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A SALT TORELANT REGULATION OF LIGHT DEACIDIFICATION IN THE LEAVES OF THE CAM PLANT KALANCHOE INTEGRIFOLIA.

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ABSTRACT

In the CAM plant **Kalanchoe integrifolia**, effects of light intensity, temperature and bicarbonate concentration on the light-dependent C-transfer from the dark synthesized malic acid to starch in the light during phase 3 of the diurnal CAM cycle have been studied. High light intensity and high temperature increased the rate of malic acid consumption and subsequent starch synthesis while higher bicarbonate concentrations were increasingly inhibitory to this C-transfer process and may stimulate light acidification. The concentration of bicarbonate necessary to make acidification possible during this period of the light time is dependent on the initial tissue acidity.

When acdified leaves were treated with DCMU, a decline in starch level which amounted to about 23 % of those of the untreated leaves was observed. This indicates that decarboxylation of malic acid in the light is partially stimulated by the photosynthetic CO_2 fixation behind closed stomata.

Possible suggestions for the regulation of light dependent C-transfer in **K. integrifolia** leaves were discussed.

INTRODUCTION

Plants which engage in CAM display a complex pattern of daily CO_2 exchange based on at least four distinct phases of autotrophic and heterotrophic CO_2 fixation biochemistry (Osmond and Hiltum, 1981 and Ting and Gibbs, 1982). These phases are dark period (phase 1); onset of illumination (phase 2, normally less than one hour); early day period (phase 3, one to several hours); and net atmospheric CO_2 uptake during the second half of the day (phase 4). During phase 1 all carbon fixed is accumulated as malic acid in cell vacuoles. In the light, phase 2 represents a transition period during which an initial CO_2 uptake occurs, while during phase 3, the previously stored malic acid is released from the vacuoles and further metabolished, i.e. light deacidification proceeds. Its metabolism includes decarboxylation and reassimilation of the resulting CO_2 by the C_3 pathway of photosynthesis; in this period, a depression of CO_3 pathway of photosynthesis; in this period, a depression of CO_2 uptake is taken place. Phase 4 is characterized by a final net CO_2 uptake and its fixation.

The mechanism of dark fixation is now clear (Osmond, 1978; Kluge and Ting, 1978 and Ting, 1985). However, the light-dependent deacidification in the next three phases has not been fully investigated. Among these three phases, only the depression of CO_2 uptake (phase 3) seems to be an essential feature of CAM. In contrast, both the initial CO_2 uptake (phase 2) and the final net CO_2 uptake (phase 4) may be missed in certain CAM plants. Sutton (1975) has shown that carbon compounds produced from decarboxylation of malic acid during phase 3 are almost completely used for starch or glucan synthesis.

The factors which prevent a futile decarboxylation of malate and refixation of CO_2 into malate during phase 3 of the diurnal CAM cycle in **Kalanchoe integrifolia** have yet to be resolved. In the current study, attention was focused on the light-dependent C-transfer from the dark synthesized malic acid to starch in the leaves of this CAM plant to elucidate how photodeacidification may be regulated.

MATERIAL AND METHODS

Kalanchoe integrifolia (Crassulaceae), a typical CAM plant was used in this study. Plants were grown and propagated as described earlier by Barakat and Khalil (1974). The plants were grown in glasshouse with approximate day/night temperatures 16/30°C under photoperiods of 11-hr light and 13-hr dark. All plants were grown under well-watered conditions, where moisture was not a limiting factor. When plants were about 4 months old they were transferred to growth chamber and allowed to adapt for 3 days to the required conditions before samples were taken up for analysis. Leaf samples were collected in triplicate at the desired time from the fourth to the six nodes from the top of the plants. Immediately an initial analysis was made. The rest of the detached leaves were dipped by their cut ends in feeding troughs containing water or the desired solutions and kept in the growth chamber under required temperature and light intensity (Khalil et al., 1984 and Khalil and Khogali, 1988).

Since measurement of photosynthetic rates in CAM tissue is complicated by the closure of stomata during deacidification resulting in negligible CO_2 fluxes, it is difficult to measure the light-limited rate of photosynthesis by means of net CO_2 fixation (Osmond et al., 1979 and Winter, 1980). Measurements of starch contents in the intact leaf tissues were then undertaken in this investigation as an indication for the photosynthetic rates during this period. Starch was estimated from the difference between the total available carbohydrates and total soluble sugars (Murata, et al., 1968). Free titratable acidity as well as malic acid were determined as previously described by Barakat and Khalil (1971). The data are expressed as meq. acid and mg starch 100 g^{-1} FW for acid and starch content respectively.

In assessing the effect of a photosynthetic inhibitor on the relative light-dependent C-transfer, detatched leaves of K. integrifolia were dipped into 10^{-5} M DCMU solution; controls without DCMU were run concurrently.

In experiments designed to determine the effect of various concentrations of bicarbonate on the process of light deacidification, the selected leaves were dipped in the bicarbonate solution which placed in small beaker and kept in cylinderical containers (15 cm x 11 cm diameter). They were flushed with CO_2-free air obtained by passing ordinary air through soda lime tower.

RESULTS AND DISCUSSION

It has been reported by Khalil and Khogali (1988) that optimum dark acid synthesis and accumulation by K. integrifolia leaves was dependent on the interplay of high intensity and high temperature of the preceding light period. However, factors which prevent a futile decarboxylation of malate and refixation of CO_2 into malate in the light as well as the light-dependent C-transfer from the malate to starch in the leaves of this CAM plant have yet to be resolved. Although the experiments presented here provided no direct indication as to the metabolic fate of malic acid decarboxylation products, determination of the contents of malic acid and starch in Kalanchoe leaves, at various temperatures in the dark and light and/or at different light intensities during phase 3 of the diurnal CAM cycle, indicated that light deacidification was not merely a temperature effect (Fig. 1 and Table 1). However, the break down of malate is not dependent only on illumination because a similar decline in acidity occurs, albeit more slowly, in K. integrifolia leaves kept in continuous dark (Fig. 1 and Khalil et al., 1984). The data in Table 1 show that malic acid consumption and subsequent starch synthesis were stimulated through the combined effects of temperature and light intensity. The higher the light intensity and temperature the greater the malic acid transported from the vacuoles, decarboxylated and transferred to starch. Light may induce the release of malic acid stored in the vacuole to the cytoplasm (Nalborczyk et al., 1975); this could be accomplished by changes in membrane permeability. Furthermore, the data in Table 1 are compatible with a report by Barrow and Cookburn (1982) that deacidification is stimulated by increasing light intensities. Although Deleens et al., (1985) concluded that, at higher temperatures, deacidification is controlled by the release of malate from the vacuole rather than by decarboxylation, it seems most probably that the rate of the two processes were decreased in K. integrifolia leaves under low temperatures. This was in agreement with the work of Medina and Osmond (1981) who found that malate accumulation in the vacuoles of K. daigremontiana was more rapid at lower temperature. So, light intensity and temperature could be used to asses the saturation characteristics of photodeacidification in Kalanchoe.

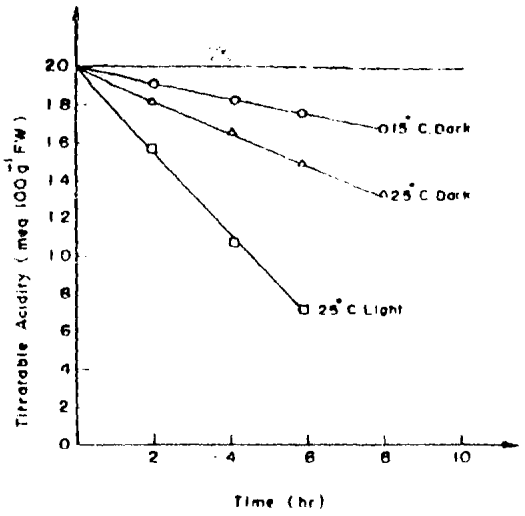

Fig. 1

Acid consumption of acidified **Kalanchoe integrifolia** leaves. Illumination with light intensity of 1400 Ft-c.

Table 1.

Effect of temperature and light intensity on malate and starch levels in acidified **K. integrifolia** leaves after 5 hr into their photoperiod (during phase 3 of the diurnal CAM cycle).

Tamp. °C	Light Intensity Ft-C					
	1000		1500		2000	
	Malic Acid	Starch	Malic Acid	Starch	Malic Acid	Starch
15	11.4	941	9.9	1080	9.3	1242
25	9.2	1230	8.0	1354	6.8	1440
35	8.2	1500	7.0	1625	6.3	1720

* Initial levels of malic acid and starch were 14.8 meq acid and 901 mg starch $100g^{-1}$ FW respectively.

Figure 2 shows that deacidification started earlier and proceeded with much faster rate in acidified leaves placed under strong light intensity (1800 Ft-c). After 5 hr of illumination acidity reached its minimum value under 1800 Ft-c, whereas only about 50 % of the malic acid had disappeared under 600 Ft-c. The reverse was exactely true for starch values where higher values for its level measured under 1800 Ft-c than under 600 Ft-c. This indicats that placing the leaves under low light intensity may inhibit the light-dependent C-transfer from malic acid to starch, probably through the suppression of photosynthesis. As starch synthesis occurred during a period after there was significant net malate degradation it is likely that the carbon required was derived from malate that accumulated during the preceding dark period.

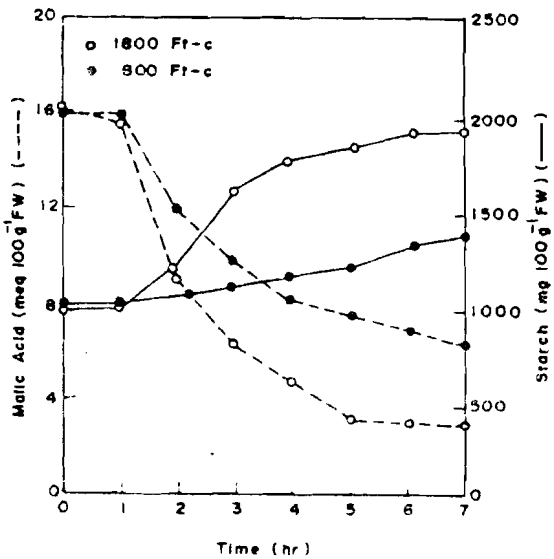

Fig. 2

Changes in starch levels as a function of malate depletion in **K. integrifolia** under two light intensities

* Zero time indicates the time at which the light was turned on after a dark period of 12 hr.

* Temperature during deacidification was about 35°C.

To obtain more evidence concerning the involvement of photosynthesis during decarboxylation, the effect of the photosynthetic inhibitor DCMU on the light deacidification and starch accumulation was examined. Leaves were dipped into 20 uM DCMU solution for at least one hour before illumination set on. After that, the leaves were placed under saturation light intensity of about 2000 Ft-c at 35°C. Figure 3 shows that both deacidification and starch accumulation was suppressed by DCMU treatment. Approximately 1200 mg 100 g^{-1} FW starch was observed in the DCMU treated leaves after 5 hr of illumination, as compared with 1960 mg 100 g^{-1} FW in the non-treated ones. Since the CO_2 produced in malate decarboxylation is removed by photosynthesis (Osmond, 1978 and Kluge and Ting, 1978), thus the internal CO_2 concentration is kept sufficiently low to permit effective continuation of decarboxylation (Barrow and Cockburn, 1982). When photosynthesis is inhibited by DCMU

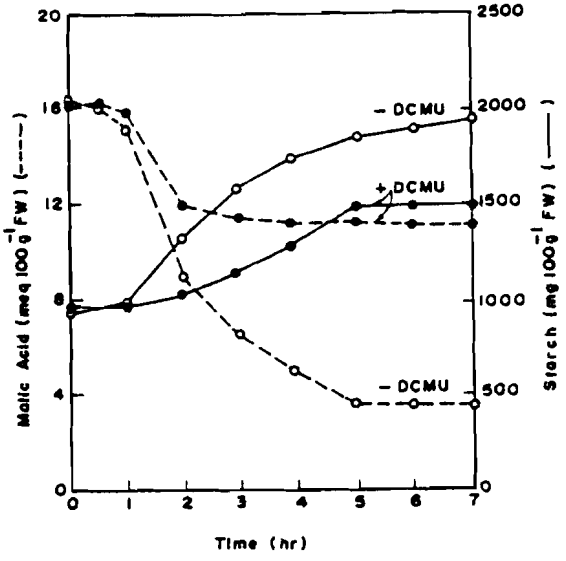

Fig. 3

Effect of DCMU on malic acid and starch levels in acidified leaves of K. **integrifolia** under saturating light intensity (2000 Ft-C).

* Temperature during deacidification was about 35°.
* Concentration of DCMU was 20 um.

or suppressed by placing leaves under low light intensity, CO_2 utilization by photosynthesis is diminished and less starch is synthesized. In this case, CO_2 concentration within Kalanchoe leaves would increase and malate decarboxylation reached equilibrium; the carbon transfer from malic acid to starch can be inhibited. Thus, a driving force for the decarboxylation of malate in these leaves may be the decrease in the CO_2 concentration of the leaves when the photosynthetic apparatus is set into operation by light. Spalding et al. (1979) and Adams and Osmond (1988) suggested that, in CAM plants, the photosynthetic apparatus is optimized for rapid and efficient photosynthesis under the high CO_2 concentrations prevailing during deacidification, rather than for photosynthesis in ordinary air.

To ensure that carbon incorporated into starch during phase 3 of the diurnal CAM cycle was limited by the rate of CO_2 released from malic acid or perhaps influenced in some other way by deacidification process during this period an experiment represented in Fig. 4 was undertaken. Deacidified leaves were obtained during late light period to ensure complete decarboxylation of any malic acid accumulated in the preceding dark period. Since CAM plants engage predominantly in C_3 photosynthetic uptake of atmospheric CO_2 during this late light period of day/night cycle (Winter, 1985), deacidified leaves were flushed with CO_2-free air to achieve the absence of external CO_2 in addition to the absence of an internal CO_2 supply (malic acid). In this case, photorespiratory CO_2 cycling is likely to be the only other source of CO_2 available. Experiments with acidified leaves were also included. As seen from Fig. 4, saturation light intensity of about 2000 Ft-c in the absence of external CO_2 would decrease the amount of starch which could be further synthesized by deacidified leaves after 6 hr of illumination. As malate is no longer a carbon source, the major carbon maintained in starch synthesis in the light is most likely derived from the external CO_2 that is associated during the experimental period. However, removal of external CO_2 was without significant effect on the amount of starch synthesized in the acidified leaves in the light during phase 3 of the diurnal CAM cycle, when malic acid decarboxylation provides internal CO_2 for photosynthesis. This was in support of the fact that stomata of CAM plants close so tightly as to preclude CO_2 exchange in the light during deacidification (Kluge and Ting, 1978, and Osmond, 1978). The internal CO_2 will be then determined by the metabolic balance between CO_2 release from malic acid decarboxylation and refixation through the C_3 pathway. It is, therefore, evident that CO_2 concentration is involved in the regulation of deacidification in the leaves of K. integrifolia. The results of the above experiments are, although insufficient, compatible with the hypothesis that CAM is a CO_2 concentrating mechanism which may help to avoid photoinhibition during phase 3 when external CO_2 is excluded by stomatal closure (Osmond, 1982, Adams et al., 1987).

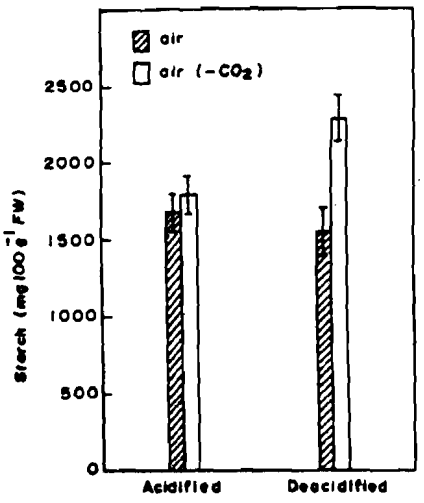

Fig. 4

Comparison of changes in starch levels in acidified and deacidified K. integrifolia leaves in the persence and absence of CO_2 during a 6 hr light. Illumination was about 2000 Ft-c and temperature was nealy 35°C.

* The initial level of starch was 910 and 1400 mg 100 g^{-1} FW for the acidified and deacidified leaves respectively.

Figure 5 shows that both the deacidified and acidified leaves responded to the addition of bicarbonate. However the concentration of bicarbonate necessary to make acidification possible in the light is dependent on the initial level of the tissue acidity. The deacidified leaves showed no net increase in malic acid with the increase in bicarbonate concentration until 2.5 m M bicarbonate were added; a threshold concentration which is approximately 10 times less than that needed by acidified leaves. However, maximal levels of acidity in the presence of bicarbonate were identical for both the acidified and deacidified leaves. This finding did not come as a surprise, since the capacity of the leaf vacuoles to maintain a high proton gradient limits the amount of acid a vacuole can hold (Luttge et al., 1982). The level of the acid would rise and accumulate in the vacuoles untill a saturation is reached which limits the maximal level of acidity in both the acidified and deacidified leaves. Within the author's present limits of knowledge, a combination of changes in the cytoplasmic malate concentration and pH are the most

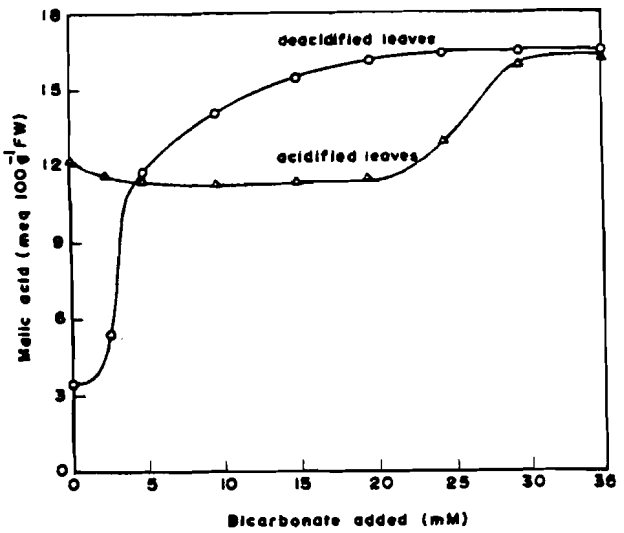

Fig. 5

Malic acid accumulation in K. integrifolia leaves as a function of added amount of bicarbonate under saturated light intensity (2000 Ft-c) and in the absence of external CO_2.

easonable for the occurrence of light acidification in Kalanchoe leaves in the presence of bicarbonate. These changes may affect the activity of both carboxylation (phosphoenolpyruvate carboxylase) and decarboxylation (NADP-malic enzyme) enzymes which constitute the principal enzymes on which CAM metabolism in genus Kalanchoe as well as a large numbers of CAM plants depend (Osmond, 1978; Reddy and Das, 1978 and Dittrich, 1979). Since the functioning of vacuolar ATP-ase in genus Kalanchoe, which is an essential part of the mechanism of acid accumulation in vivo (Luttge and Ball, 1979; Luttge et al., 1981; Smith et al., 1983 and Aoki and Nishida, 1984), is anion sensitive, i.e. stimulated by anion such as HCO_3^- (Jochem et al., 1984), it was then assumed that in the present experiment HCO_3^- may induce the accumulation of malate rather than its release from the vacuole into the cytoplasm during illumination. Malate is an activator for malic enzyme (Osmond, 1978) and a potent inhibitor of CAM PEP-carboxylase in the light (Von Willert et al., 1979 and Wedding and Black, 1986). The change in the sensitivity of PEP carboxylase occurs only after the movement of malic acid from the vacuole into the cytoplasm (Winter, 1980). It is noteworth to mention that malate is a more effective inhibitor of PEP-carboxylase at low pH (Nott

and Osmond, 1982 and Wu and Wedding, 1985). In the present experiment, the cytoplasmic malic acid concentration may not have reached an activation level of malic enzyme and/or an inhibition level of PEP-carboxylase enzyme under the threshold bicarbonate concentration leading to a delay in malate utilization and a net malic acid synthesis to occur. Malate could thus simultaneously and reciprocally modulate the hysteretic behaviour of the carboxylating and decarboxylating enzymes of the CAM plant K. integrifolia.

Bicarbonate may induce a change in the cytoplasmic pH from the normal pH that is necessary for certain cellular reactions in K. integrifolia. In this case, an increase in cytosolic pH would occur which lead to increased carboxylation by increasing the activity of PEP-carboxylase and decreased decarboxylation by decreasing the activity of NADP-malic enzyme, thereby a net synthesis of malic acid. It was concluded by Smith and Bown (1981) and Davies (1986) that these two enzymes were essential for the control of cytosolic pH in leaves of plants under normal conditions of water supply. Bicarbonate is then implicated as a causative agent in organic acid synthesis in K. integrifolia leaves when the cytoplasmic malate concentration drops and/or the pH rises under illumination.

Since vacuoles have limiting capacity for the accumulation of malic acid and that diurnal fluctuation of acidity is due to malate stored in the vacuoles, it is most probable that the amount of malic acid synthesized depend on the initial level of the tissue acidity. Information are needed on the cytosolic and malate concentrations as well as malic acid transport at the tonoplast in the light during phase 3 to elucidate these possibilities in this CAM plant.

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