Improvement of Ex Vitro Desiccation through Elevation of CO₂ Concentration in the Atmosphere of Culture Vessels during In Vitro Growth

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Abstract. Acclimatization of in vitro plantlets is one of the key steps in successful tissue culture propagation. Gaseous atmosphere during in vitro culture can influence the rate of ex vitro acclimation of the plantlets produced. In the current study, effects of elevated CO₂ concentration on the leaf water loss dynamic responses of in vitro-produced walnut leaves during ex vitro desiccation were investigated. Elevated CO₂ concentration in the headspace of culture vessels caused a considerable decrease in stomatal aperture. Although the traits related to stomatal size were not influenced by CO₂ elevation, the number of small stomata was increased, and the number of large stomata was decreased at elevated CO₂ concentration. Higher CO₂ concentration resulted in a lower transpiration rate and a higher relative water content (RWC) during ex vitro desiccation. This improvement was due to decreased stomatal aperture during the first phase of water loss. Osmotic potential (ψ_s) was decreased under an elevated CO₂ concentration, but no influence was observed on the concentration of compatible solutes. In conclusion, increasing the CO₂ concentration of culture vessel headspace can be an efficient tool for improving acclimation of in vitro-grown walnuts without negative effects on plantlet growth.

Conventional in vitro propagation can result in production of plants with low survival capacity (Crane and Hughes, 1990). Variable and often insufficient CO₂ concentration, high relative humidity (RH), and accumulation of ethylene or other gases in the headspace of the culture containers can cause problems for plantlets during and after in vitro propagation (Hazarika et al., 2004; Kozai, 2010; Lamhamedi et al., 2003; Pospisilova et al., 2007). Headspace of tissue culture vessels is characterized by high RH and low vapor pressure deficit (VPD). Low VPD, through its impact on stomatal conductance and transpiration rate, can influence water relations of in vitro plantlets (Fuchigami et al., 1981; Grout and Aston, 1977; Nogues et al., 1998). Long-term exposure to high RH decreases the closing ability of stomata because of low foliar abscisic acid (ABA) levels (stomatal malfunctioning). As a result, the capacity of leaves to control water loss decreases when plants are exposed

to conditions of increased evaporative demand (Aliniaiefard and van Meeteren, 2013, 2016; Aliniaiefard et al., 2014; Fanourakis et al., 2013; Rezaei Nejad and Van Meeteren, 2005, 2007). The consequence of low water conservation capacity of in vitro plants can be high mortality of plantlets after transferring to ex vitro conditions (Crane and Hughes, 1990; Shim et al., 2003). Various methods such as ventilation (Cui et al., 2000; Shim et al., 2003), lids permeable to water vapor (Ghashghaie et al., 1992), and others (Cha-um et al., 2003, 2010; Tanaka et al., 1992) have been used to decrease the RH in the headspace of culture vessels. However, growth retardation, low efficiency, and contamination have been reported as negative side effects of those techniques (Sallanon and Maziere, 1992).

Photosynthesis of in vitro plantlets is often restricted by low CO₂ concentration, low light irradiance and presence of high sugar concentration in the medium (Kubota, 2002). Tissue culture media are typically supplemented with sugar as the source of carbon to maintain a positive carbon balance (Kwa et al., 1995). On the other hand, an increase in CO₂ concentration in the headspace of culture vessels during darkness and decreased CO₂ during light periods have been reported (Fujiwara et al., 1987; Thomas, 1999). Low CO₂ concentration and high sugar levels limit the activity of ribulose

bisphosphate carboxylase during most of the photoperiod (Donnelly and Vidaver, 1984; Kilb et al., 1996). Therefore, plantlets are forced to develop heterotrophy or photo-mixotrophy under in vitro conditions (Fujiwara et al., 1987; Kozai et al., 1997).

Low CO₂ concentration in the atmosphere surrounding the leaf results in low internal CO₂ concentration in the substomatal cavity (C_i), producing a signal to open the stomata. Conversely, high CO₂ concentration in the atmosphere surrounding the leaf results in high C_i, causing closure of the stomata (Allaway and Mansfield, 1967; Uehlein et al., 2003). Observations in tobacco and grapevine plantlets showed stomata closed in response to high CO₂ (During and Stoll, 1996; Pospisilova et al., 1999). It has been shown that elevated CO₂ concentration can enhance the effect of ABA on stomata closure (Pospisilova et al., 2000).

Low in vitro CO₂ concentration combined with high RH in the headspace of culture vessels appears to promote an enduring open stomatal state in in vitro-produced plants (Hazarika, 2006). As a result, the water retention capacity of their leaves during ex vitro acclimation is inhibited. Because low water retention is thought to be due largely to high stomatal transpiration during ex vitro acclimation, the aims of this study were 1) to alter stomatal morphology during in vitro growth; 2) to determine the water loss characteristics of in vitro-generated leaves during ex vitro desiccation and; 3) to determine if increased CO₂ concentration during in vitro development can increase the water retention capacity of leaves during ex vitro acclimatization.

CO₂ enrichment can be achieved by growing the explants in tissue culture containers sealed with gas-permeable films, direct supply of CO₂ in the vessels by forced ventilation, or using CO₂-generating chemicals in containers (Kozai, 1991; Solarova and Pospisilova, 1997; Xiao et al., 2011). In the current study, to prevent problems due to ventilation (Sallanon and Maziere, 1992), increased CO₂ was achieved by CO₂-generating chemicals isolated from the medium. Persian walnut (*Juglans regia* L.) was chosen for this study because of 1) difficulties in its propagation through vegetative reproduction (Aviles et al., 2010); 2) the commercial importance of mass propagation of high quality, disease-free, and uniformly multiplied cultivars with desirable traits through in vitro propagation (Payghamzadeh and Kazemitabar, 2011) and; 3) difficulties in acclimatization after in vitro production.

Material and Methods

Plant materials and growth conditions. Microshoots of Persian walnut (*Juglans regia* L. cv. Chandler) (20 ± 2 mm length) were selected from previously established cultures; transferred every 3–4 weeks to fresh medium; cultured on the DKW (Driver–Kuniyuki Walnut) medium (Driver and Kuniyuki, 1984) supplemented with IBA

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(0.01 mg·L⁻¹), BAP (1 mg·L⁻¹), and sucrose (3%); and solidified with Gelrite (2.2 g·L⁻¹). Four vessels for each treatment and two explants per vessels were used. Vessels were 65 mm in diameter and 85 mm in height (≈350 mL), and each vessel contained 50 mL of DKW medium; therefore, the volume of headspace atmospheric space was 300 cm³. Plantlets (nodal explants with leafy part) were grown under photomixotrophic conditions (DKW medium supplemented with 3% sucrose). Medium pH was adjusted to 5.5 before autoclaving for 20 min at 121 °C. Work was conducted in a growth chamber with 100 μmol·m⁻²·s⁻¹ light intensity at 25 ± 2 °C under 16/8 h light/dark cycle for 30 d, without further transfer of the plant material. Growth chamber supplemented with fluorescent lamps with 15 cm vertical distance between the lamps and culture vessels.

Increasing CO₂ in the headspace of culture vessels. Plantlets were exposed to two different CO₂ concentrations in the headspace of culture vessels: a) vessels with 3 mL of mixed NaHCO₃ and Na₂CO₃ solution (3 M) in the ratio of 73/27 (v/v), respectively. The mixed solution, autoclaved and placed into 4-mL vials, released 40 ppm/m³ CO₂ in to the atmosphere of the vessel headspaces (Solarova and Pospisilova, 1997). This is the CO₂-releasing potential of the mixed solution; however, when and how it releases depends on many factors and is not possible to calculate, and b) vessels without the CO₂-releasing solution which were considered as control plantlets. Concentration of CO₂ in the headspace atmosphere of the culture vessels can also be influenced by some plant metabolic processes (e.g., photosynthesis and respiration); therefore, in many previous publications, the exact amount of CO₂ in time have not been mentioned (Joshi et al., 2009; Mitra et al., 1998; Suthar et al., 2009). Work stages are presented in Fig. 1.

Vegetative characteristics. At the end of the experiment, the microshoots were removed

from the culture medium and vegetative characteristics were measured. Shoot lengths were measured with a ruler, fresh shoot weights were taken, and then leaf areas were scanned and analyzed using ImageJ (U.S. National Institutes of Health; Bethesda, MD; <http://imagej.nih.gov/ij/>). Dry weights of shoots were obtained by placing the samples in an oven at 70 °C for 48 h. Specific leaf area (SLA), leaf water content per unit area (LWCA), leaf mass area, and leaf water content (LWC) were measured according to the equations of Aliniaiefard et al. (2016). The relative chlorophyll (Chl) content was measured with a portable leaf chlorophyll meter (SPAD; Konika Minolta).

Stomatal morphology. To investigate the differences in stomatal morphology between CO₂-treated shoots and control shoots, the lower epidermis of the second lateral leaflets from the apex (adaxial surface) of five tissue-cultured shoots for each treatment were coated by a thin layer of nail polish. After a few minutes, a strip of transparent sticky tape was applied on the dried polish. Sticky tapes were peeled from leaves and along with nail polish were mounted on microscopic slides. Images of 200 stomata taken from these epidermal strips using a light microscope (model Olympus) in combination with ImageJ (U.S. National Institutes of Health, Bethesda, MD; <http://imagej.nih.gov/ij/>) were used to measure the stomatal length, stomatal width, pore length, pore width, stomatal area, ratio of stomatal length to stomatal width, and stomatal density. (The accuracy of the scale that was used for measuring stomatal traits was 1 μm.)

Stomatal response to desiccation. The weighing of detached leaves at room temperature under controlled conditions was used to study the effect of desiccation on transpiration rate and RWC. The leaves were detached from shoots and used immediately under 50 μmol·m⁻²·s⁻¹ irradiance at room temperature and 50% RH, resulting in 1.24 kPa VPD. For leaf desiccation, after measuring leaf

area, leaves were placed upside down on a balance and gravimetrically weighed every 5 min for a duration of 90 min. Transpiration rate was calculated according to Aliniaiefard et al. (2014) based on the following equation:

$$E = \left\{ \left\{ \frac{\Delta \text{fresh weight (g)}}{\text{molar mass water} \left(\frac{\text{g}}{\text{mol}} \right)} \right\} \times 1000 \left(\frac{\text{mmol}}{\text{mol}} \right) \right\} / \left\{ \frac{\text{measurement frequency (s)}}{\text{leaf area (m}^2\text{)}} \right\}$$

To determine the RWC, the leaves were dried for 48 h at 70 °C. The RWC during the desiccation period was calculated according to Slavik (1974).

Leaf ψ_s and compatible solutes determination. For determination of ψ_s , leaves were cut into small segments, placed into Eppendorf tubes perforated with four small holes, and immediately frozen in liquid nitrogen. After being encased individually in a second intact Eppendorf tube, they were allowed to thaw for 30 min and centrifuged at 15,000 g for 15 min at 4 °C. The collected supernatant was used for ψ_s determination. Osmolarity (c) was assessed with a vapor pressure osmometer (Osmomat 030-gonotec) and converted from mosmoles kg⁻¹ to MPa according to the Van't Hoff equation (Martinez et al., 2004).

Proline concentration of leaves was spectrophotometrically measured as described by Bates et al. (1973). Free proline content (μg·g⁻¹ DW) was determined from a standard curve prepared with five standard concentrations (0–200 μg·mL⁻¹) of L-proline (Bates et al., 1973; Meloni et al., 2004).

Glycinebetaine concentration (μg·g⁻¹ DW) was estimated according to Grieve and Grattan (1983). The absorbance was determined at 365 nm with a spectrophotometer (Lambda 25-ultraviolet/VIS spectrometer). Reference standards of GB (50–200 μg·mL⁻¹)

Fig. 1. Work stages for CO₂ elevation in the atmosphere of the vessel headspaces. (A) Microshoots were cultured in the culture vessels. (B) Vials containing 3 mL of mixed NaHCO₃ and Na₂CO₃ solution (3 M) in the ratio of 73/27 (v/v), respectively, were placed in the vessels. (C) The samples were incubated and grown at 25 ± 2 °C under 16/8 h light/dark cycles for 30 d.

were prepared in 2 M sulfuric acid (Grieve and Grattan, 1983).

Statistical analysis. Data of stomatal morphology, compatible solutes, and vegetative traits were subjected to ANOVA with $P \leq 0.05$ considered as not significant. For stomatal characteristics, data obtained from one leaf were considered not independent, and a paired t test was used to find significant differences ($P \leq 0.05$) between CO₂-treated plantlets and control plantlets. For the statistical analysis of transpiration rate (E) and RWC, data were fitted using nonlinear regression with one-phase exponential decay, $E = (E_0 - \text{Bottom}) \cdot \exp(-K \cdot X) + \text{Bottom}$, where E_0 is E at time zero, K is the slope of the curve, Bottom is E when it reaches a plateau, and X is time. An F test was used for comparing the parameters of the fitted curves. The change of E as a function of RWC was fitted using a sigmoidal dose-response curve with a variable slope ($E = \text{Bottom} + \{(\text{Top} - \text{Bottom}) / [1 + 10(\text{RWC}_{50} - \text{RWC}) \cdot \text{Slope}]\}$) and an F test was used for comparing the parameters of the fitted curves. GraphPad Prism 5 for Windows (GraphPad software, Inc. San Diego, CA) was used for analyzing the data.

Results

Stomatal morphology. No significant changes in stomatal size were observed because of exposure of leaves to high CO₂ concentration in culture vessels (Table 1). However, stomatal aperture in leaves grown under high CO₂ was 34% narrower than stomatal aperture in controls and stomata were closed in leaves grown under high concentration of CO₂ (Table 1; Fig. 2). Huge heterogeneity in stomatal size was observed under both treatments (Fig. 3). However, growing plantlets under elevated CO₂ increased the number of small-sized stomata ($\leq 150 \mu\text{m}^2$), while number of stomata larger than $500 \mu\text{m}^2$ were decreased by high CO₂ concentration (Fig. 2).

Stomatal response to ex vitro desiccation. Transpiration rate (E) at the start of ex vitro desiccation was decreased by 47% in leaves grown in vitro under high CO₂ concentration (Fig. 4A; top in Table 2). Span (difference between E_0 and bottom) of the E curve in CO₂-treated plantlets was one-third the span of the E curve for control plantlets (Table 2).

Exposure of shoots to high CO₂ concentration during in vitro growth significantly influenced the RWC when leaves were exposed to ex vitro desiccation (Fig. 4B). Half-value of RWC in CO₂-treated plantlets was reached 20 min after desiccation, whereas the half-value of RWC in control plantlets occurred only 15 min after desiccation. In addition, the RWC of CO₂-treated shoots plateaued at 35%, vs. 10% for controls (bottom in Table 3). The span of the RWC curve for leaves exposed to elevated CO₂ was 33% smaller than in controls (Table 3). These results indicate that elevated CO₂ concentration in the headspace during in vitro culture

can increase water retention capacity during ex vitro acclimatization.

$E \times \text{RWC}$ curve for leaves exposed to high CO₂ concentrations in vitro was considerably lower than the $E \times \text{RWC}$ curve for controls (Fig. 4C). The RWC₅₀ (RWC at which E is midway between the top and bottom of the $E \times \text{RWC}$ curve) of the fitted curve was 81% for the leaves treated with CO₂ and 58% for controls (Table 4).

Vegetative characteristics. No significant changes in vegetative traits were observed because of CO₂ concentration in the headspace of culture vessels (Table 5).

Leaf ψ_s and compatible solute determination. Exposure of walnut microshoots to elevated CO₂ resulted in a 28% decrease in leaf ψ_s (ψ_s) relative controls (Table 5) even though the concentrations of proline and glycinebetaine in these leaves were unaffected (Table 5).

Discussion

In our study, increasing the CO₂ concentration in vitro resulted in the improvement of water retention capacity during ex vitro desiccation. Low CO₂ concentration in the headspace of culture vessels is thought to be the main factor limiting photosynthesis in plants produced in vitro. Increasing the CO₂ concentration in vitro can lead to an increase in the carboxylase and a decrease in the oxygenase activity of ribulose-1,5-bisphosphate

carboxylase oxygenase, resulting in improved carbon assimilation (Reddy et al., 2010; Rybczynski et al., 2007). The low net photosynthetic rate, and consequently low growth rate of plantlets after transplanting, is usually due to low CO₂ concentration in tightly closed cultivation vessels (Pospisilova et al., 2007). In the current study, no significant differences in vegetative growth characteristics were observed between high CO₂ and control plantlets. This could be due to limited duration (30 d) of exposure to high CO₂ concentration. Although improved content of Chl a and Chl a/b ratio and photosynthesis following exposure of in vitro plants to elevated CO₂ has been previously reported (Pospisilova et al., 2000), we did not find a significant change in chlorophyll content (SPAD) (Table 5).

In the present study, elevated CO₂ concentration in the vessel headspace altered stomatal functioning and improved ex vitro acclimatization of walnut plantlets. Similarly, stomatal conductance was decreased in tobacco plantlets treated with ABA and acclimated under elevated CO₂ concentration (Pospisilova et al., 2000). Furthermore, decreased stomatal conductance and improved plant water status were observed in tobacco plantlets after transplantation under elevated CO₂ concentration (Pospisilova et al., 1999). Also, improved stomatal function was observed in *Dianthus Caryophyllus* L. leaves grown under CO₂ applied by forced

Table 1. Stomatal features of walnut tissue-cultured plantlets (cv. Chandler) developed under two CO₂ concentrations. Micropropagated shoots were grown in jars with a CO₂-releasing solution in the headspace of the culture vessels (CO₂) or without the CO₂-releasing solution as control plantlets (C). The samples were incubated at $25 \pm 2^\circ\text{C}$ under 16/8 h light/dark cycles.

Stomatal traits	CO ₂	C	Significance
Stomatal length (μm)	24.85 ± 0.79	26.33 ± 0.51	0.1566 ^{NS}
Stomatal width (μm)	17.92 ± 0.93	19.87 ± 0.60	0.1181 ^{NS}
Pore length (μm)	14.62 ± 0.91	16.11 ± 0.31	0.1617 ^{NS}
Stomatal aperture (μm)	4.022 ± 0.18	6.018 ± 0.14	<0.0001**
Stomatal length/width	1.405 ± 0.031	1.344 ± 0.032	0.2133 ^{NS}
Stomatal density (no. mm^{-2})	352.5 ± 9.5	359.9 ± 5.69	0.5235 ^{NS}
Stomatal area (μm^2)	321.3 ± 26.84	367.1 ± 16.57	0.1849 ^{NS}

NS, **Nonsignificant or significant at $P \leq 0.01$, respectively.

Fig. 2. Stomata on abaxial surface of in vitro-grown walnut (cv. Chandler) leaves developed under ambient or elevated CO₂ concentrations. Micropropagated shoots were grown in jars (A) without CO₂-releasing solution as control plantlets or (B) with CO₂-releasing solution in the headspace of culture vessels. The samples were incubated at $25 \pm 2^\circ\text{C}$ under 16/8 h light/dark cycles.

ventilation. This corresponded with increased K^+ concentration in the guard cells and increased free ABA content (Majada et al., 1997).

Generally, heterotrophic plantlets show abnormal leaf anatomy and inadequate water control (Brainerd and Fuchigami, 1982; Galzy and Compan, 1992) and the transpiration rate at the beginning of acclimatization of in vitro plantlets is commonly very high but decreases after adaptation to ex vitro

conditions (Chaari-Rkhis et al., 2011; Hazarika, 2006; Pospisilova et al., 1999). However, if water loss can be controlled by environmental conditions and initial stomatal function improved, then survival and growth during the acclimatization period can be improved. Therefore, the objective of this article was to study the effect of elevated CO_2 concentration in culture vessel headspace on growth rate, water relations and stomatal function, all of which potentially influence the acclimatization phase and mortality of walnut tissue culture plantlets during the transfer to ex vitro condition.

Elevated CO_2 concentration in the headspace of culture vessels increased leaf stomatal density relative to controls (Table 1). Less functional stomata with wide apertures have been reported previously in leaves of plants produced under high RH and low CO_2 concentrations (Aliniaiefard and van Meeteren, 2013, 2014, 2016; Zobayed et al., 1999). Stomatal function at a high CO_2 concentration may be mediated by an increased K^+ concentration in the guard cells and the free ABA content of leaves (Majada et al., 1997; Pospisilova et al., 2000). Low foliar ABA content has been suggested as a cause of stomatal malfunctioning and high water loss in high RH-grown plants (Arve et al., 2013; Fanourakis et al., 2011; Rezaei Nejad and Van Meeteren, 2007; Santamaria et al., 1993). This is because of the limited plant ability to produce ABA owing to low evaporative

transpiration rate (i.e., in vitro condition) (Aliniaiefard and van Meeteren, 2013, 2014). RH in closed in vitro containers is kept close to 100%; thus, the plantlets are never exposed to evaporative difference which is necessary to induce the synthesis of ABA (Santamaria et al., 1993). Accumulation of ABA in leaves developed under elevated CO_2 concentration has been also reported (Majada et al., 1997; Pospisilova et al., 2000). In agreement with our results, stomatal closure in response to high CO_2 concentration was also observed in tobacco and grapevine plantlets (During and Stoll, 1996; Pospisilova et al., 1999). In this study, the number of small-sized stomata increased in response to high CO_2 (Figs. 2 and 3). Formation of more small stomata with narrow apertures resulted in decreased transpiration rate and, as a result, improved control of water loss during desiccation. It has been shown that stomatal morphology correlates with stomatal responsiveness to desiccation and that smaller stomata have a more rapid response to closing stimuli (Hetherington and Woodward, 2003). High transpiration rates during desiccation of the tissue-cultured plantlets can be due to stomatal abnormalities (Brainerd and Fuchigami, 1982; Grout and Aston, 1977; Hazarika, 2006), reduced leaf epicuticular wax (Grout and Aston, 1977; Shackel et al., 1990; Sutter, 1988), and high stomatal density (Desjardins et al., 1987; Hazarika, 2006). Our previous study (unpublished data) showed that the role of cuticular transpiration is much lower than the role of stomatal transpiration during ex vitro desiccation of in vitro-produced leaves. In the current study, the E of CO_2 -treated walnut plantlets was almost half that of the control plantlets (Fig. 4A), resulting in higher RWC during ex vitro desiccation (Fig. 4B).

The first phase of water loss during ex vitro desiccation depends on the original leaf stomatal density and aperture. Since the stomatal density of walnut in in vitro leaves was not significantly increased by high CO_2 treatment, stomatal aperture was the main determinant of water loss during the first phase of acclimatization (span in Tables 2 and 3; top in Tables 2 and 4). Stomatal functionality, another key determinant of water loss during desiccation (Aliniaiefard et al., 2014), was not a determinant factor for water loss in the current study (slope in Tables 2 and 4).

RWC50 of the $E \times RWC$ curve was 81% for the leaves of CO_2 -treated plantlets and 58% for control plantlets. This indicates that the ability of in vitro CO_2 -treated leaves to conserve their water content during ex vitro desiccation is superior to that of the control leaves (38% for CO_2 -treated leaves and just 7% in control leaves). Similarly, RWC of *Pfaffia glomerata* (Spreng.) was increased when vessel atmosphere was enriched with high CO_2 (Saldanha et al., 2013).

Compatible solutes (proline and glycinebetaine) and tissue water content were not influenced by in vitro CO_2 (Table 5). However, foliar ψ_s was decreased by the CO_2

Fig. 3. Variation in stomatal size on leaves of in vitro-grown walnut (cv. Chandler) microshoots exposed to ambient or elevated CO_2 concentrations. Micropropagated shoots were grown in jars with CO_2 -releasing solution in the headspace of culture vessels (CO_2) or without CO_2 -releasing solution as control plantlets (C). The samples were incubated at $25 \pm 2^\circ C$ under 16/8 h light/dark cycles.

Fig. 4. (A) Leaflet transpiration rate, (B) relative water content and (C) transpiration rate as a function of RWC during 1.5 h of ex vitro desiccation of leaves excised from tissue cultured walnut microshoots (cv. Chandler). In vitro-formed leaves were grown under either ambient or elevated CO_2 concentrations. Micropropagated shoots were grown in jars with CO_2 -releasing solution in the headspace of culture vessels (CO_2) or without CO_2 -releasing solution as control plantlets (C). For desiccation, the leaves of plantlets were detached and placed with their abaxial side up on a balance in an environment with 50% relative humidity, $21^\circ C$, and $50 \mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance, resulting in 1.24 kPa vapor pressure deficit. The water loss of the leaves was recorded every 5 min for a duration of 90 min.

Table 2. Parameters of curve fitting for transpiration rate (E) during 1.5 h of ex vitro leaf desiccation in tissue cultured walnut plantlets (cv. Chandler) exposed to two CO₂ concentrations. Micropropagated shoots were grown in jars with CO₂-releasing solution in the headspace of culture vessels (CO₂) or without CO₂-releasing solution as control plantlets (C). For desiccation, the leaves of microshoots were detached and placed with their abaxial side up on a balance in an environment with 50% relative humidity 21 °C, and 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ irradiance, resulting in 1.24 kPa vapor pressure deficit. The water loss of the leaves was recorded every 5 min for a duration of 90 min.

	Top	Bottom	Slope	Half-life	Span
CO ₂	2.052 ± 0.114 a	0.15 ± 0.038 a	3.06 ± 0.32 a	0.226 a	1.902 ± 0.102 a
C	3.869 ± 0.156 b	0.1195 ± 0.041 a	3.261 ± 0.23 a	0.212 a	3.749 ± 0.141 b

Table 3. Parameters of curve fitting for relative water content during 1.5 h ex vitro desiccation of leaves from tissue cultured walnut microshoots (cv. Chandler) exposed to ambient and elevated CO₂ concentrations. Micropropagated shoots were grown in jars with CO₂-releasing solution in the headspace of culture vessels (CO₂) or without CO₂-releasing solution as control plantlets (C). For desiccation, detached leaves were placed with their abaxial side up on a balance in an environment with 50% relative humidity, 21 °C and 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ irradiance, resulting in 1.24 kPa vapor pressure deficit. The water loss of the leaves was recorded every 5 min for a duration of 90 min.

	Bottom	Slope	Half-life	Span
CO ₂	35.05 ± 3.97 a	2.02 ± 0.37 a	0.343 a	75.39 ± 4.60 a
C	10.06 ± 1.31 b	2.73 ± 0.15 a	0.253 b	112 ± 2.81 b

Table 4. Parameters of curve fitting for $E \times \text{RWC}$ during 1.5 h ex vitro desiccation of leaves from tissue cultured walnut microshoots (cv. Chandler) exposed to ambient and elevated CO₂ concentrations. Micropropagated shoots were grown in jars with CO₂-releasing solution in the headspace of culture vessels (CO₂) or without CO₂-releasing solution as control plantlets (C). For desiccation, detached leaves were placed with their abaxial side up on a balance in an environment with 50% relative humidity, 21 °C and 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ irradiance, resulting in 1.24 kPa vapor pressure deficit. The water loss of the leaves was recorded every 5 min for a duration of 90 min.

	Top	Slope	RWC50
CO ₂	2.2 ± 0.214 a	0.0246 ± 0.002 a	81.77 ± 3.86 a
C	3.14 ± 0.163 b	0.0240 ± 0.003 a	58.82 ± 2.04 b

Table 5. Effects of elevated CO₂ concentration in the headspace of culture vessels on vegetative characteristics, ψ_s (MPa) and compatible solutes of walnut tissue cultured leaves (cv. Chandler). Micropropagated shoots were grown in jars with CO₂-releasing solution in the headspace of culture vessels (CO₂) or without CO₂-releasing solution as control plantlets (C). The samples were incubated at 25 ± 2 °C under 16/8 h light/dark cycles.

	CO ₂	C	Significance
Proline ($\mu\text{mol}\cdot\text{g}^{-1}\text{DM}$)	5.136 ± 0.059	4.917 ± 0.083	0.0982 ^{ns}
Glycinebetaine ($\mu\text{mol}\cdot\text{g}^{-1}\text{DM}$)	1.617 ± 0.046	1.530 ± 0.100	0.4747 ^{ns}
Osmotic potential (MPa)	-1.470 ± 0.031	-1.067 ± 0.033	0.0009**
Shoot length (cm)	5.833 ± 0.185	5.833 ± 0.088	1.000 ^{ns}
SLA ($\text{cm}^2\cdot\text{g}^{-1}$)	472.9 ± 16.70	564.1 ± 46.62	0.1565 ^{ns}
Chl content (SPAD)	21.533 ± 2.17	20.911 ± 1.24	0.8158 ^{ns}
LWCA	0.0078 ± 0.0002	0.0084 ± 0.0001	0.1489 ^{ns}
LMA	0.0021 ± 0.0000	0.0018 ± 0.0001	0.6814 ^{ns}
LWC	0.787 ± 0.004	0.823 ± 0.012	0.8158 ^{ns}

ns, **Nonsignificant or significant at $P \leq 0.01$, respectively.

SLA = specific leaf area; Chl = chlorophyll; LMA = leaf mass area; LWCA = leaf water content per unit area; LWC = leaf water content.

treatment (Table 5). ψ_s is directly influenced by sucrose content of the culture medium (de Paiva Neto and Otoni, 2003) and interferes with the water content of in vitro cultivated plant tissues (Cha-um et al., 2011). Sugar is normally used as a source of carbon to maintain positive carbon balance in the tissue culture process (Kwa et al., 1995). It appears that in the current study, the presence of both CO₂ gas and sugar as carbon sources (photomixotrophic system) resulted in a decrease in leaf ψ_s in the CO₂-treated plantlets. Several studies have shown that in vitro growth under photoautotrophic conditions and a CO₂-enriched atmosphere can lead to a greater increase in biomass accumulation than under photomixotrophic conditions (Badr et al., 2011; Kozai, 2010; Saldanha et al., 2013;

Solarova and Pospisilova, 1997). However, vegetative characteristics in the current study were not influenced by elevated CO₂ concentration. Similarly, the presence of sugar in the medium combined with high CO₂ concentration in the atmosphere of culture vessels did not result in improved growth of fern (Kwa et al., 1995). Short exposure time to the high CO₂ and also low light intensity could be reasons for the lack of growth improvement in present study.

Despite of an increase in compatible solutes, no significant treatment differences were found in proline or glycinebetaine content (Table 5). Content of compatible solutes such as proline can be increased by reducing in RWC or by structural damage in plant leaves (Taylor, 1996). In the current

study, LWC also was not altered by high CO₂ concentration (Table 5).

Improved ex vitro acclimatization due to elevated CO₂ concentration in tissue culture vessels has been shown in several plant species (Buddendorf-Joosten and Woltering, 1994; Carvalho et al., 2002; Pospisilova et al., 2000; Solarova and Pospisilova, 1997). In the present study employing leaves of in vitro-grown walnut microshoots we found exposure to increased CO₂ concentration resulted in generation of more small-sized and fewer large-sized stomata and found no negative effects on plantlet vegetative characteristics. Alterations in stomatal traits led to improved resistance to ex vitro desiccation. This improvement was mainly due to decrease in the first phase of water loss during the dynamic response of the leaf to ex vitro desiccation. Therefore, increasing CO₂ concentration during in vitro culture of walnuts can be a useful tool to reduce ex vitro water loss in a species that is particularly sensitive to desiccation during ex vitro acclimation.

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