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Alterations in growth and crassulacean acid metabolism (CAM) activity of *in vitro* cultured cactus

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Abstract

Unlike C-3 plants, cacti possess a crassulacean acid metabolism (CAM) physiology that can alter the pattern of carbon uptake and affect plant growth under artificial environmental conditions, especially in tissue culture. *In vitro*-derived plantlets of *Coryphantha minima* grew 7-fold larger than plants cultured under similar *ex vitro* conditions. Growth regulators incorporated into the culture media during shoot proliferation stage of micropropagation had a strong influence on this increased growth. Other important factors that contributed to increased growth under *in vitro* conditions were high relative humidity and sugar in the culture medium. An analysis of gas exchange and daily fluctuations of malic acid levels revealed an increase in net photosynthetic rate, in terms of carbon assimilation, by *in vitro* plants compared with that of *ex vitro* plants. This stimulated photosynthesis in the presence of an external carbon source was unexpected but apparently true for cacti exhibiting CAM physiology. Unlike CAM plants grown in *ex vitro* conditions, net CO_2 uptake by *in vitro*-cultured cacti occurred continuously in the light as well as the dark. Once regenerated, cacti were transferred to *ex vitro* conditions where the normal CAM pathway resumed with a concomitant reduction in growth and CO_2 uptake. These results showed that growth of cacti can be considerably accelerated by *in vitro* culture.

Abbreviations: CAM – crassulacean acid metabolism; PPF – photosynthetic photon flux

Introduction

Several cacti, particularly endangered species, have been successfully propagated *in vitro* by axillary and adventitious shoot proliferation using different explant sources. These include seedling epicotyls, apical tips, excised areoles or portions of callus (Mauseth, 1979; Vizkot and Jara, 1984; Ault and Bagamon, 1985; Martínez-Vázquez and Rubluo, 1989; Clayton et al., 1990; Malda, 1996). Most of these endangered species are difficult to cultivate and are naturally slow growing. For unknown reasons, *in vitro* conditions of tissue culture seem to stimulate growth rates of cacti compared to *ex vitro* conditions. This was observed with *Mammillaria woodsii* Craig in which plants grown from seed required a year or more to achieve the same size as plants grown a few months in tissue culture (Vizkot and Jara, 1984). Similar observations were reported for *Mammillaria prolifera* Miller (Minocha and Mehra, 1974); *Esposotoa huanucoensis* Ritter (Angris and Mehra, 1982) and *Cephalocereus senilis* (Haw.) Pfeiffer (Bonnes et al., 1993).

It is generally assumed that most plants cultured *in vitro*, particularly C-3 plants, experience reduced growth due to a decrease in photosynthetic activity as a consequence of several factors (Kozai, 1991). *In vitro* culture vessels are also known to restrict $CO_2$and air exchange as well as increase the relative humidity and ethylene concentration in the plant environment (Kozai, 1991). As a result, photosynthetic activity and

carbon acquisition are decreased and stomatal development, epicuticular wax development and general plant performance are altered (Sutter, 1988; Capellades et al., 1990; Jackson et al., 1991; DeYue et al., 1992; Cassells and Walsh, 1994; Santamaría and Kerstiens, 1994). To compensate for this, *in vitro* plants are cultured in a sucrose-rich media, whereby sucrose acts as the primary carbon and energy source for plant growth (Langford and Wainwright, 1988; Kozai, 1991).

The particular response of cacti to *in vitro* environments might be related to their CAM physiology. High humidity in the culture vessel might permit stomata of cacti to remain open during daylight and result in continuous CO_2 fixation during the light and dark periods. Under normal conditions, CAM plants exhibit a very short transition from nocturnal to diurnal CO_2 assimilation (Griffits, 1988). It is possible that *in vitro* cultured cacti assimilate carbon constantly, resulting in continuous and rapid growth. In this study we show that *in vitro* conditions do, in fact, stimulate the growth and alter the CAM activity of a rare and endangered cactus species, *Coryphantha minima* Baird.

Materials and methods

Plant culture

Seeds of *Coryphantha minima* were aseptically germinated, and apical tips from seedlings were transferred to culture medium containing Murashige-Skoog (MS) salts (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 0.40 mg l^{-1} thiamine–HCl, 555 μMyo-inositol, 80 mg l^{-1} adenine sulfate–2H_2O; and the combinations of 4.40 μM benzyl-adenine (BA) with 0.27 μM naphthalene acetic acid (NAA) and 46.0 μM kinetin with 5.71 μM indole acetic acid (IAA). Culture media was solidified with 8 g l^{-1} agar and adjusted to a pH 5.6 prior to autoclaving at 121°C.

For *ex vitro* culture, seeds were sown on coarse sand and seedlings transplanted to a potting mixture containing a mixture of (1:1:1) peat, perlite and coarse sand (Sanderson et al., 1986). Plants were watered every 5 days with tap water and every 15 days with 15 ml of one-half strength MS basal inorganic salt solution. Both, *in vitro* and *ex vitro* cultures were maintained in growth chambers at a temperature range of 25/27°C (day/night) and light was provided by cool white-fluorescent (90%) and incandescent lamps (10%), with a 16-h photoperiod. Cultures were maintained under a photon flux (PPF) of 120-130 μmol $m^{-2}s^{-1}$.

Growth rates of *in vitro* new shoots were evaluated under different sucrose levels (0, 1.5, 3 and 4.5%) and in culture vessels that had different closures to increase air exchange rates. Common closures for Magenta G^{TM} culture boxes were compared against modified closures having a 6-mm diameter vent covered with a 0.22-μm microporous filter (Sigma C-3430) that is purported to provide greater air exchange.

Growth measurements

Stem surface and volume were calculated by geometric formulae, assuming that plants approached a cylindrical shape. Diameter and circumference were calculated from imprints made from four cross sections of ten randomly selected plants. A regression analysis was performed to correct the diameter values, so effects of tubercles on the total volume and surface area were considered. In addition, fresh weight was also determined. Measurements were taken monthly on a minimum of four replicates per treatment. *Ex vitro* cultured plants were always measured 1 day after watering. Volume and fresh weight data were subjected to analysis of variance and initial volumes and fresh weights were examined as co-variables.

Photosynthetic activity

Photosynthetic activity was considered on the basis of net carbon fixation rates, which were determined by gas exchange and daily malic acid fluctuations. CO_2 exchange was measured on whole plants with a LI-6200 portable photosynthesis system (LI-COR Inc., Lincoln, NE), operating with closed system measurements. Assimilation chambers to in situ measure *in vitro* gas exchange, were made out of a culture vessel (Magenta^{TM} GA) holding a brush-less micro-cooling fan (Radio Shack, No. 273-244) inside. The gas analyzer sensor head was attached and sealed to the same vessel. This assemblage was directly coupled to each culture vessel containing either *in vitro* grown cacti or *ex vitro* cultured specimens planted in sand in small Petri dishes. The connection between the two vessels was through a polypropylene coupler (Sigma C-0667) and the joint seam was covered with parafilm to seal the system. One week prior to taking gas exchange measurements, mature specimens of *C. minima* were moved from the greenhouse to the growth chamber. One day prior to measurements, all *ex vitro* plants were transplanted into horticultural grade sand in the

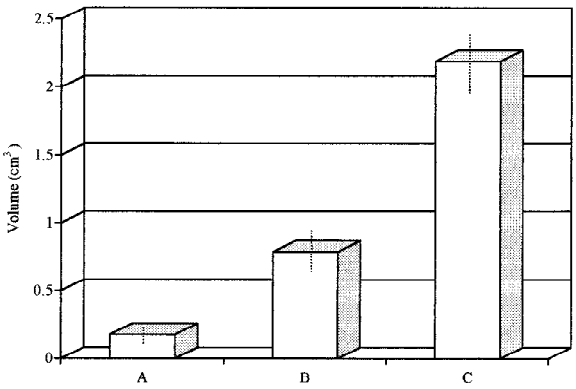

Figure 1. Size comparison of *Coryphantha minima* plantlets cultured under different conditions: (A) One year old seedling, cultured *ex vitro*; (B) 1-year-old seedling cultured *in vitro*; (C) 6-month-old new shoot developed *in vitro* with 10 mg l^{-1} kinetin and 1 mg l^{-1} IAA (n_I = 5).

bottom half of disposable polystyrene Petri dishes and irrigated with 5 ml of distilled water. The culture medium of *in vitro* culture, as well as the Petri dishes were covered with a layer of paraffin–petroleum jelly (2:1) mixture to reduce water evaporation from the sand or agar surface and eliminate CO_2 diffusion due to root respiration at the moment measurements were taken.

Gas exchange measurements were made in the growth chamber on three separate cactus samples during the middle of the dark and light periods. The assimilation chamber was placed in the growth chamber and allowed to equilibrate to growth chamber conditions before the assimilation chamber was closed and data was recorded. Each measurement consisted of three consecutive observations. A 2-ppm change in CO_2 concentration was the criterion for each observation and a time interval of about 200 sec was required to reach that change. The assimilation chamber was always installed in the same position in the growth chamber so that PPF levels and temperature were similar for all measurements. Assimilation chamber temperature and relative humidity remained constant during each measurement interval.

Diurnal fluctuation in tissue acidity was monitored from randomly selected samples (1 g tissue) of *in vitro* and *ex vitro* plants. Sampling was at the beginning, middle and end of the dark period, as well as at the middle of the light period. Acid titration was done according to Pearcy et al. (1989).

Table 1. Comparison of the fractional growth in a 3-month period of *Coryphantha minima* during different stages of micropropagation. Values are increases in volume or weight increases ± the standard error (n_i = 10)

Stage	Increase in volume (cm ^{3})	Increase in fresh weight (g)
Culture initiation	0.049 ± 0.015	0.09 ± 0.01
Shoot proliferation	0.582 ± 0.387	1.90 ± 0.98
Rooting	0.193 ± 0.074	1.32 ± 0.85
Acclimatization	0.313 ± 0.094	0.49 ± 0.24

Results and discussion

Enhanced growth during *in vitro* shoot proliferation of *Coryphantha minima* was confirmed (Figure 1). After 15 months, new shoots produced *in vitro* were almost 7-fold larger than *ex vitro* seedlings of the same age. Size differences were not as pronounced between *in vitro* and *ex vitro* seedlings, suggesting that the presence of compounds in the culture media needed for shoot proliferation influenced this enhanced growth rate. A comparison of growth during each stage of *in vitro* propagation (Table 1) showed that the greatest growth increase occurred during the shoot proliferation stage where cytokinins and auxins were used. This was also observed in a separate study with the cactus *Sclerorebutia alba* where a 15-fold increase in fresh weight occurred when the concentration of benzyladenine was raised from 0 to 1 mg l^{-1} (Dabekaussen et al., 1991).

Enhanced growth was also associated with common conditions prevailing in the tissue culture environment (i.e., a high relative humidity and low ventilation inside the culture vessels). Such conditions were modified by using vented closures, and a significant decrease in plant growth was observed compared to plants cultured in vessels capped with normal closures (see Figure 2). Since a faster drying of the agar along with the absence of condensed water in the interior of the vessels were observed in the treatments using vented closures, a possible reduction of relative humidity could be associated, and this factor may be related to growth reductions. There are documented studies reporting the influence of reduced humidity on growth, such as the reported by Shallanon and Maziere (1992) for rose plants cultured *in vitro*. In those experiments, leaf area, shoot length and shoot multiplication rates decreased when relative humidity was reduced.

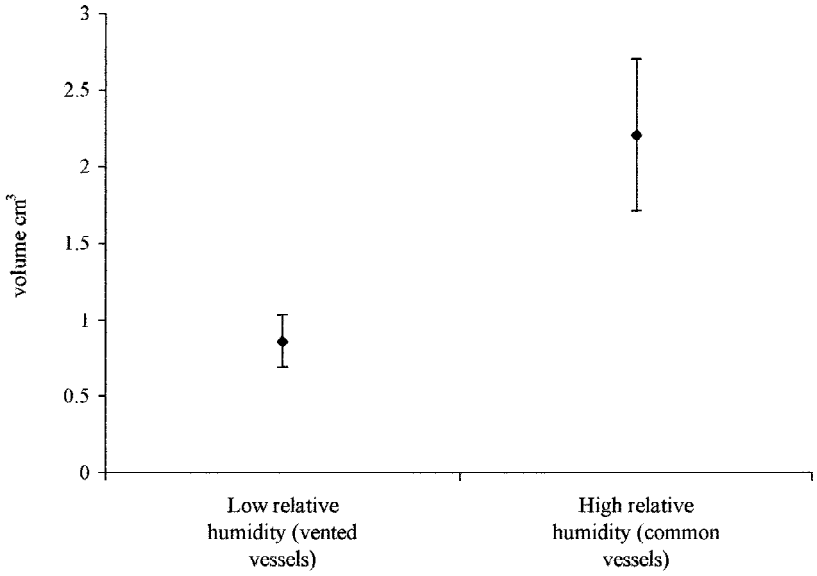

Figure 2. Comparison of average sizes of shoot volumes, after 2 months in culture under different relative humidity conditions. Plants were cultured in MS basal medium supplemented with 0.05 mg l^{-1} naphtalene acetic acid and 1 mg l^{-1} benzyladenine. Bars indicate standard errors. $p(f= 4.98/1,21) = 0.037$ (n_I = 7).

Table 2. Fractional increase in volume and fresh weight in a 45-day period in *Coryphantha minima* cultured under different levels of light and ventilation in the culture vessel (n_I = 20)

Environmental variable	Increase in volume (cm^3)	Increase in fresh weight (g)
Light		
120–200 μmol $m^{-2}s^{-1}$	0.192	0.22
390–400 μmol $m^{-2}s^{-1}$	0.394	0.45
Ventilation		
Culture vessels with normal lids	0.401	0.49
Culture vessels with vented lids	0.182	0.20

Source of variation	Fresh weight	Volume
Light	$p(f$=6.58/1,80)=0.012	$p(f$=2.17/1,80)=0.144
Ventilation	$p(f$=11.94/1,80)=0.001	$p(f$=3.78/1,80)=0.055
Light–Ventilation	$p(f$=5.52/1,80)=0.021	$p(f$=2.12/1,80)=0.144

Ventilation in the culture vessels together with light intensity also influenced *in vitro* growth of *Coryphantha minima*, interacting positively with fresh weight *in vitro* (Table 2). Increasing photon flux from 120–200 to 390–400 μmol $m^{-2}s^{-1}$increased volume and fresh weight of cactus tissue, as non-ventilated closures did, where relative humidity remains high. Similar growth enhancement at higher photosynthetic photon flux (PPF) was related to increased photosynthetic activity in *Cymbidium* orchid, a CAM plant, where net photosynthetic rates increased when PPF was raised from 35 to 102 and 206 μmol $m^{-2}s^{-1}$ (PospíŠilová et al., 1992).

Enhanced growth response in *C. minima* cultured *in vitro* appeared to be related with their carbon metabolism. Titratable acidity in *C. minima* tissue showed that night-time peaks for malic acid accumulation were greater for *in vitro* plants than *ex vitro* cultured cacti (Figure 3). This indicated that carbon dioxide fixation during the dark period in this CAM species was actually higher with *in vitro* conditions. Analogous results were observed when photosynthesis was measured in terms of gas exchange (Figure 4). Here, *in vitro* cultured plants also showed greater net CO_2 uptake rates (1.20 CO_2 μmol $m^{-2}s^{-1}$) than *ex vitro* plants (0.79 CO_2 μmol $m^{-2}s^{-1}$), a 51% increase, during the dark period when CAM plants typically accumulate CO_2 from the air. However, more extraordinary was the observation that net CO_2 uptake also occurred during the light period in plants cultured *in vitro*, when CAM plants ordinarily do not accumulate CO_2, but show a respiratory loss of CO_2. This was observed for *C. minima* plants cultured *ex vitro*. Thus, under *in vitro* conditions this cactus species appears to fix CO_2 continuously in both, light and dark resulting in an alteration of the normal CAM pattern.

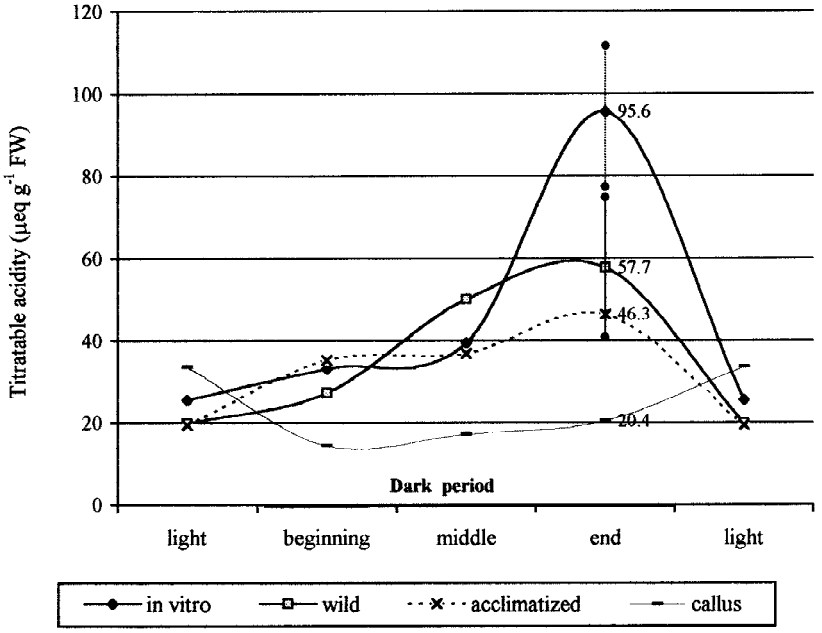

Figure 3. Comparison of daily malic acid fluctuations in *Coryphantha minima* plants cultured *in vitro* and acclimatized for 2 months versus wild plants maintained in greenhouse conditions (n_1 = 3). Bars indicate standard errors where significant differences were found.

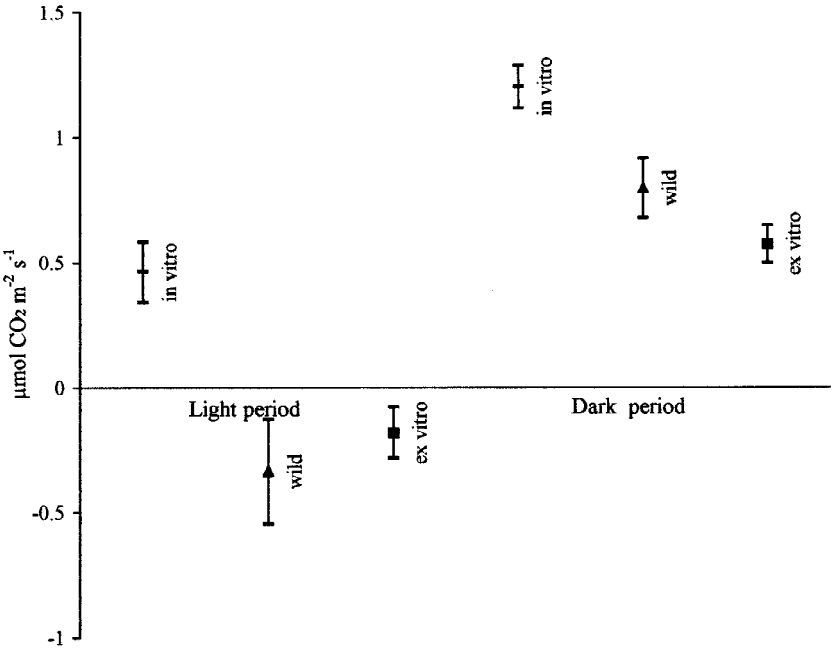

Figure 4. Diurnal and nocturnal CO_2 exchange in *Coryphantha minima* plants cultured *in vitro* compared to micropropagated plants maintained *ex vitro* for 6 months and to wild plants maintained in the greenhouse (n_1 = 6). Bars indicate standard errors.

Table 3. Effects of different sucrose concentrations in the culture medium in volume and fresh weight of *Coryphantha minima*, after 3 months in culture (n_i =6)

Sucrose concentration (%)	Increase in volume (cm^3)	Increase in fresh weight (g)
0	–*	–*
0.5	0.043 ± 0.076	0.002 ± 0.002
1.5	0.248 ± 0.057	0.098 ± 0.008
3.0	0.262 ± 0.045	0.187 ± 0.041
4.5	0.208 ± 0.026	0.060 ± 0.020

$p(f= 3.29 /3,79) = 0.024$ for volume, $p(f= 3.34 /3,79)= 0.022$.
*Plants did not survive under this treatment, and were not considered for the ANOVA.

Upon transfer to *ex vitro* conditions, plants reverted to normal CAM metabolism after 2 months of acclimatization as shown by acid fluctuations, compared to mature plants grown under greenhouse conditions. Dark period acid peaks dropped from 95.6 μeq g^{-1} FW for *in vitro* conditions, to 46.3 μeq g^{-1} FW in acclimatized plants (see Figure 3). However, not all tissue culture stages had the same influence on CAM activity in cacti. Acid fluctuation in non-green, callus cultures showed no nocturnal peaks (Figure 3). Observations by Seeni and Gnanam (1980) showed no diurnal fluctuations of titratable acidity in *Chamaecereus sylvestrii* cell suspension cultures. They proposed that cultured cells possessed small vacuoles that did not store as much organic acid in the dark. Similarly, the absence of a nocturnal acid peak in our callus cultures indicated an alteration of such CAM activity. After shoots regenerated from callus, peaks in nocturnal acidity became evident, indicating that CAM was operational. In other cacti, not grown *in vitro*, it has been shown that seedlings undergo a conversion from C-3 to CAM as seedlings germinate and shoots develop (Altesor et al., 1992). These observations demonstrate the high plasticity of carbon assimilation in obligate CAM plants, such as most of cacti.

Adjusting the sucrose concentration also affected cactus growth *in vitro* (Table 3). Growth was greatest at 3% sucrose, the typical concentration used in tissue culture. Raising or lowering this amount generally caused a reduction in growth. Plants did not survive in the absence of sucrose (Table 3). Growth was significantly reduced at 0.5% sucrose. Similar results were reported for rose where the absence of sucrose killed shoots cultured *in vitro* (Langford and Wainwright, 1988). In tobacco, reductions of sucrose concentration caused a pronounced reduction of root, stem and leaf dry mass (PospíŠilová et al., 1992).

Net CO_2 uptake by *C. minima* cultures was greater as sucrose concentrations increased from 1.5 to 3% (Figure 5). Although the dark period peak of acid accumulation was still observed in 3% sucrose, it was noteworthy that net CO_2 uptake was greater in both light and dark periods under 3 versus 1.5% sucrose. This differs with observations in *Rosa* and *Pieris* (C-3 plants), which had reduced net photosynthetic rates as sugar concentration increased above 1% (Capellades et al., 1990). Therefore, in contrast to the observation that sucrose induces photomixotrophic growth in C-3 plants, sucrose appeared to stimulate CO_2 uptake in *C. minima* cultured *in vitro*. This could be related to a possible greater stomatal opening occurring at 3 than 1.5% sucrose; which is indirectly suggested by transpiration measurements. Figure 6 indicates higher transpiration rates under 3% sucrose in the culture medium, during shoot proliferation stage versus rooting stage where 1.5% sucrose is used. It also shows that *in vitro* plants had higher transpiration rates than *ex vitro* plants; suggesting that *in vitro* cultured shoots have greater stomatal opening. In other species cultured *in vitro*, it has been shown that stomata tend to remain open or to close only partially (Brainerd and Fuchigami, 1982; Conner and Conner, 1984; Diettrich et al., 1992; Santamaría and Kerstiens, 1994). The higher rates and continuous uptake patterns of CO_2 by *Coryphantha minima* suggest such an influence on stomatal behavior. Stomatal opening in CAM plants cultured *in vitro*, needs to be verified using porometric or anatomic measurements.

The decrease in net CO_2 uptake and transpiration rates observed during acclimatization by *C. minima* plantlets (see Figures 3, 4 and 6) suggests that stomata close in response to a change in relative humidity and culture conditions. Photosynthetic data (Figures 3 and 4) indicated that acclimatized plants have lower rates than wild specimens. In contrast to cacti during acclimatization, most C-3 plants show a net increase in photosynthesis when plants are transferred from *in vitro* to *ex vitro* conditions (Donelly and Vidvaver, 1984; Capellades et al., 1990; Kozai, 1991; DeYue et al., 1992). For example, PospíŠilová et al., (1992) reported that net photosynthesis increased from 5.34 μmol CO_2 $m^{-2}s^{-1}$ to 6.48 $\mu m^{-2}CO_2 m^{-2}$ s^{-1} on *in vitro* tobacco plants acclimated after 3 weeks.

While acclimatized *C. minima* plants exhibited low overall CO_2 fixation, plant survival rates were high. It is possible that during acclimatization, stomata remain

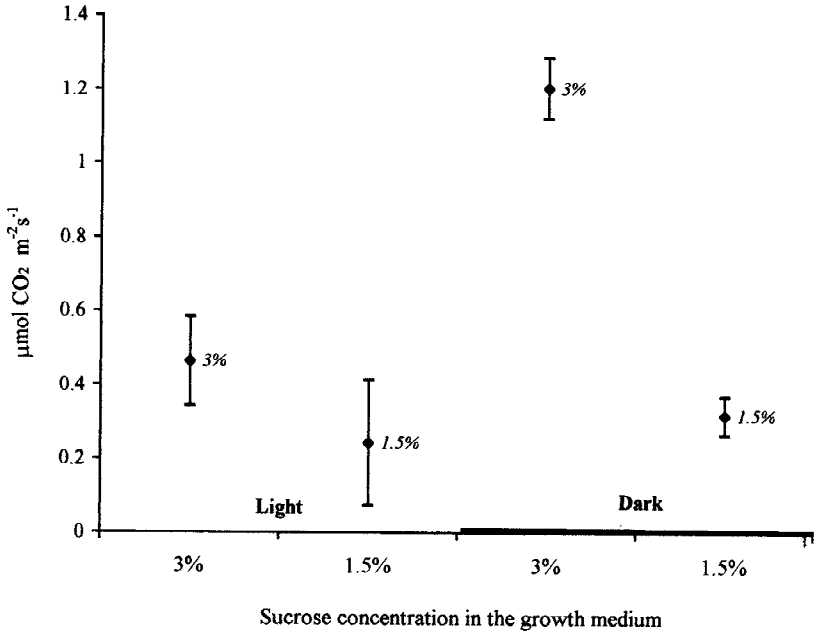

Figure 5. Diurnal and nocturnal gas exchange of *Coryphantha minima* cultured *in vitro* with different sugar concentrations in the culture medium (n_1 = 6). Bars indicate standard errors.

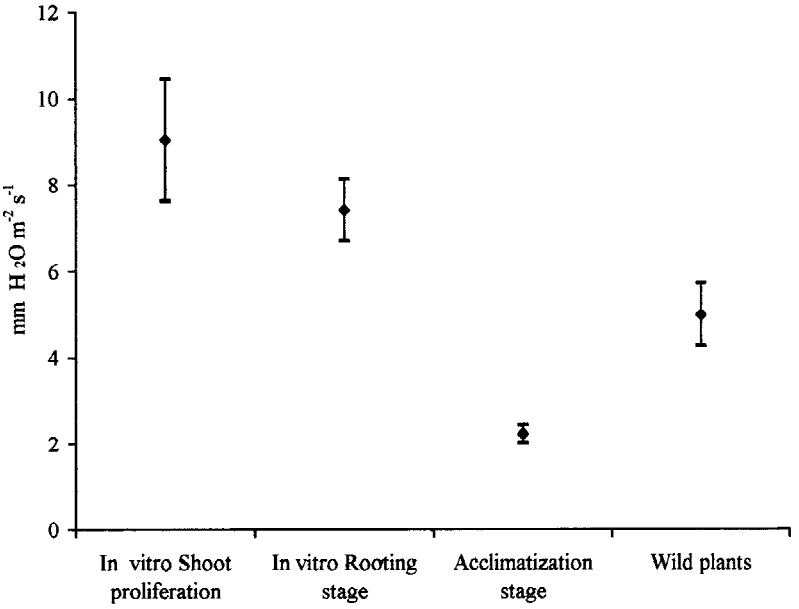

Figure 6. Comparison of transpiration rates of *Coryphantha minima* in different stages during *in vitro* culture versus wild plants maintained in the greenhouse (n_1 = 6). During shoot proliferation the culture medium was supplemented with 3.0% sucrose, whereas 1.5% was used for *in vitro* rooting. Bars indicate standard errors.

closed and internal CO_2 was re-fixed. This remains to be tested; however, it is well documented that CAM plants offer some plasticity with regard to changes in water availability, in particular as it relates to stomatal closure and re-fixation of internal CO_2 (Griffits, 1988). This may lead to enhanced abilities to overcome stress during transfer from the culture vessels to the outside environment.

In addition to the possibility of stomatal closure, the decline in net photosynthetic rates during acclimatization of *in vitro* derived cactus could also be related to the removal of the rich medium supplied by MS formulation, when plants are transferred to potting mixtures. Growth and photosynthesis rates are affected by a sudden decrease in nutrients in several species, including some C-3 plants (Bhojwani and Dhawan, 1988).

Our current understanding of plant development suggests that certain attributes of cacti, such as CAM physiology, may influence the way these plants respond to *in vitro* environments, which contrast with the response observed in typical C-3 or C-4 species.

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