



Short communication

Leaf malate and succinate accumulation are out of phase throughout the development of the CAM plant *Ananas comosus*

N. Rainha^{a,b,*}, V.P. Medeiros^a, C. Ferreira^a, A. Raposo^a, J.P. Leite^a, C. Cruz^{b,c},
C.A. Pacheco^d, D. Ponte^a, A.B. Silva^{c,e}

^a Instituto de Inovação Tecnológica dos Açores (INOVA), Estrada de São Gonçalo, 9504-540 Ponta Delgada, São Miguel, Açores, Portugal

^b Centro de Ecologia Evolução e Alterações Ambientais (cE3c), Universidade de Lisboa, Faculdade de Ciências, Campo Grande, 1749-016 Lisboa, Portugal

^c Universidade de Lisboa, Faculdade de Ciências, Departamento de Biologia Vegetal, Campo Grande, 1749-016 Lisboa, Portugal

^d Instituto Superior de Agronomia, Tapada da Ajuda, 1349-017 Lisboa, Portugal

^e Biosystems & Integrative Sciences Institute (BioISI), Universidade de Lisboa, Faculdade de Ciências, Campo Grande, 1749-016 Lisboa, Portugal

ARTICLE INFO

Article history:

Received 8 October 2015

Received in revised form

29 December 2015

Accepted 31 December 2015

Available online 6 January 2016

Keywords:

Organic acids

Malate

Citrate

Succinate

Ananas comosus

Pineapple

Crassulacean Acid Metabolism

ABSTRACT

In plants with Crassulacean Acid Metabolism (CAM), organic acids, mainly malate are crucial intermediates for carbon fixation. In this research we studied the circadian oscillations of three organic anions (malate, citrate, and succinate) in *Ananas comosus*, assessing the effect of season and plant development stage. Seasonal and plant development dependencies were observed. The circadian oscillations of malate and citrate were typical of CAM pathways reported in the literature. Citrate content was quite stable (25–30 $\mu\text{mol g}^{-1}$ FW) along the day, with a seasonal effect. Succinate was shown to have both diurnal and seasonal oscillations and also a correlation with malate, since it accumulated during the afternoon when malate content was normally at a minimum, suggesting a possible mechanistic effect between both anions in CAM and/or respiratory metabolisms.

© 2016 Elsevier Masson SAS. All rights reserved.

1. Introduction

Crassulacean Acid Metabolism (CAM) is a major example of the importance of organic acids in plant physiology. The typical nocturnal leaf acidification of CAM plants results from the fixation of atmospheric CO_2 into C4 acids (predominantly L-malate) via a sequence of enzymatic reactions: (i) carboxylation of phosphoenolpyruvate (PEP) in oxaloacetate (OAA) by PEP carboxylase (PEPC); (ii) subsequent reduction of OAA to malate by malate dehydrogenase (MDH); and (iii) storage of malate in the vacuole as malic acid. During the subsequent light period, this malic acid is remobilized into the cytoplasm and either decarboxylated or used in mitochondrial respiration.

Malate may be decarboxylated by one of two enzymes, depending on the plant species: malic enzyme (ME-type plants) or

phosphoenolpyruvate carboxykinase (PEPCK-type plants). CO_2 is then mostly incorporated into hexoses via the gluconeogenesis pathway and the Calvin Cycle. High leaf internal CO_2 concentrations increase the carbon fixation efficiency of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (for review, see Borland et al., 2011; Lüttge, 2004; Matiz et al., 2013). In the mitochondria, malate may be used as a respiratory substrate for ATP production via the tricarboxylic acids (TCA) cycle and mitochondrial electron transport chain (ETC). During the night, a relevant part of the malate resulting from nocturnal CO_2 fixation can pass through the mitochondria, in a process not completely understood. Differing diurnal malate mitochondrial utilization in ME-type and PEPCK-type plants was recently highlighted (Peckmann et al., 2012).

It has been suggested that malate decarboxylation occurs in *Ananas comosus* (Hong et al., 2004), a PEPCK-type plant, mainly by malate oxidation to oxaloacetate (OAA) in: (i) the cytosol by active MDH; and (ii) the mitochondrial matrix, with OAA being exported from the mitochondria via a malate-OAA shuttle. To a lesser extent in pineapple, malate may be oxidized into pyruvate and CO_2 in the mitochondrial matrix by malic enzyme. This mechanism,

* Corresponding author. Instituto de Inovação Tecnológica dos Açores (INOVA), Estrada de São Gonçalo, 9504-540 Ponta Delgada, São Miguel, Açores, Portugal.
E-mail address: nuno_rainha@sapo.pt (N. Rainha).

predominant in ME-type plants, may provide CO₂ and reducing power, which may result from a complete net oxidation of TCA intermediates (e.g. malate, citrate and succinate). Results obtained with pineapple mitochondria suggest a low diurnal respiration when stomata are closed (CAM phase III), though this is dependent on malate concentration (Hong et al., 2004).

Other carboxylates besides malate, such as citrate, may also exhibit diurnal patterns of leaf concentrations. Circadian oscillations in citrate have been demonstrated in two *Kalönchoe* species (Chen et al., 2002; Chen and Nose, 2004). Citrate accumulates overnight in *Mesembryanthemum crystallinum*, grown under non-stress conditions and during the early stage of salinity treatments, although to a much lesser extent than malate (Herppich et al., 1995). Some *Clusia* species accumulate high concentrations of citrate with significant diurnal oscillations, depending on environmental factors (Lüttge, 2006). Citrate role in CAM metabolism is still not completely understood. It is generally assumed that citrate's contribution to carbon gain in CAM is very limited, also it may be relevant to the TCA cycle, or to ensure the buffer capacity of the vacuoles increasing their capacity to accumulate acids in the dark period or under stress (Lüttge, 2006).

Although succinate is related to malate and TCA through many pathways (Hägerhäll, 1997; Kennedy et al., 1992; Lüttge, 2004; Niewiadomska and Borland, 2008), there are no reports of its contribution to CAM. Its diurnal variations were reported for *A. comosus* by Kenyon et al. (1985), but significant diurnal and seasonal oscillations were not described. Effects of succinate in pineapple respiratory metabolism are described (Hong et al., 2004; Peckmann et al., 2012). In this brief communication, we describe the circadian patterns and the relations between malate, citrate, and succinate leaf concentrations in the constitutive CAM plant *A. comosus* L. Merril 'Smooth Cayenne' (pineapple), in order to understand their seasonal response in plants at distinct developmental stages.

2. Plant growth conditions

In the Azores, the production cycle of pineapple (*A. comosus* L. Merr. 'Smooth Cayenne') occurs inside glass greenhouses. Total cycle length varies from 22 to 29 months. Temperatures inside greenhouses have important seasonal and daily oscillations: average temperatures in the summer are near 25 °C and during the autumn and winter are below 20 °C. Photoperiod and nebulosity also determine greenhouse thermal performance. The amplitude can be higher than 20 °C between minimum and maximum temperatures.

In this experiment, plants were maintained under a precise watering regime, with high water availability to ensure no limitations for plant development, using automatic irrigation systems with combined sprinklers and drippers. Daily evapotranspiration in pineapple greenhouse conditions had been previously determined for different seasons to be between 0.4 mm and 0.8 mm. Irrigation was then suspended 30–45 days before harvest. To avoid crop damage from excessive temperatures, slaked lime was applied to the glass roofs of the greenhouses (Rainha et al., 2013). Ten greenhouses were prepared for this study and scheduled to have plants in different development stages in each season.

Leaf samples were obtained in three different development stages: prior to flower induction (Veg); after flower induction but without flowering emergence (FI); and flowering (FL – before anthesis). Each development stage was followed during one year. Disks with 2 cm diameter were collected from the middle parts of adult leaves of 4 plants, in each development stage. Disks were immediately frozen in liquid nitrogen for further organic acid extraction and quantification. Sampling was performed on 7

occasions along the day, at the equinoxes and solstices.

3. Organic acid extraction

Frozen leaf disks (3.14 cm²) were thawed, weighed, and ground using a mortar and pestle. 5 mL of double distilled water were added to the homogenized leaf and transferred to a test tube before incubation in a water bath (30 °C) for 20 min. The samples were centrifuged at 4000g for 5 min, the supernatant collected and the procedure repeated twice with 2.5 mL of double distilled water in each extraction. The supernatants were mixed and the solution completed to 10 mL. Afterwards, 1.5 mL of each sample were transferred to a microcentrifuge tube, to precipitate non soluble components using a cooled centrifuge (4 °C) at 10 000g for 10 min. The resulting supernatant was filtered through a 0.45 µm pore size filter and transferred to vials for quantification using high performance liquid chromatography (HPLC).

4. Organic acid quantification by HPLC with photo diode array detection

The HPLC system consisted of a Dionex Ultimate 3000 HPLC series (Thermo Scientific, MA, USA) coupled to a photodiode array detector (PDA) and auto-sampler system. Detection was performed at 210 nm. Malate, citrate and succinate were separated in a Rezex ROA – Organic Acid H⁺ (8%); (300 × 7.8 mm) column (Phenomenex, Torrance, CA, USA) maintained at a constant temperature of 40 °C. The mobile phase consisted of 0.005 N sulphuric acid with 5% methanol at a constant flow rate of 0.6 mL min⁻¹. The chromatographic data for the quantitative determination was obtained using the external method and the Chromeleon 6.8 Chromatography Data System (Thermo Scientific, MA, USA).

5. Statistical analysis

Statistical analysis was performed, when appropriate, using SPSS version 17.0 for Windows. Before carrying out statistical tests, normality of the data was checked by means of the Kolmogorov–Smirnov statistic ($p > 0.05$). Means were compared by Tukey's studentized range test ($\alpha = 0.05$) or, when appropriate, t-test ($\alpha = 0.05$). Correlations were established according to the Pearson coefficient (R).

6. Results and discussion

Malate was by far the organic anion most related with CAM under all the conditions studied (Fig. 1). The observed leaf malate concentrations were of the same order of magnitude as those previously reported (Chen et al., 2002). The observed circadian oscillations in malate, with the maximum concentrations always reached at the end of the night, were typical of CAM plants. Malate consumption during CAM phase III represented 70–85% of the maximum leaf malate concentration at the end of the dark period (Fig. 2). Malate accumulation overnight was also dependent on the leaf/plant development and environmental conditions. When the environment was favourable for CAM (autumn equinox), plants in advanced phenological stages exhibited higher diurnal amplitudes of malate accumulation, suggesting that CAM amplitude may be reinforced after inflorescence emergence and development, as the new organs represent higher carbon sinks.

Citrate balance in pineapple was assumed to be energetically preferable to malic acid accumulation and may be important in the internal recycling of carbon skeletons in CAM plants under environmental stress (Borland and Griffiths, 1989; Lüttge, 1988). More recently, Chen et al. (2002) reported average citrate leaf

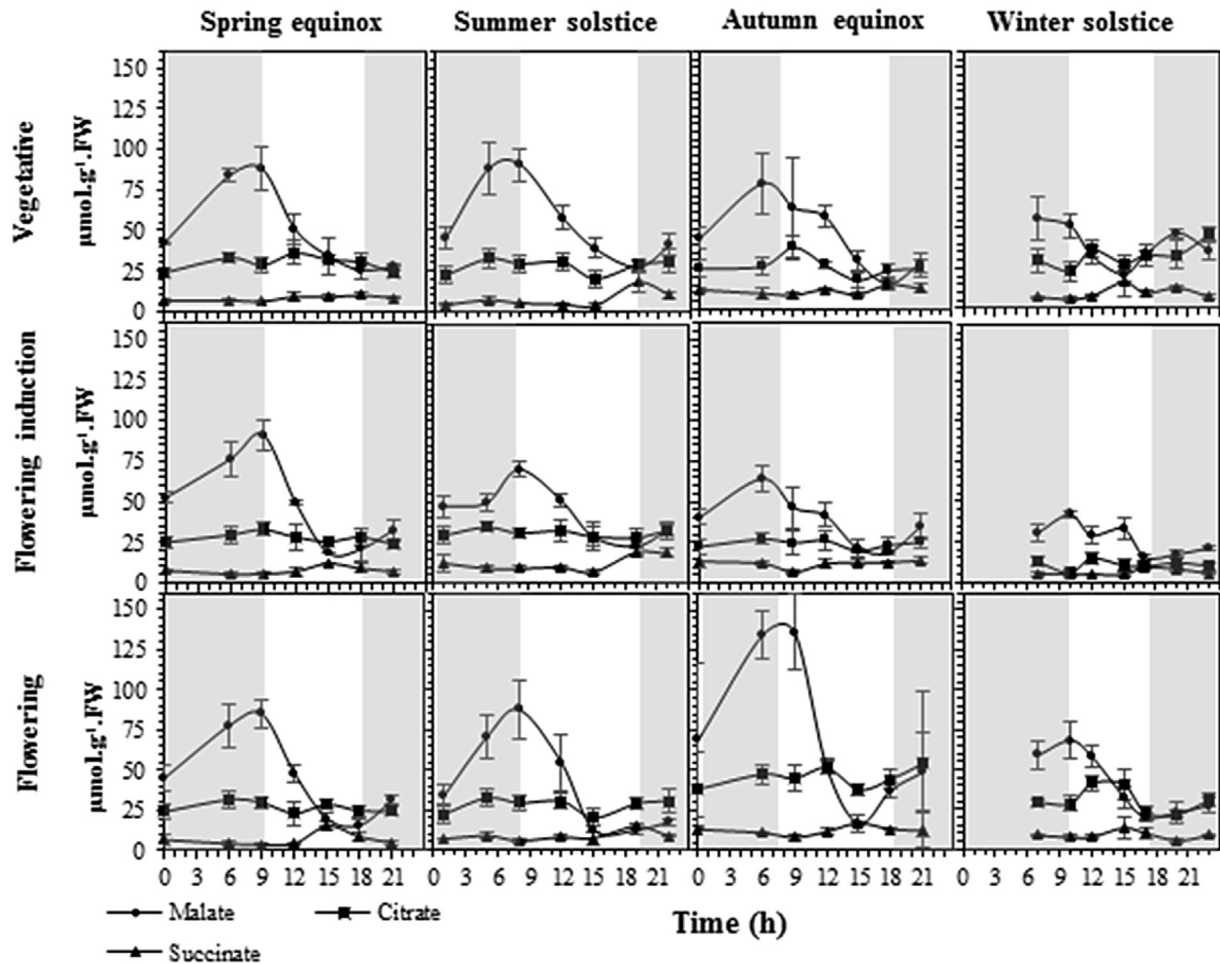


Fig. 1. Organic acid (malate, citrate and succinate) variation in adult leaves of pineapple plants grown under greenhouse conditions in three stages of development: Vegetative (Veg), after flower induction (FI) and flowering plants (FL). Results are expressed as mean \pm standard deviation of four independent measurements. Dark period is represented by grey bars.

concentration around $65 \mu\text{mol g}^{-1} \text{FW}$ (Chen et al., 2002), with little or no day/night variation (Chen and Nose, 2004). Medina et al. (1993) reported diurnal citrate oscillation in pineapple varieties and wild species grown under tropical conditions. In this study, lower concentrations ($25\text{--}30 \mu\text{mol g}^{-1} \text{FW}$) were observed in the majority of the samples, with between 10 and $45 \mu\text{mol g}^{-1} \text{FW}$ (Fig. 1). Daily citrate variations were normally below 25% of the maximum leaf citrate concentration (Fig. 2). Significant day/night oscillations of leaf citrate concentrations were only observed in flowering plants at the winter solstice (Fig. 2), with a 50% daily variation relative to the maximum concentration of leaf citrate. Average values in each season were constant for vegetative plants ($p = 0.676$), but not for plants after flower induction or for flowering plants ($p < 0.05$). These results suggest that leaf citrate concentration varies seasonally, with higher values normally occurring at the summer solstice and autumn equinox, which are more favourable for CAM plants, and lowest in the winter. Occasionally, leaf citrate concentrations exceeded those of malate at the end of the afternoon, when malate was at a minimum. Leaf citrate accumulation was significantly correlated ($R^2 = 0.438$) with malate, reflecting a similar diurnal pattern, with nocturnal accumulation and consumption during the day. However, it is not clear whether the observed correlations between the daily patterns of leaf accumulation of both acids represent a mechanistic relation in terms of CAM, considering both plant and growth conditions (Lüttge, 2006;

Maxwell et al., 1994; Rainha et al., 2013).

According to our observations succinate contribution to overall leaf acidification cannot be neglected, being the third most relevant acid determined. Other relevant acids such as isocitrate were absent or simply below quantification limits. Average leaf succinate concentrations ($7\text{--}11 \mu\text{mol g}^{-1} \text{FW}$) did not differ between plant development stages ($p > 0.05$) (Fig. 1). Nevertheless, succinate accumulation was influenced by season (maximum concentrations observed at the autumn equinox were statistically different from the other sampling periods) and time of day. Succinate leaf concentrations oscillated diurnally, being on average higher and statistically different ($p = 0.00$) during the day than those observed during the night. Succinate oscillations generally occurred near 50% of the maximum value, but diurnal concentrations may vary from 25% up to 75% (Fig. 2). Maximum concentration was observed during the light period, especially in the afternoon, normally when malate was at a minimum. The overall correlation between succinate and malate leaf concentration was $R^2 = -0.288$. At the winter solstice no correlation was observed ($R^2 = 0.084$, $p > 0.05$). During the other periods, when the amplitude of leaf malate concentration was higher, a negative correlation with succinate was observed ($R^2 = -0.433$, -0.574 and -0.388 at the autumn equinox, spring equinox and summer solstice, respectively), suggesting a metabolic dependency between the two acids in PEPCK-CAM of *A. comosus*. The diurnal oscillations in succinate exceed those reported by

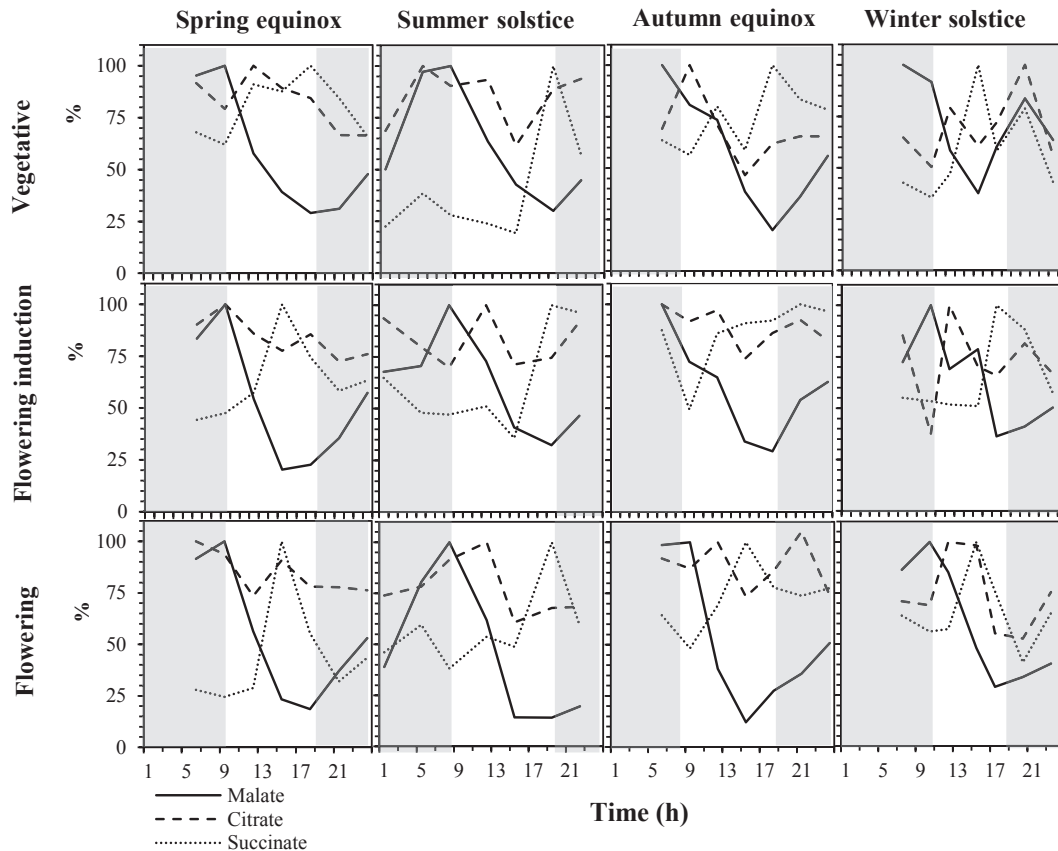


Fig. 2. Daily variations in malate, citrate and succinate percentage relative to the maximum concentration (100%) in the D leaf of pineapple plants grown under greenhouse conditions in three stages of development: Vegetative (Veg), after flower induction (FI) and flowering plants (FL). Dark period is represented by grey bars.

Kenyon et al. (1985) for pineapple and seem to adjust seasonally which is a novelty regarding succinate contribution to leaf acidification in CAM plants.

Respiratory and mitochondrial activities are important to control CAM during organic acid accumulation at night and acid decarboxylation during the day. In malic enzyme and PEPCK-type plants, mitochondria may exert their role in the control of the diurnal rhythm of malic and citric acids to a differential degree (Herppich and Peckmann, 1997; Lüttge, 2002). Some of the oscillation of citric acid (Figs. 1 and 2) may be attributed to respiratory activity in different degrees. Recently, Peckmann et al. (2012) demonstrated that in PEPCK-CAM plants such as pineapple, the mitochondria oxidize malate in a different way from mitochondria of malic enzyme-CAM plants which usually oxidize succinate and malate with rather similar rates. The two initial enzymatic machinery differ and succinate is preferably oxidized to malate, but both share the same metabolic pathways following oxidation (Peckmann et al., 2012). Additionally, pineapple mitochondria were shown to oxidize succinate more rapidly than malate suggesting that respiration during CAM phase III is low and dependent on malate concentration (Hong et al., 2004). Our results show that in CAM phase III, succinate concentration increases associated with or following malate metabolization. This is easily observed in vegetative stages, as succinate concentrations decrease with an increase in malate concentration, in accordance with the mitochondria substrate preference (Fig. 2). The diurnal increase on succinate concentration could be associated with a reduction in the respiratory activity at the mitochondrial level in the afternoon, to the effect of inhibiting metabolites such as PEP, which accumulate during the light period, or even related to the activation of alternative

oxidation pathways.

At the end of the night period, when malate concentrations are at their maximum from nocturnal fixation of CO₂ in vacuoles, succinate is at its lowest. It is possible that there is a different feedback mechanism by which succinate concentrations are kept low so that higher rates of diurnal malate metabolization occur, so that PEP molecules are newly available for CO₂ fixation in the following night period.

Contribution

Nuno Rainha, Violante Medeiros, Cristina Cruz and Anabela Silva: made the data analysis, result discussion and wrote the manuscript.

Violante Medeiros made THE leaf sampling and experimental design.

Carlos Arruda and Duarte Ponte worked on plant growth and development.

Nuno Rainha made the organic acid extraction.

Carla Ferreira, André Raposo and João Leite performed the HPLC determination of the three organic acids (malate, citrate and succinate).

References

- Borland, A., Zambrano, V.A.B., Ceusters, J., Shorrocks, K., 2011. The photosynthetic plasticity of Crassulacean Acid Metabolism: an evolutionary innovation for sustainable productivity in a changing world. *New Phytol.* 191, 619–633.
- Borland, A.M., Griffiths, H., 1989. The regulation of citric acid accumulation and carbon recycling during CAM in *Ananas comosus*. *J. Exp. Bot.* 40, 53–60.
- Chen, L., Lin, Q., Nose, A., 2002. A comparative study on diurnal changes in

- metabolite levels in the leaves of three crassulacean acid metabolism (CAM) species, *Ananas comosus*, *Kalanchoë daigremontiana* and *K. pinnata*. J. Exp. Bot. 53 (367), 341–350.
- Chen, L., Nose, A., 2004. Day-night changes of energy-rich compounds in Crassulacean Acid Metabolism (CAM) species utilizing hexose and starch. Ann. Bot. 94, 449–455.
- Hägerhäll, C., 1997. Succinate: quinone oxidoreductases. Variations on a conserved theme. Biochim. Biophys. Acta 1320, 107–114.
- Herppich, M., Herppich, B., Von Willert, D.J., 1995. Diurnal rhythm in citric acid content preceded the onset of nighttime malic acid accumulation during metabolic changes from C₃ to CAM in salt-stressed plants of *Mesembryanthemum crystallinum*. J. Plant Physiol. 147, 38–42.
- Herppich, W.B., Peckmann, K., 1997. Responses of gas exchange, photosynthesis, nocturnal acid accumulation and water relations of *Aptenia cordifolia* to short-term drought and rewatering. J. Plant Physiol. 150, 467–474.
- Hong, H.T.K., Nose, A., Agarie, S., 2004. Respiratory properties and malate metabolism in Percoll-purified mitochondria isolated from pineapple, *Ananas comosus* (L.) Merr. cv. smooth cayenne. J. Exp. Bot. 55, 2201–2211.
- Kennedy, R.A., Rumpho, M.E., Foz, T.C., 1992. Anaerobic metabolism in plants. Plant Physiol. 100, 1–6.
- Kenyon, W.H., Severson, R.F., Black Jr., C.C., 1985. Maintenance carbon cycle in Crassulacean acid metabolism plant leaves. Source and compartmentation of carbon for nocturnal malate synthesis. Plant Physiol. 77, 183–189.
- Lüttge, U., 2002. CO₂-concentrating: consequence in Crassulacean acid metabolism. J. Exp. Bot. 53, 2131–2142.
- Lüttge, U., 2006. Photosynthetic flexibility and ecophysiological plasticity: questions and lessons from *Clusia*, the only CAM tree, in the neotropics. New Phytol. 171, 7–25.
- Lüttge, U., 2004. Ecophysiology of Crassulacean Acid Metabolism (CAM). Ann. Bot. 93, 629–652.
- Lüttge, U., 1988. Day-night changes of citric acid levels in crassulacean acid metabolism: phenomenon and ecophysiological significance. Plant Cell Environ. 11, 445–451.
- Matiz, A., Miotto, P.T., Yepes, A., Freschi, L., Mercier, H., 2013. CAM photosynthesis in bromeliads and agaves: what can we learn from these plants? In: Dubinsky, Z. (Ed.), Photosynthesis. InTech, Rijeka, pp. 91–134.
- Maxwell, C., Griffiths, H., Young, A.J., 1994. Photosynthetic acclimation to light regime and water-stress by the C₃-CAM epiphyte *Guzmania monostachia*: gas exchange characteristics, photo-chemical efficiency and the xanthophyll cycle. Funct. Ecol. 8, 746–754.
- Medina, E., Popp, M., Olivares, E., Janett, H.-P., Lüttge, U., 1993. Daily fluctuations of titratable acidity, content of organic acids (malate and citrate) and soluble sugars of varieties and wild relatives of *Ananas comosus* L. growing under natural tropical conditions. Plant Cell Environ. 16, 55–63.
- Niewiadomska, E., Borland, A.M., 2008. Crassulacean acid metabolism: a cause or consequence of oxidative stress in plants? Prog. Bot. 69, 247–266.
- Peckmann, K., von Willert, D.J., Martin, C.E., Herppich, W.B., 2012. Mitochondrial respiration in ME-CAM, PEPCK-CAM, and C₃ succulents: comparative operation of the cytochrome, alternative, and rotenone-resistant pathways. J. Exp. Bot. 63 (8), 2909–2919.
- Rainha, N., Medeiros, V.P., Rodrigues, A.C., Simas, A., Arruda, C., Silva, A.B.S., Cruz, C., Ponte, D., 2013. An overview of pineapple culture in the Azores. Pineapple News 20, 9–15.