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Growth and nocturnal acid accumulation during early ontogeny of *Agave attenuata* grown in nutrient solution and *in vitro* culture

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Abstract

Dry matter production of shoots and roots and the diurnal fluctuation of titratable acidity of single leaves were investigated in the CAM plant *Agave attenuata* during the first 70 d after germination. The plants were grown either in vermiculite sub-irrigated with a nutrient solution or in *in vitro* cultures on an inorganic nutrient agar. Two types of culture tube covers were used: either airtight closures or polypropylene caps with membranes permeable to air.

In the earliest ontogenetic phases of development (cotyledon and primary leaf stage), the plants were already able to carry out considerable nocturnal organic acid accumulation. *In vitro* cultivated plants, from the beginning of their development, were also capable of diurnal acid fluctuation, though of distinctly weaker activity than the pot plants. The mean relative growth rates (RGR) of pot culture plantlets approached a third of perennial herbaceous plants. Plantlets grown in *in vitro* culture reached only half to the one quarter of the RGR of pot plants. The reduced yield could be attributed to the low CO_2 supply in the culture tubes and the less than optimal water and nutrient supply in the agar medium.

Additional key words: Crassulacean acid metabolism, dry mass, nocturnal acid accumulation, relative growth rate.

Introduction

The diurnal fluctuation of organic acids and the capacity of CAM plants for dark acidification increase during their development until maturation of the leaves and decrease as the leaves become senescent (Ranson and Thomas 1960, Winter and Smith 1996). Examples of this are species of *Agavaceae* (Nobel 1988), *Aizoaceae* (Willert *et al.* 1976, Herppich *et al.* 1992), *Cactaceae* (Altesor *et al.* 1992), *Crassulaceae* (Jones 1975, Medina and Delgado 1976, Nishida 1978), and *Piperaceae* (Ting *et al.*1993). This ageing effect is mainly dependent on an increase

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of PEP carboxylase activity during unfolding and formation of the leaves and its decline during ageing (Zima and Šesták 1985). The induction of the diurnal acid metabolic cycle, although controlled by environmental factors, is primarily determined by the developmental stage of the plant (Cushman *et al.* 1990, Cushman and Bohnert 1996).

It was the intention of this study, acting as an observational basis for questions about developmental switches in metabolic shifts, to investigate the beginning and the temporal course of the CAM capacity of *Agave attenuata* plantlets by measuring the titratable acidity of single leaves. In particular, the aim was to follow the acidity pattern of the cotyledon from germination to senescence. In the experimental set-up, plants were grown in well-watered pots. Additionally, in a parallel experimental set-up with *in vitro* grown plants, the influence of artificial conditions in the culture tubes on the CAM activity was examined.

Materials and methods

Plants and growth conditions: Plants of *Agave attenuata* Salm-Dyck (*A. cernua* Berger), a succulent species native to semiarid habitats of subtropical regions of Mexico, were grown in pots on vermiculite soaked with a nutrient solution and *in vitro* on a solid nutrient medium.

Seeds were germinated in Petri dishes on filter paper moistened with deionized water. Two days after germination, the seedlings were transferred to plastic pots (8 × 8 × 6.5 cm) containing vermiculite with 4 to 5 seedlings per pot. A modified Hoagland solution No. 2 (Hoagland and Arnon 1950), at 1/4 strength, was used. Instead of $FeSO_4 . 7 H_2O$ and tartaric acid, the iron solution of the MS medium (Murashige and Skoog 1962) was used at 1/4 concentration. The pH value was adjusted to 5.7 - 5.8. Pots were saturated with the nutrient solution weekly by immersion. In order to prevent the substrate from drying out, the pots were covered with polyethylene film, leaving holes for the plantlets.

For cultivation *in vitro*, seeds of *A. attenuata* were surface-sterilized in 70 % ethanol for half a minute and in a 5 % NaOCl solution for 15 min, and rinsed with sterile water. Single seeds were placed into culture tubes (50 cm^3) on 20 cm^3 of agar medium. The modified Hoagland solution was solidified with agar (8 g dm^{-3}). At first, all the culture tubes were closed with airtight covers (*Bellco KAP-UTS, Sigma Chemicals*, St. Louis, USA) and sealed with parafilm. On the sixth day after germination, the airtight closure of half the culture tubes was replaced by a polypropylene cover with membrane of 8 mm diameter and 0.02 mm pore width permeable to air (*SUN-CAPs, Sigma Chemicals*, St. Louis, USA).

The cultures were exposed to a 12-h photoperiod under halogen metal vapour lamps *Osram HQI-E 400 W/D*. Irradiance (PAR) was 180 - 210 μmol m^{-2} s^{-1} for the plantlets in pots and 160 - 185 μmol m^{-2} s^{-1} for the plantlets inside the culture tubes. The temperature was 28 ± 2 °C during the light period and 21 ± 1 °C during the dark period. The temperature inside the culture tubes was 1 - 2 °C higher than the ambient

air temperature. The relative air humidity in the growth room was about 30 % during the day and 40 % at night.

Five days before measurement, the plants were exposed to the following controlled conditions in a growth cabinet (*Ecophyt VEPHL 5/1350, Heraeus Vötsch*, Balingen, Germany): 12 h photoperiod, irradiance (PAR) 330 μmol m^{-2} s^{-1} outside and 290 μmol m^{-2} s^{-1} inside the culture tubes, light source 16 cool-white fluorescent tubes (*Osram L65W/20R*) and 3 incandescent lamps (100 W). Temperature during the light period was 25 °C, and during the dark 15 °C. External relative air humidity was about 30 % (day) and 40 % (night). Air flow was 0.2 m s^{-1}.

Growth rate: Fresh and dry matter (oven-dried to constant mass at 80 °C) of 5 to 6 plants of the pot cultures and *in vitro* cultures, respectively, were determined at about fortnightly intervals from germination. Cotyledons, leaf rosettes and roots were examined separately. The mean relative growth rate (Fisher 1921, Květ *et al.* 1971, Hunt 1990) was calculated using the equation $RGR = (\ln W_2 - \ln W_1)/(t_2 - t_1)$, where W_1 and W_2 are the dry masses of plantlets at the times t_1 and t_2. Values represent averages of all the samples weighed together.

Determination of titratable acidity: At the same intervals, 8 plants were sampled at the end of the dark period and at the end of the following light period. The leaves were separated from the plant rosette and sorted according to their age. They were then frozen at about -40 °C, homogenised in distilled water at a ratio of 1:30, and centrifuged. The supernatant was titrated against 0.005 M NaOH until the indicator (phenolphthalein solution, 10 cm^3 dm^{-3} sample) changed colour at pH 8. The strong dilution of the cell sap and the extremely low concentration of the NaOH permitted working with very small sample volumes (1 to 3 cm^3). Replicate measurements were carried out, and the means and standard deviations of the titration acidity were calculated for each successive leaf generation and expressed in μeq(H^+) g^{-1}(f.m.). It was not possible to calculate the acidity values per leaf area for seedlings and small leaf rosettes. The difference between titration acidity at the end of the dark period and the end of the following light period gives a measure of the nocturnal acid accumulation (ΔH^+). The average ΔH^+ for the total rosette was calculated from the titration acidities of the leaf generations.

Results

Plant development: The growth and development of the *Agave attenuata* plantlets showed a clearly different course in the three cultivation types. Only in the unfolding of the cotyledon no difference was noticeable between the treatments, within a week the cotyledon attained its final size.

The seedlings grown in the pots (Fig. 1) developed into well-rooted rosettes within 10 weeks. Their average dry matter was 33 mg per plantlet. Thus, the biomass increased from about 4 mg dry matter at the cotyledon stage by 8.5 times within 53 d.

For *in vitro* cultures, two different culture conditions were established, starting on the sixth day after germination, by using the variants with *KAP-UTS* and *SUN-CAP*

covers. For plants grown in tubes closed by *KAP-UTS*, the primary leaf remained very thin. Each of the successive leaves appeared later than in the other two kinds of cultivation and their growth was poor (Fig. 2*A*). After 10 weeks, the seedlings from tubes closed by *KAP-UTS* produced a maximum of 3 successive leaves and the average dry biomass of the plantlets (4.4 mg) reached only about 13 % of the dry

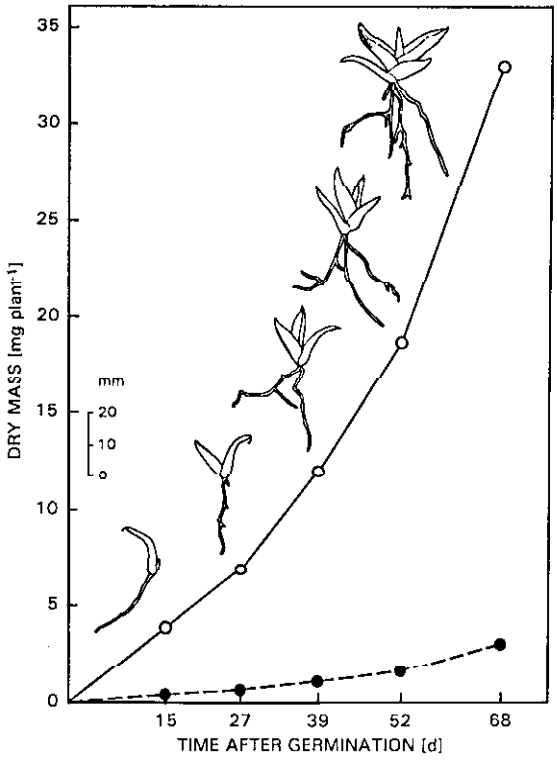

Fig. 1. Biomass increase of *Agave attenuata* growing on vermiculite soaked with nutrient solution. *Circles* - total dry matter per plantlet, *dots* - dry matter of roots per plantlet. Means of 5 - 6 samples. *Length scale* [mm] for natural size of the plantlets

matter production of the plants grown in the pot culture. The plantlets grown in tubes closed by *SUN-CAPs* had formed a maximum of 4 small leaves with characteristically formed dentated leaves (Fig. 2*B*). Their average dry matter (14.9 mg) was about 45 % of the total dry matter of the pot plants. From the cotyledon stage until the end of the experiment, biomass increased by 1.8 times (*KAP-UTS*) and 4.3 times (*SUN-CAPs*).

Root matter of pot grown *Agave attenuata* was on average 10 % of the total plant dry matter. Plantlets grown in tubes with *KAP-UTS* developed very long, unbranched roots, with 16 - 17 % of the total plant dry matter. For plants grown with *SUN-CAPs*, the proportion of roots increased from 11 % at the cotyledon stage to 19 % within the following weeks. At the end of the experiment, as the growth of the plantlets stagnated (Fig. 2*B*), the root matter attained 27.8 % of the total dry matter. In

vermiculite soaked with a nutrient solution, the minerals are more easily available than in a nutrient agar medium, therefore the higher root:shoot ratio of *in vitro* cultivated plantlets indicate a suboptimal availability of water and nutrients.

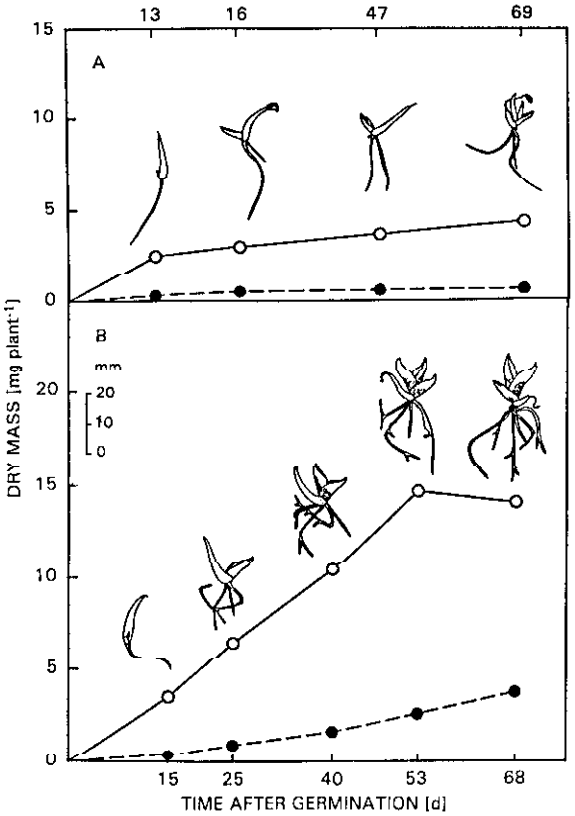

Fig. 2. Biomass increase of *Agave attenuata* growing *in vitro* (*A*) Tubes covered with conventional airtight closures (*KAP-UTS*) and (*B*) with air permeable closures (*SUN-CAPs*). *Circles*: total dry matter per plantlet, *dots*: dry matter of roots per plantlet.

Relative growth rate: During the first stage of the seedlings development (unfolding of the primary leaf), the mean RGR for the pot culture was about 0.049 g g^{-1} d^{-1}. Up to the fourth leaf stage it was on average 0.04 g g^{-1} d^{-1}. The RGR of plants cultivated *in vitro* with *SUN-CAPs* was between 0.04 - 0.02 g g^{-1} d^{-1}. With *KAP-UTS*, during the formation of the primary leaf, it was 0.014 g g^{-1} d^{-1} and later it was 0.01 - 0.008 g g^{-1} d^{-1}, therefore considerably smaller than the RGR of the nutrient solution culture. Nobel (1988) quotes a mean RGR of about 0.027 g g^{-1} d^{-1} for 10-d-old seedlings of *Agave deserti* grown under wet conditions in the field, older seedlings grow slower. Succulents in a temperate climate, in which CAM plants predominantly assimilate carbon *via* the C_3 pathway (Schuber and Kluge 1981, Wagner and Larcher 1981), approach higher relative growth rates. For example, species of *Sedum* reach between 0.04 - 0.15 g g^{-1} d^{-1} (Grime and Hunt 1975, Woodward and Pigott 1975).

Diurnal organic acid fluctuation: In order to make possible the study of developing CAM activity in the growing rosette, leaves were analysed separately according to their ontogenetic sequence (cotyledon, primary leaf and successive leaves).

In the *pot culture*, a distinct diurnal fluctuation of acid accumulation in the cotyledon was already noticeable at the time of the first measurement, 13 d after germination (Table 1). The nocturnal acid accumulation of the cotyledon, which at the time of the first measurement had reached its final size, was 67.6 % of the maximum value measured on the 26th day after germination [176 μeq g^{-1}(f.m.)]. After 10 weeks, the nocturnal acid accumulation of ageing cotyledons was on average still about 86 % of the maximum value (Fig. 3).

For the rosette leaves it was possible to reveal a diurnal acid dynamic about a week after their appearance. The primary leaf became visible around the 10th day after germination for most experimental plants, but due to its small size it could not be analysed separately for the titrations carried out on the 13th day after germination. On the 26th day after germination (2 weeks after unfolding), the primary leaf reached half of its final mass and the nocturnal acid accumulation at this stage was about

Table 1. Titrable acidity at the end of the light and dark period, and nocturnal acid accumulation during leaf ontogeny of *Agave attenuata* growing on vermiculite soaked by a nutrient solution. Means ± S.D. (n = 8 plantlets).

Sequence of leaves	Plant age [d]	Mean fresh mass of leaf [g]	Organic acidity end of light period ΔH^+ [μeq g^{-1}(f.m.)]	Organic acidity end of dark period ΔH^+ [μeq g^{-1}(f.m.)]	Acid accumulation during dark period ΔH^+ [μeq g^{-1}(f.m.)]
Cotyledon	13	0.0449	34.8 ± 0.2	153.9 ± 6.9	119.1 ± 4.9
	26	0.0487	26.0 ± 0.3	201.9 ± 1.1	175.9 ± 0.8
	40	0.0500	23.8 ± 0.8	198.8 ± 3.7	174.8 ± 2.7
	53	0.0428	23.4 ± 0.7	187.4 ± 4.4	164.0 ± 3.2
	70	0.0360	23.5 ± 0.3	175.7 ± 3.0	152.2 ± 2.1
Primary leaf	26	0.0382	24.5 ± 0.2	151.6 ± 4.9	127.1 ± 3.5
	40	0.0721	23.8 ± 1.8	190.7 ± 2.9	166.9 ± 2.4
	53	0.0662	24.6 ± 1.9	208.9 ± 3.9	184.3 ± 3.1
	70	0.0729	23.3 ± 0.5	217.8 ± 10.3	194.5 ± 7.3
2nd rosette leaf	26	0.0038	32.7 *	36.3 *	3.6 *
	40	0.0575	26.6 ± 0.6	152.8 ± 3.8	126.2 ± 1.4
	53	0.0917	33.0 ± 1.7	194.9 ± 5.3	161.9 ± 3.9
	70	0.1168	31.0 ± 1.6	235.5 ± 3.7	204.5 ± 2.8
3rd rosette leaf	40	0.0038	29.7 *	45.2 *	15.5 *
	53	0.0510	34.4 ± 1.0	129.5 ± 3.8	95.1 ± 2.8
	70	0.1454	34.7 ± 2.1	209.3 ± 7.1	174.6 ± 5.2
4th rosette leaf	70	0.0602	36.8 ± 0.9	116.2 ± 4.6	79.4 ± 1.6

* no replicates

Table 2. Maximum acid accumulation, ΔH^+, [μeq g^{-1}(f.m.)] of leaves of *Agave attenuata* grown in pots and in *in vitro* culture. Numbers in brackets - days after germination with highest activity.

Leaves	Pot culture	*In vitro* culture with *SUN-CAP* cover	*In vitro* culture with *KAP-UTS* cover
Cotyledon	175.9 ± 3.2 (26)	101.7 ± 8.1 (26)	54.7 ± 6.5 (13)
Primary leaf	194.5 ± 7.3 (70)	83.7 ± 6.6 (26)	39.2 ± 0.4 (70)
2nd rosette leaf	204.5 ± 2.8 (70)	116.1 ± 1.1 (40)	71.0 * (70)
3rd rosette leaf	174.6 ± 5.2 (70)	83.3 ± 4.6 (70)	37.0 * (70)

* no replicates

65 % of the maximum activity. The successive rosette leaves could be investigated from a very early stage of development. The third leaf, still folded, already showed signs of minimal nocturnal acid accumulation [15.5 μeq g^{-1}(f.m.)] at the first measurement. During the following 2 - 3 weeks these leaves reached their full CAM activity.

The minimal cell sap acidities measured at the end of the light period changed little throughout the leaf development. For cotyledon and the primary leaf they

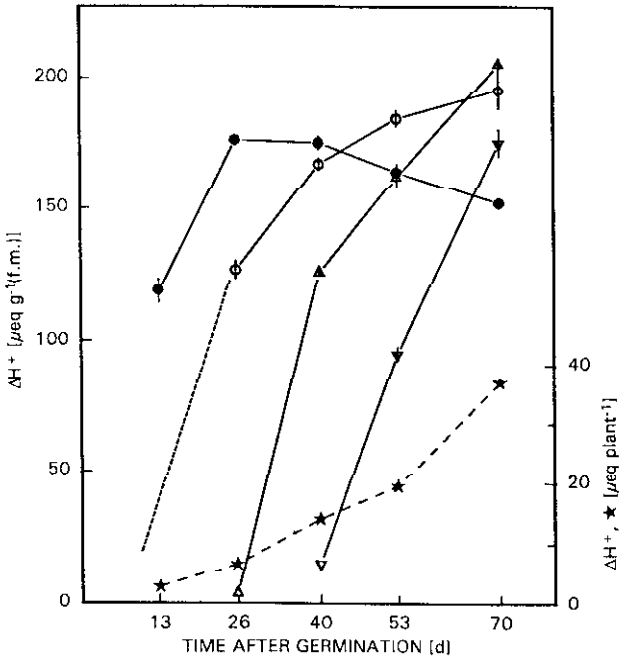

Fig. 3. Nocturnal accumulation of titratable acids of *Agave attenuata* as a function of plant age (days after germination). Plants from pot cultures with nutrient solution. Individual leaves (*left scale*): cotyledon (*dots*), primary leaf (*circles*), successive rosette leaves (*triangles*). Total nocturnal acid accumulation per seedling or rosette (*asterisks, right scale*). Means ± S.D., n = 8 leaves.

remained within a narrow region of 23 - 25 μg g^{-1}(f.m.). The values measured for successive rosette leaves were mainly between about 30 - 35 μg g^{-1}(f.m.).

The capacity for nocturnal acidification was considerably lower for plants cultivated *in vitro* than for pot plants. The maximum nocturnal acid accumulation per g fresh matter of leaves of *in vitro* grown plants covered with air-permeable *SUN-CAPs* reached about half the value of pot grown cultures. For *in vitro* grown plants with airtight *KAP-UTS* it was only 15 - 35 % (Table 2).

Discussion

Existing studies on the ontogeny of the CAM point to a dominance of the C_3 pathway of photosynthesis during early developmental stages. Nobel (1988) reports CO_2 gas exchange measurements on *Agave deserti* that show a gradual shift from C_3 to CAM typical gas exchange for the period of the 25th to the 400th day after germination. Altesor *et al.* (1992) demonstrated that *Ferocactus recurvus* and *Opuntia pilifera* developed a significant nocturnal acid accumulation from 10 to 14 weeks on, and *Neobuxbaumia tetezo* from the 20th week after germination. However, these authors observed a weak trend of nocturnal acid accumulation in seedlings during the first 3 weeks after germination, which disappeared after the cotyledons withered. This indicates, that the cotyledons of some *Cactaceae* are able to exhibit CAM activity. A nocturnal accumulation of malic acid in cotyledons of *Mesembryanthemum crystallinum*, which only appeared under salt stress, was shown by von Willert *et al.* (1992).

In our studies on well-watered *Agave attenuata*, the CAM activity occurred with the very beginning of the ontogenetic development. The cotyledons already showed a high capacity for acidification (see Tables 1 and 2, Fig. 3) and a typical age-related course until senescence. During the first four weeks of its existence, the cotyledon is the primary source of nocturnal acid accumulation in the seedlings (Table 3). Only later, when the activity of acid accumulation in the cotyledon begins to decrease, and its biomass proportion (fresh mass) of the rosette becomes reduced, is the dark

Table 3. Nocturnal accumulation of titrable organic acids of individual leaves (ΔH^+, μeq per leaf) and of the sum of leaves per plant (ΔH^+, μeq per rosette) of *Agave attenuata* grown in pots for 13, 26, 40, 53 and 70 d. Contribution of single leaves to the whole rosette is shown as percentage value.

Leaves	13 d	26 d	40 d	53 d	70 d
Cotyledon	5.35 (100 %)	8.57 (63.7 %)	8.74 (31.1 %)	7.02 (18.0 %)	5.48 (7.4 %)
Primary leaf	-	4.86 (36.2 %)	12.03 (42.8 %)	12.20 (31.3 %)	14.18 (19.3 %)
2nd rosette leaf	-	0.01 (0.1 %)	7.26 (25.9 %)	14.85 (38.2 %)	23.89 (32.4 %)
3rd rosette leaf	-	-	0.06 (0.2 %)	4.85 (12.5 %)	25.39 (34.4 %)
4th rosette leaf	-	-	-	-	4.79 (6.5 %)
Total	5.35 (100 %)	13.44 (100 %)	28.09 (100 %)	38.92 (100 %)	73.73 (100 %)

carbon fixation mainly distributed over the successive leaves. *In vitro* cultivated plants were also, from the beginning of their development, capable of diurnal acid fluctuation, though of distinctly weaker activity than the pot plants. This is particularly obvious if one calculates the nocturnal acid accumulation of entire rosettes at the end of the experiment comparing pot grown rosettes with those grown *in vivo* (Table 3).

A central problem in the *in vitro* cultures is the strongly reduced gas exchange between the inside of the culture tubes and the surrounding air. Experiments showed that low CO_2 concentrations in closed airtight culture tubes are mainly responsible for the reduced growth rates (Pospíšilová *et al.* 1992, Čatský *et al.* 1995). Although C_3 plants show CO_2 accumulations of 1500 mg m^{-3} due to dark respiration activity, very low levels during the light period are reached (Solárová 1989). This is why permeable membrane filter caps (Kozai 1988, Kozai *et al.* 1988) were used with the culture tubes in addition to conventional airtight caps. Comparing various types of culture tube closure (Solárová *et al.* 1994), the permeable membrane filters proved to be the best with regard to gas exchange and irradiance properties. However, the higher gas exchange rate also leads to water loss in the culture tubes. In tubes with *SUN-CAPs*, the air humidity is lower and the nutrient medium dries out increasingly. Within 10 weeks, the agar medium in tubes with *SUN-CAPs* shrank by 2 cm, which corresponds to a water loss of about 14 %. With this kind of culture, the nocturnal acidification of the primary leaf and the second successive leaf decreased after the midpoint of the cultivation period (see Table 2) and the growth of plantlets stopped already from the 7th week on (see Fig. 2*B*). This seems to indicate a reduced water potential in the substrate.

An indication of unfavourable conditions in both kinds of *in vitro* culture was also the early decline of the CAM activity of the cotyledons and the premature senescence. Around the 30th day after germination, the first yellowing cotyledons were noticeable for the *in vitro* cultures. On the 40th day, half of the plants in tubes with *KAP-UTS* showed withered cotyledons, in tubes with *SUN-CAPs* one fourth. Whereas in pot cultures, the first signs of senescence of the cotyledons started around the 47th day after germination, during the first 60 d, senescence was restricted to about 11 % of the individuals, and only on the 70th day, the cotyledons of about 45 % of the seedlings were senescent.

In conclusion, our results indicate that seed grown, well-watered plants of *Agave attenuata* were, at a very early stage of ontogeny, capable of considerable CAM activity. The cotyledon and successive rosette leaves of *Agave attenuata* consist of parenchymatic cells. All of these mesophyll cells contain chloroplasts, therefore CAM activity can take place in the entire leaf (Kluge and Ting 1978). Accordingly, a fluctuation of titratable organic acidity of up to 8 times that of the lower level was observed between the end of the light period and the end of the dark period for the cotyledons, the primary leaves and the successive rosette leaves. A diurnal acid fluctuation was also established for *in vitro* grown plantlets. However, according to the varying CO_2 permeability of the culture tube closures, the fluctuation was only about 4 % (*KAP-UTS*) to 14 % (*SUN-CAPs*) of the capacity of pot grown plantlets. The comparison of this ratio with the proportion of dry matter production, with

reference to the pot culture, of *KAP-UTS* (13 %) and *SUN-CAPs* (45 %), indicates that, for *in vitro* cultures, the dark fixation of CO_2 is of less importance for biomass production than daytime C_3 photosynthesis.

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