions.

Materials and methods

Two successive experiments with the same experimental design and environmental conditions were performed to compare culture response to photoautotrophy and photomixotrophy. They were carried out in a growth chamber at 210 ± 5 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) and 24 ± 1 °C. A 8-h photoperiod was used since in other experiments with the same apple rootstock (data not published) this photoperiod induced a culture growth very similar to those obtained with 16-h photoperiod.

The initial explants were shoot tips collected from actively proliferating shoot clusters of apple (*Malus pumila* hybrid *paradisiaca* × Northern Spy) rootstock MM 106 grown at irradiance of 50 ± 5 μmol m^{-2} s^{-1}, 8-h photoperiod and on DKW (Driver and Kuniyuki 1984) medium, supplemented with 2 mg dm^{-3} 6-benzylaminopurine (BA), 0.2 mg dm^{-3} gibberellic acid (GA_3), 0.06 mg dm^{-3} indole-3-butyric acid (IBA), and 4.0 g dm^{-3} agar, pH 5.5. The same growth medium was used in these experiments.

Infrared sensors connected to a computer were used to monitor the CO_2 inside the culture vessels. CO_2 concentration readings were taken 15 times per min throughout a 16-d culturing period. Sensors were placed in the culture head space of hermetically sealed 1-dm^3 glass jars containing 200 cm^3 of growth medium. To avoid CO_2 concentration increases over the highest range of sensor reading during dark respiration, we could not use more than 5 shoot tips per vessel; thus they were carefully chosen for homogeneous vigour, fresh mass and leaf number and area. To furtherly reduce experimental variability, the whole amount of growth medium was prepared at once and distributed to each culture experimental jar just after sterilization.

Two sucrose concentrations were tested: 30 g dm^{-3} (30S) and 0 g dm^{-3} (0S). In the attempt to avoid the effects of different osmotic potential between the two media, preliminary experiments were performed to test the efficacy of mannitol and sorbitol as osmotic potential regulators. Since the first compound was clearly toxic to leaf tissues and the second stimulated culture growth, the experiments were conducted without modifying the osmotic potential of the two media, which differed from each other by 0.3 MPa.

Enrichment of exogenous CO_2 was performed to both the cultures grown on 30S (30S+CO_2) and on 0S (0S+CO_2) by injecting the gas into the culture jars with a syringe when its concentration approximated 1 000 μmol mol^{-1}. The amount of CO_2 to add each time was preliminary calculated so that CO_2 concentration never exceed about 11 000 μmol mol^{-1}. Control jars were not enriched with CO_2 (30S-CO_2 and 0S-CO_2).

After 16 d of growth, culture fresh and dry mass, number of neo-formed axillary shoots, node number per cluster and leaf area were recorded. Data were analysed statistically by *ANOVA* and mean differences were compared by the Tukey' test. The experiments were repeated twice.

Results and discussion

Results obtained in both the experiments were quite similar one another. Cultures grown on 0S-CO_2 exhibited a remarkable CO_2 uptake as early as the first day of culture when CO_2 appeared already to be a limiting factor for some hours of light. The daily trend of CO_2 uptake and emission showed rather uniform light-dark fluctuations throughout the experiment (Fig. 1), with CO_2 concentrations ranging between around 300 and 1 600 μmol mol^{-1}.

In the 30S-CO_2 treatment, the amount of CO_2 taken up or emitted by the cultures during light or darkness periods respectively, exhibited pronounced differences throughout the culturing cycle, the concentrations ranging from about 300 to 4 000 μmol mol^{-1} (Fig. 1). The daily ratio between CO_2 produced during the dark period and that uptaken during the following light period, was almost always above 1 (data not shown) with the only exception of a few days (from the 5th to the 9th day) during which the amount of photosynthesised CO_2 was higher than, or equal to that produced during dark respiration. CO_2 became limiting for the first time on the 6th day from the beginning of the experiment. Very similar trends were observed in a previous research with plum cultures (unpublished data).

The different CO_2 dynamics observed between the 0S-CO_2 and 30S-CO_2 treatments during the early days of culture, appears related to the different capacity of the two growth media to satisfy the energy requirement of the cultures. Such a requirement was likely increased by the early activation of axillary meristems as a

consequence of the cytokinin present in the growth medium. The cultures grown in the presence of sucrose had presumably less need to resort to photosynthetic activity for their growth, as on the contrary occurred to the cultures grown on the sugar-less medium. Thus, the 30S-CO_2 treated cultures, metabolizing the saccharides added to the culture medium, produced amounts of CO_2 higher than those of 0S-CO_2 treated cultures during dark respiration. This hypothesis would be supported by the progressive increases in CO_2 night emission observed in plum cultures during the first week of the culturing cycle in the presence of increasing cytokinin concentrations (unpublished data).

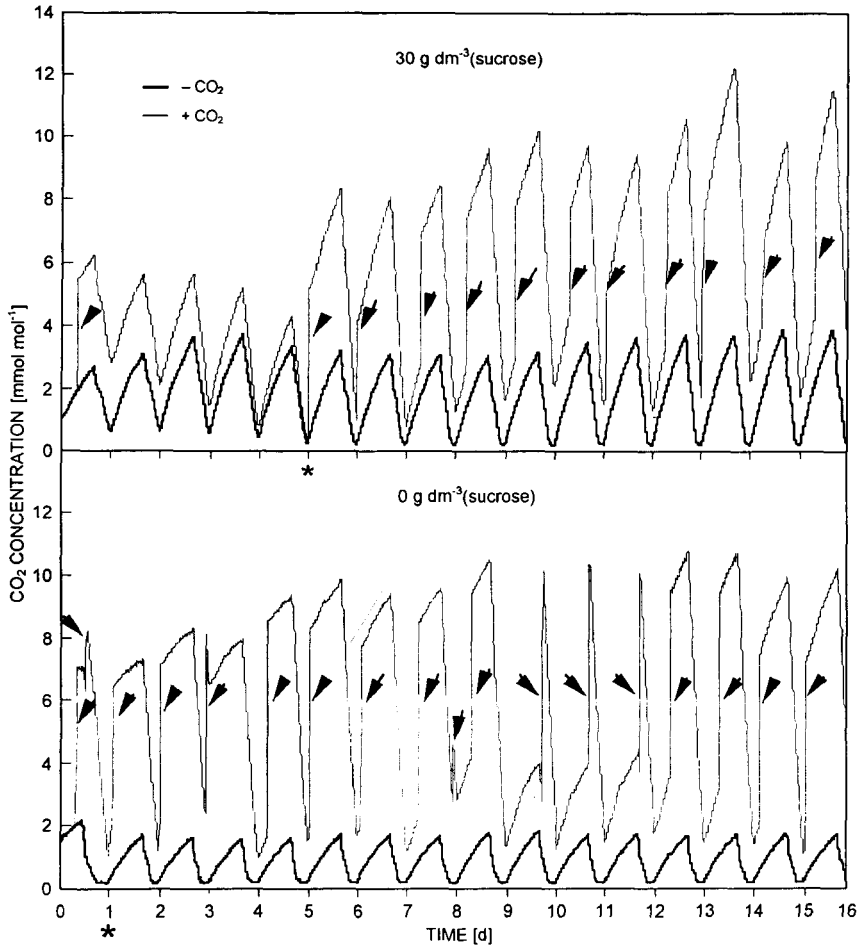

Fig. 1. Day-night dynamics of CO_2 monitored in vessels containing proliferating MM 106 apple cultures grown for 16 d in the presence of 30 g dm^{-3} or 0 g dm^{-3} sucrose, with or without CO_2 enrichment, at 210 ± 5 μmol m^{-2} s^{-1} PPFD and 8-h photoperiod. The *arrows* and *asterisks* indicate CO_2 injections times and the first time CO_2 is limiting, respectively.

After the 4th day of culture, the amount of CO_2 taken up during the light periods by the 30S-CO_2 treated cultures become higher for a few days than that of CO_2 emitted during the dark periods. Saccharide depletion in the growth medium did not seem to be a triggering factor of such behaviour since other research (Kozai and Iwanami 1988, Figueira and Janick 1994) demonstrated that *in vitro* cultures of some species utilized, over a period of about 30 culturing days, only a small fraction of the initial carbohydrate amount added to the gelled medium. It is more than likely, instead, that this result was related to some changes in the culture growth dynamics as the conspicuous leaf area expansion and the increase in metabolising tissues mass, occurred in plum cultures from the 5th to the 7th culturing day, would demonstrate (Morini, unpublished data).

The uptake of the exogenous CO_2 injected into the culture vessels also differed between the two saccharide treatments (Fig. 1). Throughout the experimental period, 0S+CO_2 cultures needed to be supplied with CO_2 every day as CO_2 assimilation was so high, even on the first day of culture, that its availability was strongly reduced after just a few hours of light. In contrast, in 30S+CO_2 the initial supply of CO_2 was progressively taken up over about 4 - 5 d, demonstrating that the culture saccharide requirement during this period was mainly satisfied by the sucrose added to the culture medium. After the first 5 culturing days, the CO_2 added daily to the vessels was

almost completely utilized by the cultures during the following 8 h of light; thus, daily injections of CO_2 were performed. From this time onward the dynamics of CO_2 was very similar to that observed in the 0S+CO_2 treatment. The difference in exogenous CO_2 uptaken observed after 4 - 5 d of culturing, even in this case, was presumably determined by the changes in the culture quantitative growth as above mentioned. This behaviour also confirm that photoautotrophy and photomixotrophy can be complementary, as hypothesized previously (Infante 1988), and that the cultures could resort to one or to other process according to the level of environmental factors and energetic compound availability. Nevertheless, when the growth conditions are suitable to both the processes, photomixotrophy would seem to be preferred by the cultures, as the data on the sucrose or exogenous CO_2 contribute to culture growth reported below, would seem to demonstrate.

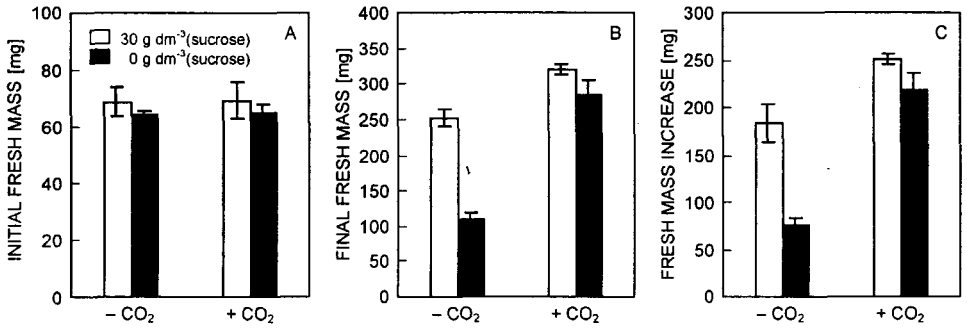

Fig. 2. Variations in fresh mass of apple cultures, recorded after 16 d of growth in the presence of 30 g dm^{-3} or 0 g dm^{-3} sucrose, with or without CO_2 enrichment. *A* - initial fresh mass; *B* - final fresh mass; *C* - fresh mass increase. *Vertical bars* represent SE of the mean, $n = 5$.

Photoautotrophic growth was remarkable in the cultures grown on the sugar-less medium. The final FM value was very satisfactory being intermediate to those of the 30S+CO_2 and 30S-CO_2 treatments (Fig. 2). According to data from the literature, the photosynthetic capacity of *in vitro* cultures increases by reducing saccharides availability in the growth medium (Grout and Price 1987, Langford and Wainwright 1987) and a culture photoautotrophic growth even more pronounced than that determined by growth medium sucrose, could be obtained by suppressing sucrose completely (Kozai 1991). Some authors (*e.g.* Grout and Price 1987) related the increase in photosynthetic activity to a less negative effect of sugar on activity and/or synthesis of RuBisco, but also other factors might be involved in such a behaviour. As suggested for mineral ions (Lumsden *et al.* 1990, Williams 1993), a localised depletion of saccharides around the culture surface could take place as well and the supplying of sucrose to the cultures through the growth medium could be more or less efficient according to the rate of diffusion which mainly depend on agar type and water availability (Debergh *et al.* 1983).

Cultures submitted to 30S+CO_2 showed the highest FM value while those grown on 0S-CO_2 were the lightest. Culture FM increases (Final $_{FM}$ - Initial $_{FM}$) in the various treatments (Fig. 2) confirmed these results. It is worth noting that also the 0S-CO_2 treated cultures showed an increase in FM which accounted for 71.8 % of the initial FM. Since the growth medium did not contain saccharides and the photosynthetic activity was practically irrelevant without CO_2 enrichment, this culture growth increase was presumably due to a "residual energy" arisen from saccharides stored in the tissues during the preceding culture cycle in the presence of sucrose in the growth medium.

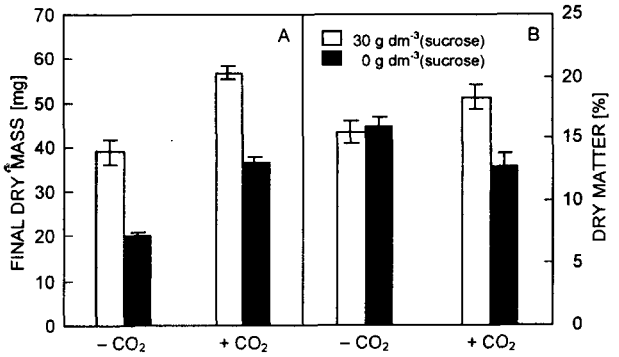

Fig. 3. Final dry mass (*A*) and dry matter (*B*) content in apple cultures grown for 16 d in the presence of 30 g dm^{-3} or 0 g dm^{-3} sucrose, with or without CO_2 enrichment. *Vertical bars* represent SE, $n = 5$.

Culture DM final value was highest in the 30S+CO_2 treatment (Fig. 3) and lowest in 0S-CO_2. In the 0S+CO_2 and 30S-CO_2 treatments, intermediate and very similar values were obtained. As for FM, also DM in 0S-CO_2 cultures markedly increased during the experiment, the final value being about 3.5 times higher than the initial one (data not shown). Dry matter content at the end of the experiments ranged from 13 to 19 % among the treatments (Fig. 3) and was much higher than that (9.1 %) recorded in cultures normally grown with 30 g dm^{-3} sucrose and 50 μmol m^{-2} s^{-1} PPFD. This difference was likely determined by the different metabolism

Table 1. Growth parameters in apple cultures grown for 16 d in the presence or the absence of sucrose in the growth medium and with or without CO_2 enrichment. Different letters within column indicate statistically different values according to Tukey' test ($P = 0.05$).

Treatments	Shoot number [explant^{-1}]	Node number [explant^{-1}]	Leaf area [cm^2 explant^{-1}]	Mean leaf area [cm^2]
0S–CO_2	1.3 b	4.6 b	1.93 c	0.42 c
0S+CO_2	3.0 a	11.3 a	6.32 b	0.56 b
30S–CO_2	2.9 a	10.2 a	6.73 b	0.66 b
30S+CO_2	3.4 a	11.6 a	10.20 a	0.88 a

induced in the cultures by the higher irradiance as also observed in other works (Infante *et al.* 1989). Dry matter content proved to be statistically lower in the 0S+CO_2 treated cultures compared to sucrose-enriched treatments (Fig. 3), possibly as a result of the higher culture water uptake consequent the lower osmotic potential of the sugar-less medium.

As regards other culture growth parameters (Table 1), shoot and node number per cluster did not vary in the presence of sucrose and/or CO_2 enrichment while leaf area exhibited a significantly higher values in the 30S+CO_2 treated cultures. This result would account for the higher FM and DM increases observed in the cultures of the latter treatment and would suggest a favourable effect of a high CO_2 availability on leaf development.

The contribution to culture growth of exogenous CO_2 greatly differed between the treatments (Fig. 4): in 30S+CO_2 treated cultures, the increase due to CO_2 enrichment was about one fifth of the final FM while in 0S+CO_2 treatment it was about four fifths. The presence of sucrose in 30S+CO_2 treated cultures accounted for more than half of the final FM. The trends of DM values in both the treatments were similar to those obtained for FM.

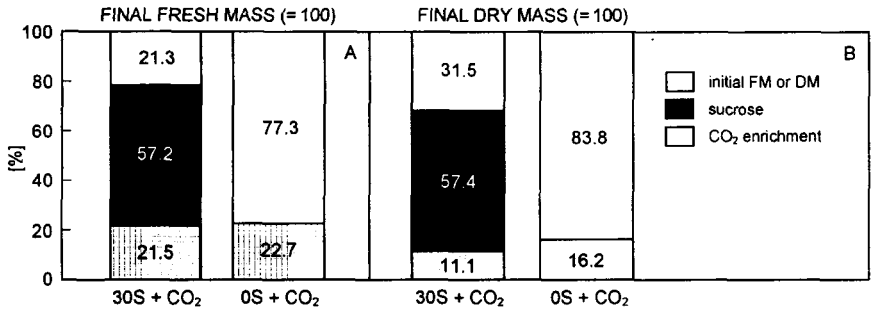

Fig. 4. Contribution of sucrose and/or CO_2 added to culture vessels, to final fresh (*A*) and dry (*B*) mass of TMM 106 apple cultures grown for 16 d in the presence of 30 g dm^{-3} or 0 g dm^{-3} sucrose and CO_2 enrichment. The values are expressed as percentage of final fresh or dry mass.

The total quantity of CO_2 added to culture vessels during the entire trial period and the culture FM increase due to CO_2 enrichment were 44.17 μmol and 68 mg in the 30S treatment and 103.96 μmol and 218 mg in the 0S treatment (Fig. 2). On the basis of these data, the increase in culture FM due to of 1 μmol of CO_2 was higher in the 0S+CO_2 than in 30S+CO_2 treated cultures, the values being 2.09 and 1.54 mg FM respectively. On the contrary, in terms of DM, the increase due to 1 μmol of CO_2 was about 1.5 times higher in the sugar-enriched treatment where this parameter was 0.41 mg DM against 0.29 mg DM of the sugar-less treatment. According to these results, cultures grown on 30S seemed to draw greater advantage from exogenous CO_2 and produced the highest quantity of dry matter (Fig. 3) but it is not known if this result was entirely due to exogenous CO_2 metabolism (autotrophic growth) or also to a higher culture uptake of growth medium sucrose (mixotrophic growth). FM increase in *Prunus cerasifera* plum cultures grown on medium containing 30 g dm^{-3} sucrose, submitted to daily injections of CO_2 and supplemented with 150 μmol m^{-2} s^{-1} PPFD and 16-h photoperiod, was also observed in other experiments (Morini, unpublished data). In this case, the FM increase originated from exogenous CO_2 was 25.7 % of the total value and it was expressed by a larger leaf area and a higher number of axillary shoots. Regardless the unknown effects on the culture growth of a possible interaction between the higher availability of CO_2 and the utilization of sucrose from the growth medium, certainly the 30S+CO_2 treated cultures gained benefit from CO_2 enrichment, particularly during the periods of CO_2 deficiency as observed in the 30S-CO_2 treatment in the second half of the culturing cycle (Fig. 1). Interestingly, the culture growth benefited as well even when such periods were removed by the application of light-dark regimes (4-h light/2-h dark) shorter than the standard one (16/8) (Morini *et al.* 1992, 1993), possibly as a consequence of a more efficient culture saccharides management and/or photoreceptors (phytochrome) activation.

From these results we conclude that the photoautotrophic growth in the absence of sucrose in proliferating MM 106 apple cultures was satisfactory, the values of FM and DM being equal to those obtained with

sugar-enriched medium. Moreover, the induction of photoautotrophy substantially improved the growth also in cultures grown on sugar-enriched medium. Since in these experiments the photoperiod applied was shorter (8 h) than that normally used (16 h), we may expect even higher photoautotrophic growth increases due to CO_2 enrichment with the application of longer photoperiods.

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