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Short communication

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

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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 μmol 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.

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,

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predominant in ME-type plants, may provide CO_2 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 *Mesnnbryanthnnum 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 cm^2) 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 4000*g* 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 centrifuged (4 °C) at 10 000*g* 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, Torrence, 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–Smirnoff 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