Article

Low CO₂ Levels Are Detrimental for In Vitro Plantlets through Disturbance of Photosynthetic Functionality and Accumulation of Reactive Oxygen Species

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Abstract: Photosynthesis of plantlets in tissue culture containers is not considered important, compared to photosynthesis of ex vitro plants, due to the exogenous source of carbohydrates present in tissue culture media. However, CO₂ starvation can generate a burst of reactive oxygen species (ROS). We examined this phenomenon in tissue culture, since CO₂ levels may become very low during the light period. The research was carried out with lily scales, regenerating adventitious bulblets, and with *Arabidopsis* seedlings. CO₂ starvation was achieved by placing a small vial of concentrated KOH solution in the culture container. CO₂ removal reduced the growth of regenerated lily bulblets by 33% or 23%, with or without sucrose in the medium, respectively. In *Arabidopsis* seedlings, CO₂ removal decreased growth by 50% or 78% in the presence or absence of sucrose in the medium, respectively. Therefore, the addition of sucrose as a replacement for photosynthesis resulted in only partial recovery of growth. Staining with nitroblue tetrazolium (NBT) showed little to no ROS in ex vitro growing seedlings, while abundant ROS were detected in seedlings grown under in vitro CO₂ starvation. Seedlings grown under normal tissue culture conditions (no CO₂ withdrawal) showed low levels of ROS. In lily tissue culture, CO₂ starvation decreased the maximum quantum efficiency of photosystem II (F_v/F_m) from 0.69 to 0.60, and in *Arabidopsis* from 0.76 to 0.62. F_v/F_m of ex vitro lily and *Arabidopsis* seedlings was 0.77 and 0.79, respectively. This is indicative of a disturbance in photosynthesis functionality and the occurrence of in vitro stress under reduced CO₂ concentrations. We conclude that poor growth, in the absence of CO₂, was partly due to strongly reduced photosynthesis, while the detrimental effects were most likely due to a burst of ROS.

Keywords: lily; arabidopsis; tissue culture; CO₂ starvation; ROS

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1. Introduction

Although plants are dependent on photosynthesis to absorb light energy and obtain carbon and energy for development, reports on the evaluation of photosynthesis of in vitro plantlets are relatively rare [1]. The microenvironment in tissue culture containers is very different from the ex vitro environment in which plants normally grow. Apart from organic nutrition (sucrose), high external levels of plant growth regulators, and low light intensity, the tissue culture environment is characterized by a very unusual atmosphere: extremely high relative humidity (RH, 98–99.5%), high levels of organic gases, such as ethylene, and fluctuating high and low levels of CO₂ and O₂ [2,3]. During the light period, the CO₂ concentration in an air-tight vessel with green plantlets is often lower than 100 µL L^{−1} which is much lower than the normal atmospheric CO₂ concentration of 400 µL L^{−1}, and may even reach 50 µL L^{−1} [4,5]. Even in loosely capped vessels, or vessels capped with gas permeable film, the concentration is often lower than 200 µL L^{−1} [6]. During the dark period, CO₂ concentration increases up to 3000–9000 µL L^{−1}. The unusual

atmosphere in the headspace of culture vessels imposes several disorders, such as stomatal malfunctioning and photosynthesis disturbance, in the plant samples growing in vitro [7]. Generally, it is expected that the low CO₂ concentration during the light period, along with the low light intensity, restrain photosynthesis. Poor photosynthesis in vitro may also be caused by the addition of sugar to the medium [8]. A reduction of sucrose enhances net photosynthesis, e.g., in coconut [9], and rain tree [10] plants. However, the addition of sugar is necessary for successful tissue culture. Despite the increase of photosynthesis, the omission of sucrose reduces the growth of plantlets dramatically, e.g., in grape [11] and Mountain ash [12] plantlets. Expectations are that a very low CO₂ level during exposure to light is deleterious for plants, due to the lack of electron acceptors in the photosynthetic electron transport chain [13]. Furthermore, there is a general belief that, due to the presence of sugar in the medium, there is no need for photosynthesis in vitro. However, it has been confirmed that removal of the two main inputs of photosynthesis (light and CO₂) are detrimental for production of plants in vitro [6]. In accordance, CO₂ enrichment in vitro increased the shoot growth and multiplication rate in *Celastrus paniculatus* [14]. In the course of CO₂ starvation, all endogenous electron acceptors become reduced, leaving oxygen as the main available electron acceptor. Oxygen can serve as an electron acceptor in the Mehler reaction [15]. Products of the Mehler reaction, such as superoxide, hydroxyl radicals, and hydrogen peroxide (reactive oxygen species, ROS) are toxic, attack vulnerable macromolecules, including chlorophyll, and bring about bleaching [16]. In the present study, we aimed to investigate growth, photosynthetic performance, and the occurrence of oxidative stress in tissue cultures of *Arabidopsis* seedlings (as a model plant, it is easy growing both in vitro and ex vitro) and lily bulblets, regenerating from scale explants under in vitro CO₂ starvation. For this aim, we have used chlorophyll-a fluorescence to detect maximum quantum efficiency of photosystem II (F_v/F_m), as an indicator of the damage to the photosynthetic apparatus, and nitroblue tetrazolium (NBT) staining for detection of ROS.

2. Materials and Methods

2.1. Growth Conditions

Field-grown bulbs (circumference 18–20 cm) of *Lilium* cv. Santander were harvested, cold-treated to break dormancy, and stored at −1.0 °C until use. The procedure for lily tissue culture was performed according to [17]. Scales were surface-sterilized for 30 min in 1% (*w/v*) sodium hypochlorite (NaClO), rinsed for 1, 3, and 10 min with sterile water, and stored until use in sterile water (on average for 1–2 h). Two explants of 7 × 7 mm were cut from the scales and placed with the abaxial side on 30 mL solidified medium in plastic culture tubes (6.5 cm diameter). The medium was composed of macro- and microelements, as according to Murashige and Skoog [18]: 30 g L^{−1} sucrose, or no sucrose; 0.4 mg L^{−1} thiamine; 100 mg L^{−1} myo-inositol; 7 g L^{−1} Microagar; and 0.05 mg L^{−1} NAA (α-naphthaleneacetic acid). All chemicals were obtained from Duchefa (Haarlem, The Netherlands). Thirty scale explants per treatment were cultured for 12 weeks at 25 °C and 30 μmol m^{−2} s^{−1} light (Philips TL 33) for 16 h per day.

2.2. Arabidopsis Seedling Growth

Arabidopsis thaliana (Col-0) seeds were sterilized with 70% (*v/v*) ethanol for 1 min, and in 2% (*w/v*) sodium hypochlorite for 15 min. They were subsequently rinsed three times for 10 min with sterilized distilled water. Sterile seeds were transferred to ten Petri dishes with half-strength Murashige and Skoog salt mixture, including vitamins [18], supplemented with 30 g L^{−1} sucrose and solidified with 7 g L^{−1} Microagar. Seeds were stratified in the dark for 3 d at 4 °C, after which they were cultured in a growth chamber with 16 h light/ 8 h dark (30 μmol m^{−2} s^{−1}, Philips TL33) at 21 °C. After 7 days, the seedlings were transferred to fresh medium with 30 g L^{−1} sucrose, or without sucrose, and then incubated for two weeks in a growth chamber with 16 h light/8 h dark (30 μmol m^{−2} s^{−1}, Philips TL33) at 21 °C. Thirty *Arabidopsis* seedlings were used per treatment.

2.3. Removal of CO₂ from the Headspace

A small vial was placed on the medium next to the explants (Figure 1). This vial contained 3 mL 20% KOH and a piece of filter paper, standing vertically, to increase the contact surface between the KOH solution and the atmosphere in the headspace. CO₂ was removed from the headspace according to the reaction $\text{KOH} + \text{CO}_2 \rightarrow \text{KHCO}_3$. The concentrated KOH solution also reduced the relative humidity [19]. Therefore, a saturated KCl solution [20] was used in the control. Both the 20% KOH and saturated KCl resulted in ca. 85% relative humidity (RH). Thirty lily scale explants and thirty *Arabidopsis* seedlings were used per treatment.

Figure 1. Container with lily scale explants on a nutrient medium with 3% sucrose and a vial of KOH to remove CO₂. Bar = 10 mm.

2.4. Mapping of Maximum Quantum Efficiency of Photosystem II Using Chlorophyll Fluorescence

Lily *in vitro* plantlets and *Arabidopsis* *in vitro* seedlings, obtained as described above, were cultured *ex vitro* in 8 cm pots filled with potting soil, or in tissue culture containers as described above, under control and low CO₂ conditions, in media with 3% sucrose. After *ex vitro* and *in vitro* growth (12 weeks for lily and 3 weeks for *Arabidopsis*), the leaves were used for measurements in a chlorophyll fluorescence imaging system (FluorCam, Photon System Instruments, Brno, Czech Republic). Intact leaves, still attached to the plants in tissue culture containers or pots, were dark-adapted for 20 min. After dark adaptation, intact plants (*in vitro* or *ex vitro*) were immediately used to measure F_v/F_m . In the FluorCam imaging system, an 8 bit, 5123512 pixel CCD camera, equipped with an F1.2/2.8–6 mm objective, was used to record fluorescence images. F_v/F_m was calculated using a custom-made protocol. Images were recorded during short measuring flashes in darkness. These flashes were applied using two panels, each containing 345 orange light-emitting diodes. At the end of the short measuring flashes, a 250 W halogen lamp produced a 1s saturating light pulse, with an intensity of $2500 \mu\text{mol m}^{-2} \text{s}^{-1}$, causing a transitory saturation of photochemistry. After reaching steady-state, two successive records of fluorescence data were digitized and averaged, one during short flashes during darkness (F_0), and the other (F_m) during the saturating light pulse. From these two images, F_v ($F_v = F_m - F_0$) and F_v/F_m were calculated. The average values of F_v/F_m per image were obtained using version five FluorCam software [21].

2.5. Visualization of Superoxide Radicals

Arabidopsis in vitro seedlings were cultured ex vitro in 8-cm pots filled with potting soil, or in tissue culture containers, as described above, in control and low CO₂ conditions, in media with 3% sucrose. Superoxide radicals were visualized by staining 3 week old *Arabidopsis* seedlings, ca. 8h after the start of the light period, with nitroblue tetrazolium (NBT) solution according to Van Den Dries et al. [22], with minor modifications. Seedlings with roots were transferred into a 0.1% (w/v) NBT solution containing 50 mM phosphate buffer (pH 7.8) and 10 mM NaN₃. The seedlings were vacuum-infiltrated for 5 min and kept for 45 min in the dark at room temperature. Stained seedlings were then bleached in acetic acid—ethanol 80% (1/4) (v/v) at 100 °C for 30 min, and stored in 50% ethanol until photographs were taken.

2.6. Statistical Analysis

Fresh weight (FW) was recorded after 12 weeks (scale explant culture; FW bulblets, FW leaves, FW roots, and FW scale explant), or after 3 weeks (*Arabidopsis* seedling culture; FW shoots and FW roots). Thirty explants were used for each observation. In the figures, the means are shown $\pm$ SE. Differences were evaluated with the Student *t*-test: *, $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$.

3. Results

3.1. CO₂ Removal Reduces Growth in Lily and *Arabidopsis*

Lily scale fragments cultured in vitro on nutrient medium display different kinds of growth. They regenerate bulblets with scales that may or may not carry leaves. In addition, roots, which are usually not connected to the shoots, regenerate. Finally, the scale explant may itself increase in size, and may generate callus tissue. We measured the FW of all four tissues: bulblets, leaves, roots, and scales. The addition of sucrose to the nutrient medium strongly increased the growth of all tissues. Removal of CO₂, when no sucrose had been added, reduced growth (Figure 2) by: 23% in lily bulblets, 21% in leaves, 34% in roots, and 25% in scale explants. Removal of CO₂ caused a larger reduction in growth when 3% sucrose had been added.

Figure 2. Effect of CO₂ removal on lily growth: bulblets (a), leaves (b), roots (c), and scale explants (d) in medium with 3% sucrose or without sucrose. *, $p \leq 0.05$ and **, $p \leq 0.01$.

Bulblet fresh weight (FW) decreased by 33%, leaf FW by 68%, root FW by 70%, and scale explant FW by 79% as a consequence of CO₂ removal. Removal of CO₂ has a detrimental effect in lilies. The tissue culture system of lilies, however, differs from most other systems, since scale explants may serve as a rich source of nutrients and as a ‘buffer’ for the regenerating plantlet. We therefore performed a similar experiment with *Arabidopsis* seedlings. We obtained comparable results to the lily experiments, though values were more extreme (Figures 3 and 4). It should also be noted that the seedlings bleached when CO₂ was removed from the headspace (Figure 4a,b), and that they died soon after the period of observation.

Figure 3. Effect of CO₂ removal on the growth of *Arabidopsis* seedlings, (a) FW shoot and (b) FW root in medium with 3% sucrose and without sucrose: *, $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$.

Figure 4. *Arabidopsis* seedlings cultured for 7 days on medium with (3%) or without (0%) sucrose. A small vial with 3 mL 20% KOH was placed in the container to remove CO₂ from the headspace (a,b). As a control for the drop in RH from the concentrated KOH solution, a small vial with saturated KCl was added when no vial with KOH was included (c,d). Bar = 10 mm.

3.2. CO₂-Low Conditions Disturbs the Physiology of the In Vitro Plants

Arabidopsis seedlings cultured on medium with sucrose, but without CO₂ in the headspace, were tiny and had bleached leaves (Figure 4). The bleaching indicates oxidative stress. The occurrence of ROS was visualized by NBT staining (Figure 5). When CO₂ was removed, the seedlings colored deep blue, showing abundant formation of ROS, whereas the in vitro seedlings with CO₂ showed very little blue staining. *Arabidopsis* seedlings grown in ex vitro conditions showed only faint and sparse blue staining.

Figure 5. *Arabidopsis* seedlings stained with NBT (a) grown ex vitro, (b) grown in vitro under control conditions, and (c) grown in vitro under conditions when (most) of the CO₂ had been removed. Bar = 10 mm.

ROS also damages the photosynthetic system. Photosynthetic performance can be properly evaluated by determining F_v/F_m . In general, a higher F_v/F_m was observed in *Arabidopsis* compared to lily plants (Figure 6). In both plants, in vitro plants had a lower F_v/F_m than ex vitro plants (Figure 6). Furthermore, in vitro F_v/F_m strongly decreased in CO₂-poor conditions, in both lily and *Arabidopsis* plants (Figures 6 and 7).

Figure 6. F_v/F_m in lilies (a) and *Arabidopsis* (b) grown ex vitro, in vitro, and when (most) of the CO₂ was removed from the headspace. Different letter indicate significant differences between treatments ($p < 0.05$).

Figure 7. Image of F_v/F_m of lilies and *Arabidopsis* grown ex vitro (a,d), in vitro with 3% sucrose (b,e), and in vitro with 3% sucrose in a CO₂-poor environment (c,f).

4. Discussion

When most of the CO₂ was removed from the headspace by placing a vial of concentrated KOH solution in the container, we observed a strong inhibition of growth. In the case of a nutrient medium with 3% sucrose, this was unexpected, as the assumption was that sucrose in the medium supplies organic nutrition, and should support adequate growth. Since it has been reported that CO₂ starvation brings about an ROS burst [13,23], we presumed that the inhibition of growth was, at least to some extent, caused by this detrimental effect of CO₂ withdrawal. When CO₂ is not available, other electron acceptors become reduced; particularly oxygen, resulting in toxic, activated oxygen species (ROS). In agreement with this, CO₂ removal from the headspace caused severe bleaching of *Arabidopsis* leaves and reduced the photosynthetic functionality. Growth declined drastically as a result of oxidative stress. In *Arabidopsis thaliana* cultured in CO₂ deprived conditions, in vitro plantlet growth was 3.4 times lower than in aerated cultures [24]. In lilies, bleaching was less pronounced (not shown), though fluorescence dropped even more than in *Arabidopsis* (Figure 6). Growth reduction in lily plants was more severe in leaves than in bulblets. This may be because the leaves are a site of abundant chlorophyll, while bulblets contain less chlorophyll (Figure 2; also see *Arabidopsis* in Figure 4). Moreover, staining with NBT showed abundant ROS formation when CO₂ had been removed by a concentrated KOH solution.

Tissue culture is usually carried out in the light, and the plant material in question is chlorophyllous. Under these conditions, photosynthesis inevitably occurs, although at a reduced rate. Indeed, in standard tissue culture conditions, the expected curves for CO₂ and O₂ during a daily regime of light and dark have been observed: high CO₂/low O₂ levels in the dark and low CO₂/high O₂ levels during the day [4]. It might often be that CO₂ levels are so low that O₂ is reduced, leading to ROS production. Indeed, we observed a slight ROS presence in tissue-cultured plants under normal CO₂ environment. Here,

it should be noted that the amount of plant material in the container (6 small seedlings) was much less than in common tissue culture practices, indicating that in the practice of tissue culture, the detrimental effect is probably much larger.

A reduced electron transport flux through the photosynthetic electron chain, and subsequently ROS formation, has also been reported in the presence of high sugar in the medium, even under normal light conditions [25]. Regarding high sucrose concentration, contradictory effects on photosynthesis activity and ROS formation have been reported. Several authors report that high sucrose concentrations in the medium are deleterious for photosynthetic activity [5,26]. However, sucrose has also been reported to stimulate photosynthesis in *in vitro* plantlets [27,28]. ROS formation can also be stimulated [29] or decreased [30] by sugar.

A study on gene expression in *in vitro* and *ex vitro* tomato leaf tissues showed that stress-related genes and ROS scavenging enzymes were up-regulated in *in vitro* tomato plants. Superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR) enzymes eliminate toxic superoxide and hydrogen peroxide by conversion to water. The expression of APX and GR were higher in tomato leaf tissues *in vitro*, compared to *ex vitro*, which leads to adaptation and acclimatization to *in vitro* conditions [31]. Up-regulation of ROS scavenging enzymes in tomato leaf tissues, grown *in vitro*, showed that plants react to overcome stressful *in vitro* conditions by removing detrimental free radicals. Furthermore, upregulation of SOD1 and SOD2 were shown to occur in *Arabidopsis thaliana*, cultured *in vitro* in CO₂ deprivation conditions, which indicated the accumulation of ROS compared to aerated cultures [25]. In a severe stressful condition of an almost CO₂-free condition in *Arabidopsis*, this leads to an increased and high amount of ROS formation (Figure 5c), extremely damaged *Arabidopsis* seedlings (Figure 4a,b), and, after a few weeks, death of the seedlings. A higher growth rate of *ex vitro* plants, compared with *in vitro* plantlets, has been also observed, e.g., in lily [32] and walnut [20] plants. The lesser growth of *in vitro* plantlets might be related to the damaging effect of ROS on the growth of *in vitro* plantlets compared with *ex vitro* plants.

Connecting all the negative effects of CO₂ deprivation directly to oxidative burst may be deceptive. For instance, ethylene can also induce an oxidative burst, leading to acceleration of senescence [33]. It has been reported that CO₂, through up-regulation of ACC (precursor of ethylene)-synthase, can enhance ethylene production [34]; on the other hand, it is well-known that CO₂ inhibits ethylene action, and due to this inhibitory effect CO₂ is widely used in the horticultural industry to maintain the quality of ethylene-sensitive products [33]. The inhibitory effects of ethylene on growth of *in vitro* plants has also been reported [33,35]. Therefore, it is rational to construe that, due to CO₂ deprivation in the present study, no inhibitory obstacles existed to retard the ethylene action, which resulted in oxidative burst and disturbance of photosynthesis. Further study is needed to determine the concentrations of ethylene and CO₂ in the headspace of culture vessels during *in vitro* growth of plantlets, and to elucidate the role of ethylene under CO₂ deprivation.

5. Conclusions

In the present study, we confirmed the detrimental effects of CO₂ deprivation on production of plantlets *in vitro*. Disturbance in photosynthetic functionality occurred by *in vitro* CO₂ deprivation in both lily and *Arabidopsis* plantlets. However, it is not known whether the contribution of photosynthesis is substantial or marginal for plantlets under *in vitro* conditions. Staining with NBT confirmed higher ROS production in *Arabidopsis* seedlings under *in vitro* conditions, and an oxidative burst by CO₂ deprivation under the same conditions, when compared to the level of ROS in seedlings grown *ex vitro*. Therefore, poor growth due to CO₂ deprivation *in vitro* can be, to some extent, due to disturbances in photosynthesis and to a larger extent to a ROS burst.

Author Contributions: N.A. made substantial contributions to conception and design, carried out the experiments, performed the statistical analysis, and drafted the manuscript. S.A. measured the photosynthetic performance, performed statistical analysis, and carried out revision of the manuscript. R.G.F.V. supervised the research, contributed to the conception and design of the experiment, as well as the scientific discussion, and performed critical revision of the final manuscript. All authors have read and agreed to the published version of the manuscript.

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