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Nitrate enhancement of CAM activity in two *Kalanchoë* species is associated with increased vacuolar proton transport capacity

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Abstract

Among species that perform CAM photosynthesis, members of the genus *Kalanchoë* have been studied frequently to investigate the effect of environmental factors on the magnitude of CAM activity. In particular, different nitrogen sources have been shown to influence the rate of nocturnal CO_2 fixation and organic-acid accumulation in several species of *Kalanchoë*. However, there has been little investigation of the interrelationship between nitrogen source (nitrate versus ammonium), concentration, and the activity of the vacuolar proton pumps responsible for driving nocturnal organic-acid accumulation in these species. In the present study with *Kalanchoë laxiflora* and *Kalanchoë delagoensis* cultivated on different nitrogen sources, both species were found to show highest total nocturnal organic-acid accumulation and highest rates of ATP- and PPi-dependent vacuolar proton transport on 2.5 m*M* nitrate, whereas plants cultivated on 5.0 m*M* ammonium showed the lowest values. In both species malate was the principal organic-acid accumulated during the night, but the second-most accumulated organic-acid was fumarate for *K. laxiflora* and citrate for *K. delagoensis.* Higher ATP- and PPi-dependent vacuolar proton transport rates and greater nocturnal acid accumulation were observed in *K. delagoensis* compared with *K. laxiflora*. These results show that the effect of nitrogen source on CAM activity in *Kalanchoë* species is reflected in corresponding differences in activity of the tonoplast proton pumps responsible for driving sequestration of these acids in the vacuole of CAM-performing cells.

Keywords: crassulacean acid metabolism (CAM), nitrogen, organic-acids, proton pumps, vacuole.

Abbreviations: ATP, adenosine triphosphate; BTP, bis-TRIS-propane; CAM, crassulacean acid metabolism; DW, dry weight; MDH, malate dehydrogenase; NO, nitric oxide; NR, nitrate reductase; OAA, oxaloacetate; PEPC, phospho*enol*pyruvate carboxylase; PPi, inorganic pyrophosphate.

Introduction

Crassulacean acid metabolism (CAM) is a photosynthetic pathway characterized by nocturnal CO_2 fixation performed by the enzyme phospho*enol*pyruvate carboxylase (PEPC), the accumulation of organic-acids during the night (mainly in the form of malic acid), and a high efficiency of water use (Osmond 1978; Borland et al. 2011, 2014; Cushman 2001). At least 343 genera from 35 plant families are capable of performing CAM photosynthesis (Silvera et al. 2010; Smith and Winter 1996). Among these genera, *Kalanchoë* species from the eponymous family Crassulaceae have been one of the largest groups studied (Kluge and Ting 1978; Santos and Salema 1991; Kluge et al. 1991; Osmond 1978; Widmann et al. 1990, 1993). It has been suggested that most species from the genus *Kalanchoë* are able to perform CAM, and also a correlation has been established between the capacity to perform

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CAM and the climate (especially aridity) of the habitats where the individual species grow naturally (Kluge et al. 1991).

The influence of nutrient availability on the expression of CAM photosynthesis has been relatively little studied, but nitrogen supply is one factor thought to affect the CAM activity. *Kalanchoë blossfeldiana* has been investigated in several studies to determine how nitrogen availability affects CAM photosynthesis (Ota 1988a,1988b; Ota and Yamamoto 1991), and it has been observed that the expression of CAM photosynthesis was greater in plants supplied with 1.0 m*M* nitrate compared with 1.0 m*M* ammonium. However, this species exhibited the highest CAM expression when both nitrogen sources were removed from the nutrient solution (nitrogen starvation) and a water deficit was imposed (Ota 1988a), suggesting that N-depletion is an important factor in maximizing CAM performance in *K. blossfeldiana*. In another study with *K. blossfeldiana*, plants cultivated with 5.0 m*M* nitrate showed higher CAM activity compared with those cultivated with 5.0 m*M* ammonium in the absence of imposition of water deficit (Ota 1988b), suggesting that both nitrogen source and concentration are important factors influencing CAM expression in *Kalanchoë* species. In *Kalanchoë pinnata*, another much investigated CAM species, growth over a wider range of different nitrate concentrations (0.6 to 24.0 m*M*) showed that plants on higher nitrate concentrations possessed higher PEPC activity when compared with plants on lower nitrate concentrations (Winter et al. 1982). All of these studies support the idea that both the chemical source of inorganic nitrogen sources and the concentration of available nitrogen influence the magnitude of CAM expression.

On compartmental grounds, and in order to maintain cytoplasmic homeostasis, almost all the malic acid synthesized in CAM plants as a result of dark CO_2 fixation must be transported into the vacuole at night and remobilized in the following light period (Smith et al. 1996). Many studies investigating the transport properties of the vacuolar membrane have used another model CAM plant, *Kalanchoë daigremontiana*, for example to characterize the ATP- and PPi-dependent proton pumps at the tonoplast membrane and to investigate the pathway of malate transport (Bettey and Smith 1993; Cheffings et al. 1997; Hafke et al. 2003; Pantoja and Smith 2002; White and Smith 1989; White et al. 1990). White and Smith (1989) observed that the rate of proton pumping into the vacuole by the tonoplast H^+-ATPase or H^+-PPiase was constrained by transport of a charge-balancing anion, and that in the CAM plant *K. daigremontiana* the distinctive sequence of tonoplast anion permeability was fumarate^{2-} > malate^{2-} > chloride$^-$. Another study with *Nicotiana tabacum* (Solanaceae), a C_3 species, showed the influence of both nitrogen source and concentration on proton transport across the vacuole and on malate accumulation (Lüttge et al. 2000): the highest malate accumulation and the maximum ATP-dependent malate transport into the vacuole were observed when the species was cultivated in the presence of high nitrate concentrations (10.0 or 20.0 m*M*).

These prior studies raise some important questions about the degree to which the capacity for transport of organic-acids at the vacuolar membrane is coordinated with the magnitude of CAM expression. Would a higher vacuolar H^+ transport capacity be accompanied by greater organic acid

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accumulation during the night in CAM species? Would nitrogen source and concentration affect the magnitude of CAM photosynthesis by changing proton transport rates into the vacuole and associated nocturnal organic-acid accumulation? A possible approach to address these questions would be to investigate H^+ transport across the tonoplast in other *Kalanchoë* species that exhibit different levels of organic-acid accumulation to model species such as *K. daigremontiana* studied to date. Another approach would be to investigate the effects of alternative nitrogen sources at different concentrations and the extent to which changes in proton and organic-acid transport across the vacuole are correlated with higher or lower CAM expression in *Kalanchoë*. Thus, the present study is focused on a comparison of nitrate and ammonium as contrasting nitrogen sources, supplied at two different concentrations (2.5 m*M* or 5.0 m*M*), both of which are lower than the toxic concentration (6.5 m*M*) observed during preliminary experiments performed with *Kalanchoë laxiflora* and *K. delagoensis* (data are not shown). The aim of these experiments is to establish how nitrogen nutrition influences the expression of CAM and the associated capacity for vacuolar organic-acid accumulation, as well as studying the effect of alternative nitrogen sources on CAM activity. This investigation specifically sought to establish the relationship between ATP- and PPi-dependent proton transport across the vacuolar membrane and the organic-acids accumulated at night under these different nutritional conditions.

Materials and Methods

Plant material and growth conditions

Mature plants of *Kalanchoë laxiflora* Baker and *Kalanchoë delagoensis* Eckl. and Zeyh. (commonly referred to in the physiological and biochemical literature by its later synonym *Kalanchoë tubiflora* Raym.-Hamet) were obtained from the garden of the Department of Botany at the University of São Paulo, São Paulo, Brazil. Plants were transferred to controlled environment chambers and maintained under a photosynthetic flux density (PFD) of approximately 200 µmol m^{-2} s^{-1} of photosynthetically active radiation (measured at the surfaces of the uppermost leaves), 12 h photoperiod, day/night air temperature of 25/22ºC, and day/night relative humidity of 60/70%.

Plants were cultivated in pots (13 cm diameter and 7 cm height) containing fine sand, one plant per pot, and were watered daily with distilled water without supplementary nutrients for 2 weeks. After this acclimation period, experiments were performed for 60 days and each species was divided into six treatments based on nitrogen sources and their concentrations: (1) nitrogen-deficient (no supplementary nitrogen); (2) 2.5 m*M* ammonium + 2.5 m*M* nitrate; (3) 2.5 m*M* ammonium; (4) 2.5 m*M* nitrate; (5) 5.0 m*M* ammonium; and (6) 5.0 m*M* nitrate. The nitrogen concentrations selected were designed to be below any toxic level based on previous results obtained by Ota (1988a, 1988b) with *Kalanchoë blossfeldiana*, in which toxicity effects were observed under 10 m*M* but not under 5 m*M* ammonium (whereas toxicity effects were not observed for nitrate at either concentration). In addition,

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a preliminary test using 6.5 or 5.0 m*M* ammonium or nitrate was performed with *K. delagoensis* and *K. laxiflora* (data not shown). This test showed toxicity effects when *Kalanchoë delagoensis* plants were kept under 6.5 m*M* ammonium, such as, necrosis, which decreased growth and caused the plants to lose most of their leaves. For this same species, 6.5 m*M* nitrate also showed a decrease in the growth. For *Kalanchoë laxiflora*, the plants kept under 6.5 m*M* nitrate showed a decrease in their growth compared with the plants kept in 5.0 m*M* nitrate. For this same species, higher ammonium and nitrate concentrations (6.5 m*M*) caused early senescence (data are not shown). In the light of these preliminary results, we chose a lower concentration of nitrate and ammonium (5.0 m*M*).

Each plant received 30 ml of the appropriate nutrient solution with different nitrogen sources and their concentration every second day for a total of 60 days based on full-strength Hoagland solution (Hoagland and Arnon 1950). After 60 days, leaf samples were taken from six plants from each treatment and were used to determine proton transport rates in isolated tonoplast vesicles and nocturnal organic-acid accumulation.

Tonoplast vesicle isolation

Fully expanded leaves from *K. laxiflora* and *K. delagoensis* were harvested 1–1.5 hours after commencement of the light period. Tonoplast fractions from the mesophyll tissue were isolated in accordance with White and Smith (1989) with minor modifications. The leaf margins were excised and the mesophyll tissue (approximately 80 g fresh mass) was sliced and suspended in 250 mL of ice-cold extraction buffer containing 450 m*M* mannitol, 3.0 m*M* $MgSO_4$, 10.0 m*M* ethylenediaminetetraacetic acid, disodium salt (Na_2EDTA), 10 m*M* dithiothreitol (DTT), 0.5% (w/v) polyvinylpyrrolidone (PVP-40) and 100 m*M* tris(hydroxymethyl)aminomethane (Trizma® base) buffered to pH 8.0 with HCl. After homogenization in a commercial blender, the homogenate was filtered through two layers of cheesecloth and the filtrate centrifuged at 18 000 × *g* for 20 min. The resulting supernatant was centrifuged at 80 000 × *g* for 60 min, yielding a microsomal pellet that was resuspended in a glycerol-containing medium consisting of 1.1 *M* glycerol, 1.0 m*M* Na_2EDTA, 10 m*M* Tricine buffered to pH 8.0 with bis-tris-propane (BTP) and 2.0 m*M* DTT. The membrane suspension was layered onto a 23% (w/v) sucrose cushion made up in glycerol medium and the sucrose step gradient centrifuged at 100 000 × *g* for 70 min. Tonoplast vesicles were carefully removed from the interface by aspiration using a Pasteur pipette, resuspended 1 : 1 in glycerol medium buffer, and pelleted at 100 000 × *g* for 50 min. The resulting pellet was finally gently re-suspended using a paintbrush in a small volume of glycerol medium. All steps were performed at 4°C. Preparations were snap frozen in liquid nitrogen, stored at −80°C, and were used within one month. Vesicle yield did not show any significant differences among the N treatments used in this study.

Proton-transport assays

Measurement of proton transport into isolated tonoplast vesicles was conducted using a quinacrine-fluorescence-quenching method according to White and Smith (1989) with minor modifications. Initial rates of H^+ transport into the vesicles at 25ºC were determined from the initial rates of fluorescence quenching following addition of 3.0 m*M* Tris-ATP or 500 µ*M* Na_4PPi to the reaction medium in buffered solution. For assays of ATP-dependent H^+ transport, the reaction medium contained 3.0 µ*M* quinacrine, 6.0 m*M* $MgSO_4$, 0.3 m*M* Na_2EDTA, 150 m*M* mannitol and 25 m*M* BTP buffered to pH 8.0 with Mes. For measurements of PPi-dependent H^+ transport, the reaction medium was identical except that the $MgSO_4$ concentration was increased to 7.5 m*M* and the medium also contained 100 m*M* K-Mes. Charge-balancing anions (fumaric acid, malic acid, or citric acid buffered to pH 8.0 with BTP), 50 m*M* potassium nitrate (inhibitor of vacuolar membrane), or 100 µ*M* sodium orthovanadate plus 100 µ*M* sodium azide (inhibitor of non-vacuolar membrane) were used in the ATP- and PPi-dependent proton transport assays. Approximately 3 µg of tonoplast protein per reaction was used in all of the ATP- and PPi-dependent proton transport assays. Fluorescence quenching was measured using a model Perkin-Elmer LS-55 luminescence spectrometer (Llantrisant, UK) with excitation at 422 nm and emission at 495 nm, both with a slit width of 5 nm. Relative quinacrine fluorescence intensity (%*F*) was calculated based on the maximum slopes obtained after addition of ATP or PPi to calculate the rate of proton pumping in each preparation.

Protein determination

Protein concentration was measured using the method described by Bradford (1976), with bovine serum albumin as the standard.

Organic-acid quantification

To determine the nocturnal accumulation of organic-acids (Δ citrate, Δ fumarate and Δ malate), samples (100 mg fresh mass) from fully developed leaves from six individual plants were collected 1 hour after the start of the light period (dawn), and 1 hour before the end of the light period (dusk). Samples were ground in liquid nitrogen and subsequently homogenized with 500 µL of MCW solution (methanol, chloroform and water, 12 : 5 : 1) containing benzoic acid as a standard (1 mg mL^{-1} of methanol). Samples were then incubated at 60ºC for 30 min. All samples were centrifuged at 16 000 × *g* at 4ºC for 10 min. The supernatant was collected (50 µL) and dried for 1 h at 60ºC in a CentriVap Vacuum Concentrator® (Labonco, Kansas City, USA). The dried sample was then re-suspended in 25 µL of pyridine and 25 µL of *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) and incubated in a dry bath for 1 h at 92ºC. An aliquot of 1 µL of the incubated sample was used to quantify organic-acids by gas chromatography coupled with a chromatographic system (Shimadzu-QP2010SE- Kyoto, Japan), an Agilent DB5MS column (30 m, D.I. 0.25 mm, I.T. 0.5 µ*M*), with helium as a carrier gas in a 1 mL min^{-1} flux and an auto sampler (Shimadzu AOC-20i). The column

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remained at 100ºC for 6 min, with a temperature ramp from 100ºC to 300ºC at a rate of 6ºC min^{-1}. The injector temperature was 290ºC, total flux 24 mL min^{-1} and linear velocity 37.2 cm s^{-1}. Standard curves for citric, fumaric and malic acids were used to determine the concentrations of individual organic-acids in the samples. Results are expressed as millimoles per gram of dry weight (mmol g^{-1} DW).

Data analysis

All data are presented as mean values ± standard deviation (s.d.). One-way ANOVA was used to analyze the results. Significant differences among the different anions or treatments in the same species were tested using the Tukey–Kramer test at $P < 0.05$ using JMP 5.01 software (SAS Institute 2002). The difference between the inhibitors in the same treatment and species was tested by Student's *t*-test at 5% significance ($P < 0.05$).

Results

Proton transport rates in the presence of inhibitor of vacuolar or non-vacuolar membranes

To determine the relative contribution of vacuolar and non-vacuolar membranes in these two species kept in different nitrogen treatments, we used a specific inhibitor of vacuolar membranes (V-ATPases) (KNO_3) or non-vacuolar membranes (non V-ATPases) (NaN_3 plus Na_3VO_4) (Table 1). In all of the nitrogen treatments, KNO_3 caused a substantial (up to 99 %) inhibition of ATP-dependent proton transport. When the isolated tonoplast membranes were tested in the presence of NaN_3 plus Na_3VO_4, there was a small (up to 32 %) decrease in proton transport, but this degree of inhibition was much less than that caused by KNO_3 (Table 1). These results indicate that most of the extracted vesicles obtained are indeed derived from the tonoplast. All of these nitrogen treatments, for both *Kalanchoë* species, showed an inhibition percentage of ATP-dependent proton transport, in the presence of KNO_3, higher than 54% (Table 1). On the other hand, in the presence of NaN_3 plus Na_3VO_4, all of the other treatments showed an inhibition percentage between 2 and 32% when a specific inhibitor of non V-ATPases was added (Table 1).

ATP-dependent proton transport rates

ATP-dependent proton transport rates (%F) in tonoplast vesicles prepared from *K. laxiflora* and *K. delagoensis* leaves were assayed in the presence of fumarate, malate and citrate anions and were calculated from the initial slope of the quinacrine fluorescence quenching reaction as shown in Appendixes S1 and S2 in Supporting Information.

Fumarate supported the highest rates of vesicle acidification in all treatments in both species, followed by malate (not considering the control). In the presence of fumarate, *K. laxiflora* plants cultivated in 2.5 m*M* nitrate presented the highest ATP-dependent proton transport rates (about 4061%

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min^{-1} mg protein^{-1}: Table 2), followed by plants cultivated in the presence of 2.5 m*M* ammonium+2.5 m*M* nitrate, nitrogen-deficient, 2.5 m*M* ammonium, 5.0 m*M* nitrate and 5.0 m*M* ammonium (Table 2). For comparison, ATP-dependent proton transport in the presence of 2.5 m*M* nitrate (highest rates) was about 9 times higher than in the presence of 5.0 m*M* ammonium (lowest rates). For *K. delagoensis*, in the presence of fumarate, the highest proton transport rates were also observed in plants cultivated in 2.5 m*M* nitrate, 4172% min^{-1} mg protein^{-1}, followed by 2.5 m*M* ammonium+2.5 m*M* nitrate, 2.5 m*M* ammonium, nitrogen-deficient, 5.0 m*M* nitrate and 5.0 m*M* ammonium. When this *Kalanchoë* species was cultivated in 2.5 m*M* nitrate, ATP-dependent H^+ transport was about 3.5 times higher than when the species was in the presence of 5.0 m*M* ammonium.

In the presence of malate, the H^+ transport rates showed a similar pattern for both *Kalanchoë* species. *Kalanchoë* plants cultivated with 2.5 m*M* nitrate presented the highest proton transport rates, while the plants kept under 5.0 m*M* ammonium showed the lowest rates (Table 2). In the presence of citrate, the proton transport rates in *K. laxiflora* and *K. delagoensis* did not show any discernible pattern. Generally, ATP-dependent proton transport in all of the treatments in *K. delagoensis* showed significantly higher rates while in the presence of fumarate or malate compared with when in the presence of all of the treatments for *K. laxiflora* (Table 2).

PPi-dependent proton transport rates

PPi-dependent proton transport rates (%F) were calculated in a similar way from the quinacrine fluorescence quenching reactions as shown in Appendixes S3 and S4 in Supporting Information.

The pattern of PPi-dependent H^+ transport rates in the presence of all of the anions was different when compared with ATP-dependent proton transport in *K. laxiflora* and *K. delagoensis*. For *K. laxiflora*, in the presence of fumarate, the highest H^+ transport rates were observed in 2.5 m*M* nitrate, 2124% min^{-1} mg protein^{-1}, while the lowest rate was observed in the nitrogen-deficient treatment (Table 3). For *K. delagoensis*, in the presence of the same anion, the highest proton transport rates were observed in 2.5 mM nitrate, and the lowest rates in 5.0 m*M* ammonium (Table 3).
In the presence of malate, the highest rates were observed in both *Kalanchoë species* cultivated with 2.5 m*M* nitrate (Table 3). In the presence of citrate, *K. laxiflora* plants cultivated in 2.5 m*M* ammonium+2.5 m*M* nitrate showed the highest proton transport rates (Table 3). On the other hand, for *K. delagoensis*, the highest PPi-dependent proton transport rates were observed in the presence of 5.0 m*M* ammonium and 2.5 m*M* nitrate (Table 3).

The variations in the PPi-dependent proton transport rates in these species were also dependent upon the different nitrogen sources and their relative concentrations. In *K. laxiflora* and *K. delagoensis*, ATP- or PPi-dependent proton transport rates presented a control (no anion used) higher than zero, and in most of the treatments the rates in this control were higher than the rates measured in the presence of (Table 2). Generally, ATP- or PPi-dependent proton transport rates were higher in the

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presence of fumarate than when in the presence of malate or citrate in both *Kalanchoë* species, and in all of the treatments. Also, this transport seems to exhibit a preference for ATPase rather than PPiase, because the H^+ transport rates in the presence of ATP were always higher than in the presence of PPi for both species.

Nocturnal organic-acid accumulation

Nocturnal citrate, fumarate and malate accumulation was measured in the leaves of *K. laxiflora* and *K. delagoensis* (Fig. 1). In all of the nitrogen treatments for both *Kalanchoë* species, the highest nocturnal organic acid accumulation was observed for malate. For example, in *K. laxiflora* plants cultivated with 2.5 m*M* nitrate, the amount of malate accumulated during the dark period was about 8 times higher than fumarate and about 72 times higher than citrate in this same treatment. Plants from this same species treated with 5.0 m*M* nitrate or 5.0 m*M* ammonium exhibited the lowest amount of malate accumulated during the night (Fig. 1A). *K delagoensis* plants cultivated in 2.5 m*M* nitrate, the treatment that showed the highest amount of organic acids accumulated during the night, exhibited nocturnal malate accumulation of about 20 times higher than fumarate, and about 4 times higher than citrate. On the other hand, the lowest nocturnal malate accumulation was observed in 5.0 m*M* ammonium (Fig. 1B). In *K. laxiflora*, fumarate showed the second highest nocturnal accumulation, while in *K. delagoensis* citrate was the second most accumulated organic acid in all of the nitrogen treatments (Fig 1).

The rank order of total organic acids (malate + fumarate + citrate) accumulated during the night in *K. laxiflora* under different nitrogen treatments was as follows: 2.5 m*M* nitrate > 2.5 m*M* ammonium+2.5 m*M* nitrate = nitrogen-deficient > 2.5 m*M* ammonium ≥ 5.0 m*M* nitrate = 5.0 m*M* ammonium (Fig. 1A). For *K. delagoensis*, this order showed a slight difference compared with *K. laxiflora*: 2.5 m*M* nitrate > 2.5 m*M* ammonium + 2.5 m*M* nitrate > 2.5 m*M* ammonium > nitrogen-deficient > 5.0 m*M* nitrate > 5.0 m*M* ammonium (Fig. 1B). As shown in the previous results, nocturnal organic-acid accumulation varied according to the species, nitrogen sources and their concentrations. *K. delagoensis* exhibited higher nocturnal total organic acids accumulation, about 3.5 times, compared with *K. laxiflora* (Fig. 1).

Discussion

Although several studies have demonstrated the influence of nutrients on CAM expression, our study is the first to show, to our knowledge, that the rates of vacuolar proton and anion transport in CAM plants, and the associated nocturnal accumulation of malate, fumarate and citrate, are affected by growth of plants at differing concentrations and sources of inorganic nitrogen in two *Kalanchoë* species, *K. laxiflora* and *K. delagoensis*.

Winter and Holtum (2011) demonstrated an influence of KNO_3 on CAM photosynthesis in the inducible species *Calandrinia polyandra*. It was observed that after 77 days without nutrients this

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species showed a slight dark CO_2 fixation. However, after 20 m*M* KNO_3 was provided to the soil, a loss of nocturnal CO_2 fixation was noted within 48 hours. In other words, the authors observed that soil nutrient supply affects the balance between C_3 and CAM photosynthesis. Ota and Yamamoto (1991) previously observed in *K. blossfeldiana* a higher degree of CAM expression when this plant was grown in the presence of nitrate. Our results corroborate this study, as we observed in *K. laxiflora* and *K. delagoensis* that the highest concentrations of organic acids, mainly malate, were accumulated during the night in the plants kept under 2.5 m*M* nitrate (low concentration). Maftoun et al. (1980) reported that *Kalanchoë verticillata* and *K. laxiflora* grew equally well on NO_3^- and NH_4^+ as N source, while *Sedum telephoides* showed reduced leaf growth in the presence of NH_4^+. Therefore, CAM species seem to show different preferences for inorganic nitrogen sources in regards to growth. In our investigation we verified in *K. laxiflora* and *K. delagoensis* that the highest ammonium concentration (5.0 m*M*) decreased the overall accumulation of organic-acids (malate, fumarate and citrate) during the night. It is probable that even 5.0 m*M* ammonium would show slight toxicity effects to *K. laxiflora* and *K. delagoensis* compared with lower ammonium concentration (2.5 m*M*), and this toxicity might be responsible for the lowest rates of nocturnal organic-acids accumulation in these species. In *K blossfeldiana*, a higher ammonium concentration (10.0 m*M*) presented toxicity effects such as necrosis in the basal portion of the stem of this species (Ota 1988a). Britto et al. (2001) explained NH_4^+ toxicity as a result of the high energetic cost associated with pumping this ion back out of the cells, after entering at high rates in ammonium-sensitive species.

Generally, when the results from this study are combined with the observations made in earlier publications with *Kalanchoë* species, the data support the theory that species from the genus *Kalanchoë* show a higher degree of CAM expression when cultivated on NO_3^- compared with NH_4^+ (Ota 1988a, 1988b; Ota and Yamamoto 1991). One hypothesis for the preference for nitrate over ammonium could be a higher production of nitric oxide (NO) in the presence of NO_3^- compared with NH_4^+ in these *Kalanchoë* species. NO could be a signal to increase CAM expression in these species, as it is known that nitrate reductase (NR), an enzyme that uses NO_3^- as its substrate, is one of the main enzymes responsible for increasing NO production by plants tissues (Hao et al. 2008; Zhao et al. 2009). The presence of NO_3^- could thus increase NR activity and this enzyme could increase NO production in the leaves of *K. laxiflora* and *K. delagoensis*. Freschi et al (2010a), studying *Ananas comosus*, reported that the up-regulation of CAM photosynthesis was accompanied by increases in NO production and endogenous levels and that decreased CAM expression coincided with a decrease in NO emission. On the other hand, the metabolic consequences of NO_3^- nutrition seem to differ among *Kalanchoë* species. For *K. pinnata*, a high nitrate concentration (24.0 m*M*) increased PEPC activity and, consequently CAM expression, by about 3-fold when compared with a low concentration (0.6 m*M*) (Winter et al. 1982). For *K. blossfeldiana*, a slight increase in nocturnal malate accumulation was observed in the presence of a high nitrate concentration (10.0 m*M*), but the CO_2 absorbed during the night tended to decrease at this high concentration (10.0 m*M*) (Ota and Yamamoto 1991). In the

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present study, *K. laxiflora* and *K. delagoensis* appeared to show a preference for lower nitrogen concentrations (2.5 m*M*) regardless of the source of nitrogen. Another revealing result is that these species prefer either the combination of nitrogen sources (2.5 m*M* ammonium + 2.5 m*M* nitrate) or the nitrogen-deficiency, rather than the high concentration of each nitrogen source individually.

In addition to showing the influence of nitrogen sources and their concentrations on CAM photosynthetic expression, this study is also significant in that it differentiates between which species of organic acids are accumulated in the vacuole during the night in these *Kalanchoë* species. *K. laxiflora* exhibited the highest accumulation of malate, followed by fumarate and citrate during the night. Other studies have shown high (daytime) fumarate accumulation in *Arabidopsis thaliana* (Chia et al. 2000). However, the reason that some plants preferentially accumulate fumarate over other organic acids is still unclear. This organic acid is an important component of the tricarboxylic acid cycle and it can be metabolized and used to provide energy and carbon skeletons via anaplerotic pathways (Chia et al. 2000). Fumarate can be synthesized from malate by fumarate hydratase, and the reverse reaction with loss of water can convert fumarate into malate (Chia et al. 2000). Conversion of malate to pyruvate via NAD(P)-malic enzyme in the mitochondria or cytosol is under the control of fumarate. When fumarate accumulates, the conversion of malate to pyruvate is facilitated (Igamberdiev et al. 2001). Finally, pyruvate, as well as entering the TCA cycle, can be converted to phospho*enol*pyruvate (PEP), which is then carboxylated to oxaloacetate (OAA) and reduced to malate, which is accumulated in the vacuole in CAM plants during the night (Igamberdiev and Eprintsev 2016). Osmond et al. (1988) observed a labeling of [1-^{13}C] malate by virtue of label randomization between C-4 and C-1 by fumarase during the dark period in *K. daigremontiana*. At the beginning of the dark period, a decrease in fumarate content was observed in *K. daigremontiana* leaves, while the malate content showed an increase during this time. In this way, as previously observed for *K. daigremontiana*, fumarate could contribute to increasing the accumulation of malate into the vacuole of *K. laxiflora*, which would explain the high nocturnal malate accumulation observed in this species. On the other hand, *K. delagoensis* showed a higher nocturnal accumulation of citrate into the vacuole, compared with fumarate. For this *Kalanchoë* species, the nocturnal citrate storage could be a way to generate reducing power at night and survive under severe environmental conditions. Lüttge (1988) showed that citric acid accumulation is more energetically favorable than malic acid. In addition, citrate represents a way to generate reducing power at night without causing a loss of carbon, since 3 mol NADH is generated per mol of formed citrate (Lüttge 1988, Freschi et al. 2010b, Pereira et al. 2013). Furthermore, the greater storage of citrate at night in *K. delagoensis* plants kept under 2.5 m*M* nitrate could be another alternative to generate reducing power from citrate synthesis for NR activity, as previously observed for the CAM bromeliad species, *Tillandsia pohliana* (Freschi et al. 2010b). In this way, a higher CAM expression in *K. delagoensis* compared with *K. laxiflora*, could also be due to the high nocturnal citrate accumulation into the vacuole, which may help to increase NR activity by

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providing reducing power to this enzyme and consequently increase the production of NO by NR, which could be a signal to increase CAM expression in *K. delgoensis* leaves.

This study has shown that higher nitrate concentrations (5.0 m*M*) decrease CAM expression in *K. laxiflora* and *K. delagoensis*. Studies have previously reported that NO_3^- could inhibit the tonoplast ATPase (Smith et al. 1984). Therefore, *Kalanchoë* plants kept under a high nitrate concentration (5.0 m*M*) would not allow the movement of organic acids and protons into the vacuole, because this nitrate concentration seems to be sufficient to decrease ATP-dependent proton transport and, consequently, decrease CAM expression. However, low nitrate concentrations seem to have no inhibitory effect on the V-ATPase activity. Brüx et al. (2008) reported that *A. thaliana* plants grown in the presence of 1 m*M* nitrate did not reduce V-ATPase activity. In *K. laxiflora* and *K. delagoensis*, the highest ATP-dependent proton transport was observed in the plants kept under 2.5 m*M* nitrate. Thereby, the nitrate inhibitory effect on V-ATPase activity seems to be directly related to nitrate concentration, as well as dependent upon the species. Thereby lower nitrate concentrations (2.5 m*M*) does not seem to inhibit V-ATPase activity compared with higher nitrate concentrations (5.0 m*M*) in these *Kalanchoë* species used in this study. In regard to proton and organic-acids transport into the vacuole, White and Smith (1989) reported very similar ATP- and PPi-dependent proton transport rates in *K. daigremontiana*, and these rates were higher in the presence of fumarate compared with malate. In *K. laxiflora* and *K. delagoensis*, a significantly higher ATPase-dependent proton transport in the presence of fumarate or malate was observed over PPiase-dependent transport. Moreover, fumarate was more effective than malate and citrate in all of the treatments, performed on both species, in supporting ATP- or PPi-dependent vesicle acidification, indicating a preferential selectivity of the vacuolar anion channel for fumarate over malate in *K. laxiflora* and *K. delagoensis*. However, this does not imply that more fumarate will be accumulated than malate in the vacuole. As was shown in our study, there is not more fumarate than malate available in the cytosol, restricting the fumarate capable of being accumulated in the vacuole of both *Kalanchoë* species. For PPi-dependent vesicle acidification, in *K. laxiflora*, fumarate was more effective than the other two organic acids tested in all of the treatments. In *K. delagoensis*, fumarate was more effective than citrate in all of the treatments, but it was only more effective than malate in 2.5 m*M* ammonium and in higher nitrate and ammonium concentrations. ATP-dependent proton transport in *K. delagoensis,* in the presence of fumarate or malate, was significantly higher than in *K. laxiflora*.

The contribution made by PPiase and V-ATPase in the vacuolar proton transport reported for *Mesembryanthemum crystallinum* showed a large difference, since the V-ATPase activity was about nine times higher than the PPiase when these plants were CAM induced by salinity stress. Bremberger et al. (1988) suggested that tonoplast ATPase is a more important enzyme than H^+-PPiase in driving the nocturnal accumulation of organic acids in this CAM plant. Our results corroborate this study, as we observed a higher proton transport in the vacuole in the presence of ATP rather than PPi for both *Kalanchoë* species. Besides a higher nocturnal organic acids accumulation in *K. delagoensis* compared

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with *K. laxiflora*, the quinacrine fluorescence-quenching experiments for both species showed that the permeability of the tonoplast membrane for the anions used in this study appears to be higher in *K. delagoensis* rather than their permeability in *K. laxiflora*. This result is consistent with a higher nocturnal organic acids accumulation into the vacuole of *K. delagoensis* compared with the accumulation into the vacuole of *K. laxiflora*. Although it has not been evaluated in these *Kalanchoë* species, it is important to raise the questions of the effects of anions concentrations and carbohydrate supply on the organic acids accumulation into the vacuole. Lüttge et al. (2000) observed the influence of concentration and combination of different ions on the proton transport rates in *Kalanchoë daigremontiana*. An increase of malate concentration from 20 to 50 m*M* caused a significant increase in H^+ transport rate. In addition, this same study showed that the addition of a chloride anion after adding a malate anion to the tonoplast vesicles reduced the relative rates of H^+ transport compared to when malate was presented without chloride (Luttge et al. 2000). Other compounds, such as storage carbohydrates, also affect the acids accumulation into the vacuole. Chen and Nose (2004) showed that starch-degrading CAM species exhibited a higher V-PPiase activity compared with monosaccharide-degrading CAM species, which seem to show a preference for V-ATPase to the energization of the vacuole. The starch-degrading CAM species are able to generate malate by phosphorylase activity (PPiase) and through this enzyme they can save energy (ATP). However, the effects of anions concentrations and carbohydrate supply on the organic acids accumulation into the vacuole have not been studied in *K. delagoensis* and *K. laxiflora*. Therefore, more studies are necessary to better understand how they affect CAM photosynthesis in relation to proton and anion transport rates as well as organic acids accumulation into the vacuole in these *Kalanchoë* species.

In conclusion, this study has provided evidence that both ATP- and PPi-dependent proton transport rates are influenced by inorganic nitrogen sources (simultaneous or individual occurrence) by their concentrations (2.5 or 5.0 m*M*) and by the *Kalanchoë* species itself. Moreover, we have shown, to our knowledge for the first time in *Kalanchoë* species, that there is a slight ATP- and PPi-dependent proton transport in the absence of any anion, that can be considered as a background, and also that there is a preference for ATPase rather than PPiase to the proton transport in *K. laxiflora* and *K. delagoensis*. Finally, our data indicate that a low nitrate concentration (2.5 m*M*) increases the nocturnal organic-acids accumulation, increases ATP- and PPi-dependent proton transport into the vacuole in both *K. laxiflora* and *K. delagoensis,* and is responsible for the highest CAM expression in both *Kalanchoë* species; while a higher nitrate concentration (5.0 m*M*) decreases the amount of organic-acids accumulated during the night, and also seems to have an inhibitory effect on ATP-dependent proton transport into the vacuole in both *K. laxiflora* and *K. delagoensis*. In addition, this study has shown a higher CAM expression in *K. delagoensis* compared with *K. laxiflora*. This could be due to a higher nocturnal citrate accumulation into the vacuole as observed to *K. delagoensis* rather than *K. laxiflora*. The higher citrate levels may help to increase NR activity by providing reducing

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power to this enzyme and consequently increase the production of NO by NR. In this way, NO could be a signal to increase CAM photosynthesis in *K. delagoensis* in the presence of 2.5 m*M* nitrate.

Author Contributions

P.N.P conducted all of the experiments. H.M, J.A.C.S and P.N.P conceived the project. J.A.C.S assisted in developing experimental protocols and H.M supervised the experiments. P.N.P wrote the article with contributions from the other authors. All authors read and approved the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 ATP-dependent proton transport monitored by the quenching of quinacrine fluorescence in the tonoplast vesicles of *Kalanchoë laxiflora*.

Appendix S2 ATP-dependent proton transport monitored by the quenching of quinacrine fluorescence in the tonoplast vesicles of *Kalanchoë delagoensis.*

Appendix S3 PPi-dependent proton transport monitored by the quenching of quinacrine fluorescence in the tonoplast vesicles of *Kalanchoë laxiflora*.

Appendix S4 PPi-dependent proton transport monitored by the quenching of quinacrine fluorescence in the tonoplast vesicles of *Kalanchoë delagoensis.*

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Table 1 Effect of ATPase inhibitors on initial rates of ATP-dependent proton transport into isolated tonoplast vesicles from leaves of *Kalanchoë laxiflora* and *Kalanchoë delagoensis* plants kept in nitrogen-deficient conditions (used as a control), 2.5 m*M* ammonium + 2.5 m*M* nitrate, 2.5 m*M* ammonium, 2.5 m*M* nitrate, 5.0 m*M* ammonium and 5.0 m*M* nitrate. Proton transport was measured as the initial rate of quinacrine-fluorescence quenching in the presence of 50 mM fumarate following addition of 3 mM ATP to the suspension of tonoplast vesicles as described in Materials and methods. Rates of proton transport were quantified as % relative fluorescence quenching min^{-1} mg $protein^{-1}$. Measurements were made in the presence of inhibitors of vacuolar H^+-ATPase (50 mM KNO_3) or non-vacuolar H^+-ATPases (0.1 mM NaN_3 plus 0.1 mM Na_3VO_4) in comparison with the control (no inhibitors). Results are expressed as means (± s.d.) for three independent preparations. Values in parentheses give the percentage inhibition relative to the control for each species; asterisks indicate significant differences between each inhibitors (KNO_3 or NaN_3 + Na_3VO_4) and the control in the same treatment in each species by Student's *t*-test at 5% significance.

	Specific activity (% min^{-1} mg $protein^{-1}$) (inhibition relative to control)		
Kalanchoë laxiflora	**Control**	**+ KNO_3**	**NaN_3 + Na_4VO_3**
Nitrogen-deficient	3279 ± 30.8 A	161 ± 3.5 C (95%) *	2857 ± 21.7 B (13%) *
2.5 m*M* Ammonium + 2.5 m*M* Nitrate	4501 ± 76.2 A	171 ± 4.8 C (96%) *	3487 ± 43.3 B (23%) *
2.5 m*M* Ammonium	2781 ± 19.1 A	92.0 ± 18.9 C (97%) *	2254 ± 78.9 B (19%) *
2.5 m*M* Nitrate	4498 ± 33.4 A	58.6 ± 6.5 C (99%) *	4396 ± 31.9 B (2.3%)
5.0 m*M* Ammonium	697 ± 27.5 A	71.8 ± 3.2 C (90%) *	612 ± 26.4 B (13%) *
5.0 m*M* Nitrate	1119 ± 47.2 A	89.8 ± 6.2 C (92%) *	838 ± 94.4 B (25%) *
Kalanchoë delagoensis	**Control**	**+ KNO_3**	**NaN_3 + Na_4VO_3**
Nitrogen-deficient	3735 ± 54.6 A	459 ± 50.9 C (88%) *	3010 ± 30.8 B (19%) *
2.5 m*M* Ammonium + 2.5 m*M* Nitrate	3936 ± 136.1 A	203 ± 47.5 C (95%) *	2659 ± 86.2 B (32%) *
2.5 m*M* Ammonium	3673 ± 97.0 A	232 ± 31.2 B (94%) *	3580 ± 108.7 A (2.4%)
2.5 m*M* Nitrate	4757 ± 146.7 A	269 ± 23.5 B (94%) *	4622 ± 168.7 A (2.8%)
5.0 m*M* Ammonium	311 ± 28.2 A	141 ± 13.4 C (54%) *	237.8 ± 13.1 B (23%) *
5.0 m*M* Nitrate	1964 ± 132.4 A	264 ± 57.0 C (86%) *	1628 ± 55.6 B (17%) *

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Table 2 Effect of charge-balancing anion on initial rates of ATP-dependent proton transport into isolated tonoplast vesicles from leaves of *Kalanchoë laxiflora* and *Kalanchoë delagoensis* plants kept in nitrogen-deficient conditions (used as a control), 2.5 m*M* ammonium + 2.5 m*M* nitrate, 2.5 m*M* ammonium, 2.5 m*M* nitrate, 5.0 m*M* ammonium and 5.0 m*M* nitrate in dependence on the balancing carboxylate anion. Proton transport was measured as described in Table 1 in the presence of three different carboxylate anions (fumarate, malate, or citrate, each supplied as their BTP-salt at 50 mM) in comparison with the control (no added carboxylate anion). Rates of proton transport were quantified as % relative fluorescence quenching min^{-1} mg $protein^{-1}$; values are expressed as means (± s.d.) for three independent preparations. Different capital letters indicate values that were significantly different among species using the same anion (Tukey–Kramer test; $P < 0.05$). Different lower case letters indicate values that were significantly different among different carboxylate anions in the same species (Tukey–Kramer test; $P < 0.05$).

	Specific activity (% min^{-1} mg $protein^{-1}$)			
Kalanchoë laxiflora	**Fumarate**	**Malate**	**Citrate**	**Control**
Nitrogen-deficient	3040 ± 9.6 C a	1053 ± 15.3 C b	38.8 ± 7.6 D d	274 ± 6.8 A c
2.5 m*M* Ammonium + 2.5 m*M* Nitrate	3113 ± 14.9 B a	1516 ± 15.3 B b	53.08 ± 9.0 CD b	208 ± 7.0 C c
2.5 m*M* Ammonium	2131 ± 12.6 D a	522 ± 6.9 D b	126 ± 7.7 AB c	21.7 ±2.3 F d
2.5 m*M* Nitrate	4061 ± 24.2 A a	1623 ± 12.0 A b	142 ± 10.7 A d	239 ± 5.1 B c
5.0 m*M* Ammonium	445 ± 7.7 F a	149 ± 8.4 F c	73.0 ± 6.2 C d	190 ± 10.5 D b
5.0 m*M* Nitrate	878 ± 16.4 E a	210 ± 6.0 E b	111 ± 8.1 B c	104 ± 3.5 E c
Kalanchoë delagoensis	**Fumarate**	**Malate**	**Citrate**	**Control**
Nitrogen-deficient	2809 ± 8.2 D a	1424 ± 6.8 C b	73.3 ± 5.8 C d	145 ± 4.02 C c
2.5 m*M* Ammonium + 2.5 m*M* Nitrate	3776 ± 5.3 B a	1810 ± 6.7 B b	107 ± 9.1 B d	243 ± 3.5 A c
2.5 m*M* Ammonium	3715 ± 5.0 C a	1310 ±10.0 D b	142 ± 7.4 A d	220 ± 12.2 B c
2.5 m*M* Nitrate	4172 ± 15.5 A a	2088 ±6.9 A b	108 ± 10.4 B c	108 ± 7.0 D c
5.0 m*M* Ammonium	1207 ± 8.9 F a	531 ± 7.6 F b	21.4 ± 1.0 E d	67.2 ± 5.4 E c
5.0 m*M* Nitrate	2009 ± 5.2 E a	774 ± 9.9 E b	45.2 ± 8.3 D d	26.7 ± 1.2 F c

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Table 3 Effect of charge-balancing anion on initial rates of PPi-dependent proton transport into isolated tonoplast vesicles from leaves of *Kalanchoë laxiflora* and *Kalanchoë delagoensis* plants kept in nitrogen-deficient conditions (used as a control), 2.5 m*M* ammonium + 2.5 m*M* nitrate, 2.5 m*M* ammonium, 2.5 m*M* nitrate, 5.0 m*M* ammonium and 5.0 m*M* nitratein dependence on the balancing carboxylate anion. Proton transport was measured as described in Table 1 in the presence of three different carboxylate anions (fumarate, malate, or citrate, each supplied as their BTP-salt at 50 mM) in comparison with the control (no added carboxylate anion). Rates of proton transport were quantified as % relative fluorescence quenching min^{-1} mg $protein^{-1}$; values are expressed as means (± s.d.) for three independent preparations. Different capital letters indicate values that were significantly different among species using the same anion (Tukey–Kramer test; $P < 0.05$). Different lower case letters indicate values that were significantly different among different carboxylate anions in the same species (Tukey–Kramer test; $P < 0.05$).

	Specific activity (% min^{-1} mg $protein^{-1}$)			
Kalanchoë laxiflora	**Fumarate**	**Malate**	**Citrate**	**Control**
Nitrogen-deficient	315 ± 7.1 E a	203 ± 8.5 C b	33.2 1.7 C d	51.8 ± 4.5 D c
2.5 m*M* Ammonium + 2.5 m*M* Nitrate	688 ± 3.7 B a	536 ± 6.8 B b	219 ± 8.4 A c	87.3 ± 5.3 C d
2.5 m*M* Ammonium	511 ± 10.2 C a	220 ± 12.1 C b	25.3 ± 4.6 C d	51.0 ± 3.8 D c
2.5 m*M* Nitrate	2125 ± 25.0 A a	753 ±15.3 A b	75.4 ± 3.9 B d	126 ± 5.2 A c
5.0 m*M* Ammonium	404 ± 13.9 D a	119 ± 5.1 D b	20.8 ± 0.7 C c	106 ± 5.2 B b
5.0 m*M* Nitrate	523 ± 8.9 C a	142 ± 8.1 D b	87.7 ± 7.8 B c	73.3 ± 6.7 C c
Kalanchoë delagoensis	**Fumarate**	**Malate**	**Citrate**	**Control**
Nitrogen-deficient	829 ± 16.5 D a	906 ± 54.9 C a	34.4 ± 0.7 CD b	70.2 ± 3.4 DE b
2.5 m*M* Ammonium + 2.5 m*M* Nitrate	1125 ± 21.8 B a	1057 ± 10.0 B a	23.4 ± 2.43 D b	49.8 ± 13.3 E b
2.5 m*M* Ammonium	1025 ± 5.0 C a	854 ± 10.0 C b	42.0 ± 7.1 CD d	90.6 ± 9.2 CD c
2.5 m*M* Nitrate	1280 ± 6.7 A a	1303 ± 11.9 A a	66.3 ± 1.53 AB c	108 ± 13.1 BC b
5.0 m*M* Ammonium	468 ± 10.1 E a	405 ± 8.3 E b	82.9 ± 11.9 A d	141 ± 7.9 A c
5.0 m*M* Nitrate	846 ± 10.8 D a	674 ± 1.5 D b	49.7 ± 10.0 BC d	120 ± 8.5 AB c

For Peer Review

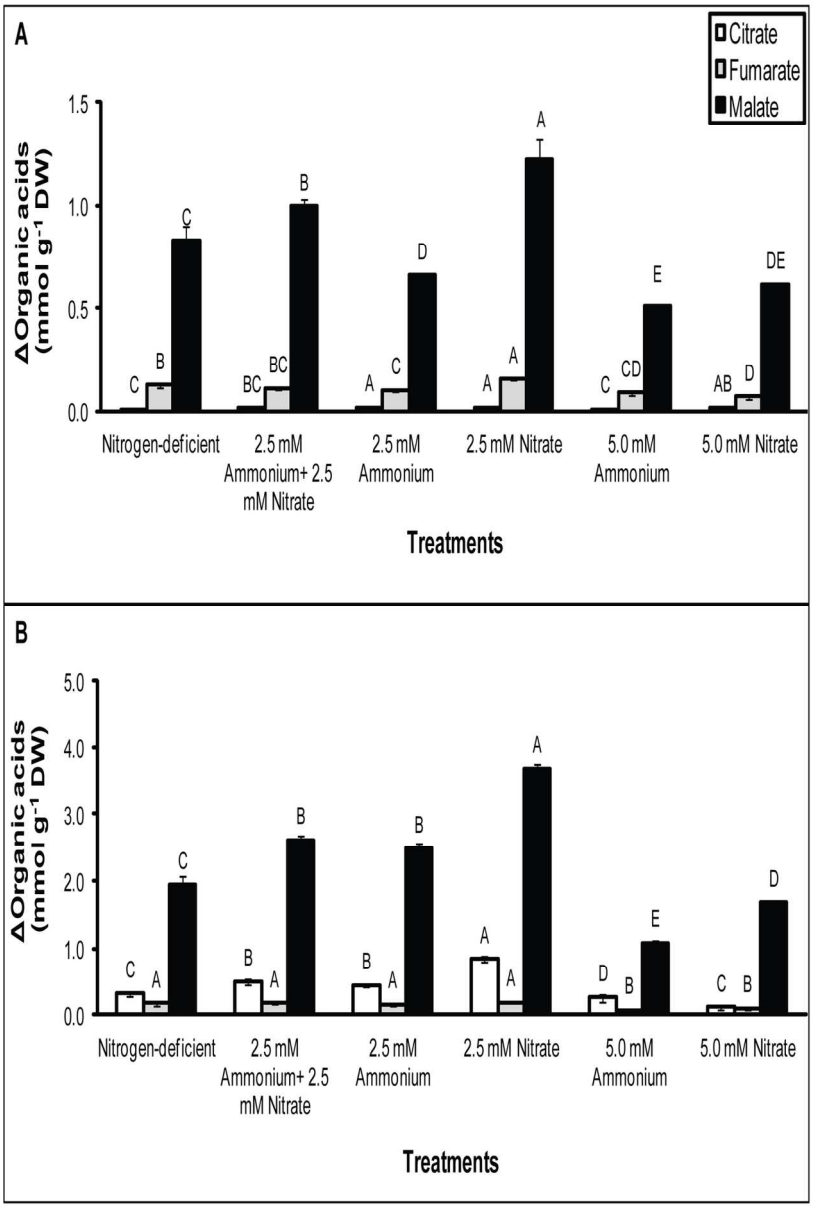
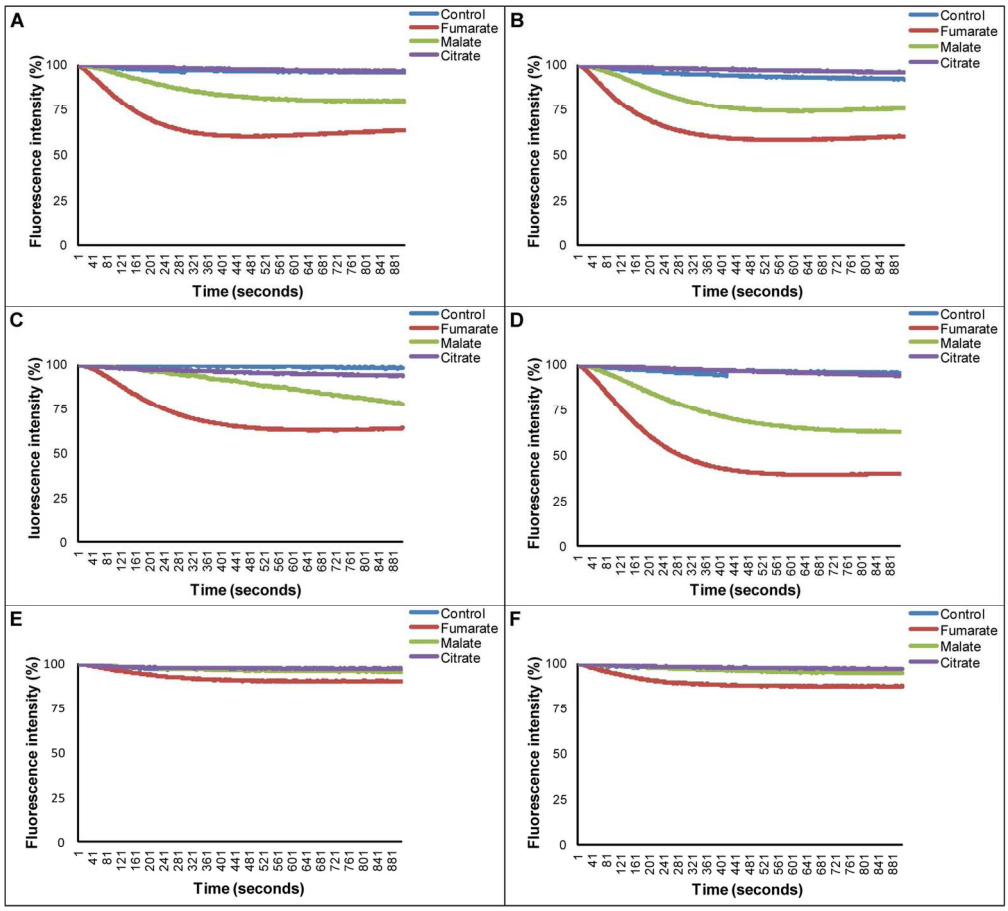


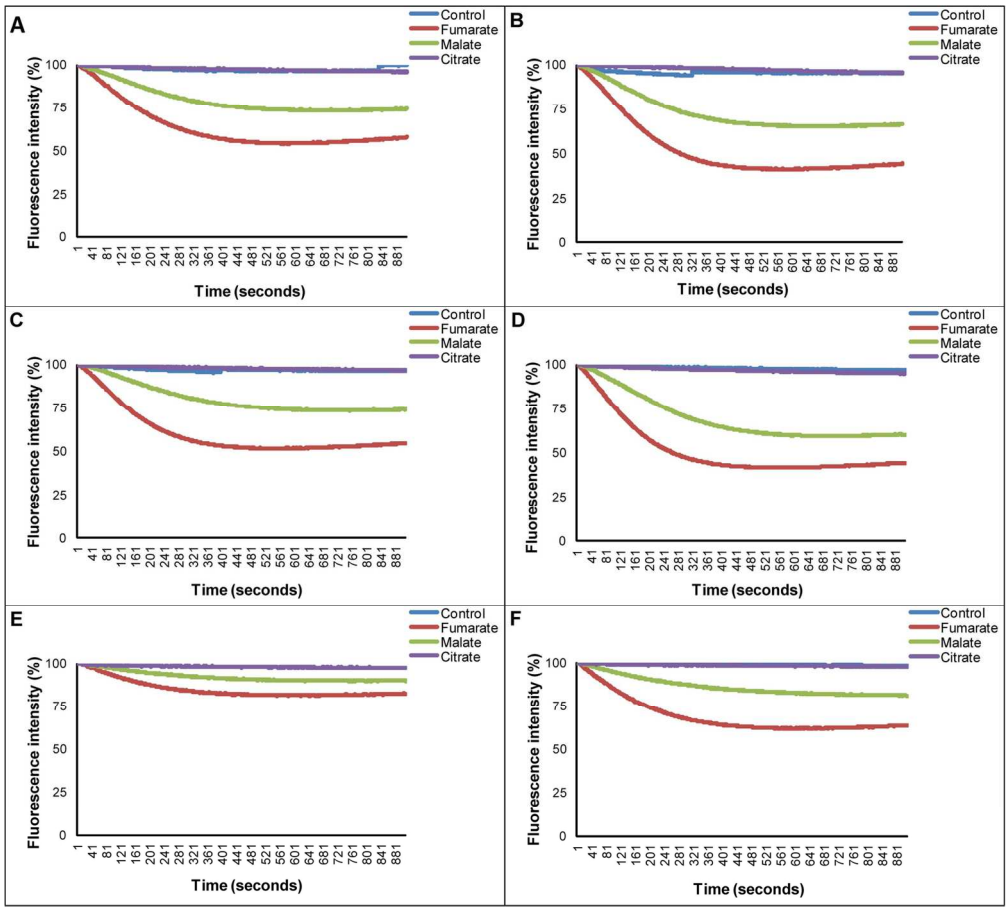
Figure 1. Nocturnal accumulation of citrate, fumarate and malate in the leaves of (A) Kalanchoë laxiflora plants and (B) Kalanchoë delagoensis plants kept under nitrogen-deficient conditions (used as a control), 2.5 mM ammonium + 2.5 mM nitrate, 2.5 mM ammonium, 2.5 mM nitrate, 5.0 mM ammonium and 5.0 mM nitrate. Values for Δmalate and Δcitrate were calculated as the difference in malate and citrate concentration, respectively, in samples harvested at dawn minus those harvested at dusk as described in Materials and methods. Values are means (± s.d.) of 3 samples, with each sample consisting of material pooled from three individual plants. Different letters indicate values that were significantly different among treatments for the same organic-acid (Tukey–Kramer test; $P < 0.05$). DW = dry weight.

119x178mm (300 x 300 DPI)



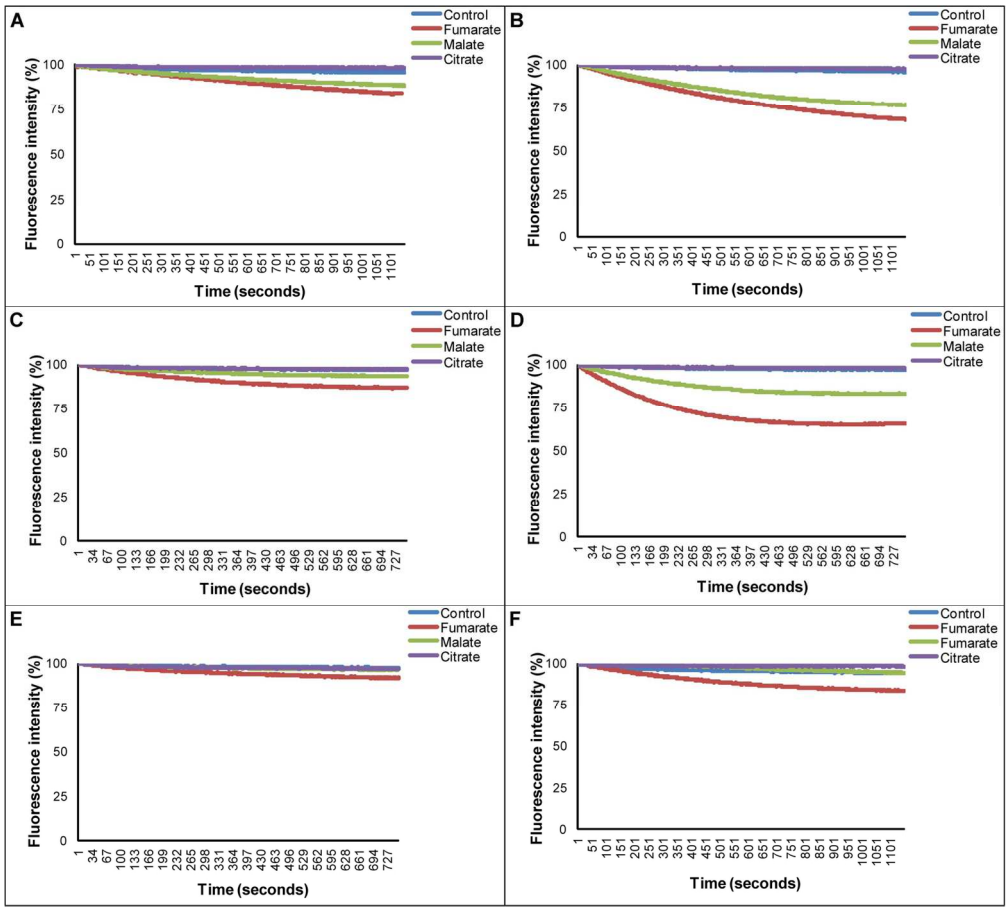
Appendix S1 ATP-dependent proton transport into isolated tonoplast vesicles monitored by quinacrine-fluorescence quenching (expressed as a percentage of the starting value) in control conditions (no added carboxylate anion), or in the presence of 50 mM fumarate, 50 mM malate, or 50 mM citrate. Tonoplast vesicles were isolated as described in Materials and methods from leaves of Kalanchoë laxiflora plants in nitrogen-deficient conditions (used as a control), 2.5 mM ammonium + 2.5 mM nitrate, 2.5 mM ammonium, 2.5 mM nitrate, 5.0 mM ammonium and 5.0 mM nitrate (A–F , respectively). Proton transport was initiated by the addition of ATP (3.0 mM) at the start of the time-course. Results are from one experiment representative of a total of three.

143x129mm (300 x 300 DPI)



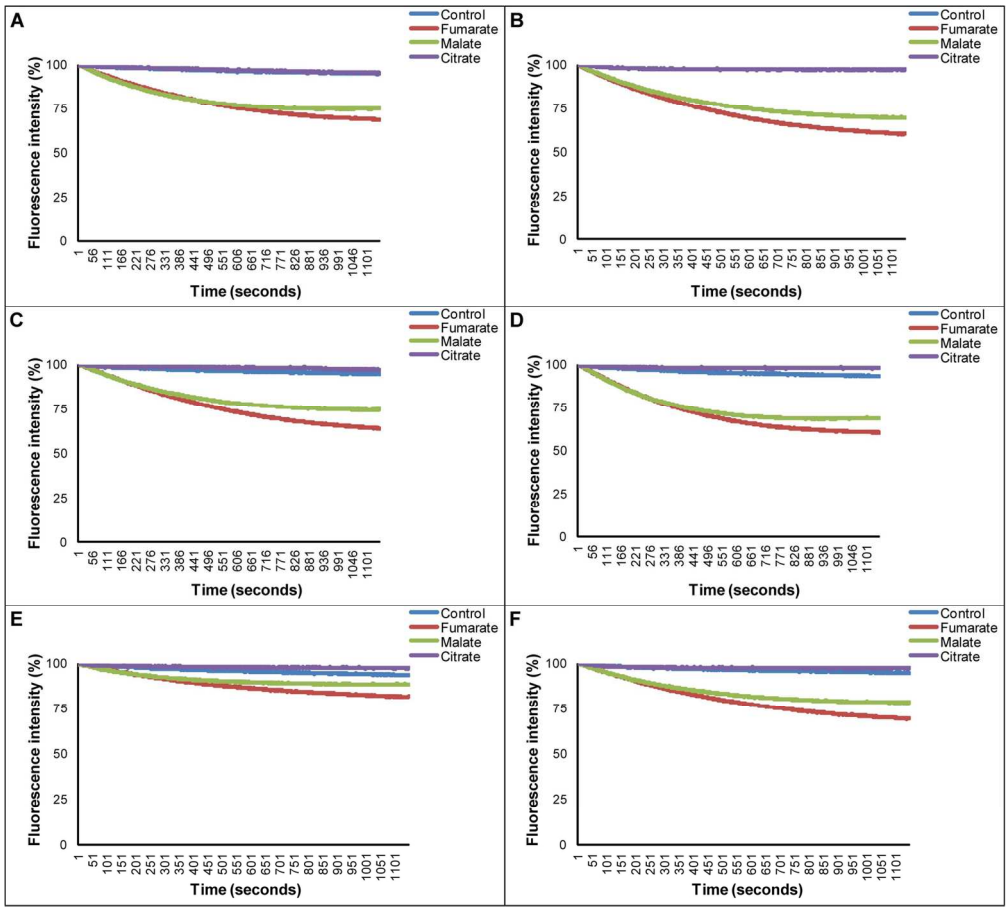
Appendix S2 ATP-dependent proton transport into isolated tonoplast vesicles monitored by quinacrine-fluorescence quenching (expressed as a percentage of the starting value) in control conditions (no added carboxylate anion), or in the presence of 50 mM fumarate, 50 mM malate, or 50 mM citrate. Tonoplast vesicles were isolated as described in Materials and methods from leaves of Kalanchoë delagoensis plants in nitrogen-deficient conditions (used as a control), 2.5 mM ammonium + 2.5 mM nitrate, 2.5 mM ammonium, 2.5 mM nitrate, 5.0 mM ammonium and 5.0 mM nitrate (A–F , respectively). Proton transport was initiated by the addition of ATP (3.0 mM) at the start of the time-course. Results are from one experiment representative of a total of three.

143x129mm (300 x 300 DPI)



Appendix S3 PPi-dependent proton transport into isolated tonoplast vesilces monitored by quinacrine-fluorescence quenching (expressed as a percentage of the starting value) in control conditions (no added carboxylate anion), or in presence of 50 mM fumarate, 50 mM malate, or 50 mM citrate. Tonoplast vesicles were isolated as described in Materials and methods from leaves of Kalanchoë laxiflora plants in nitrogen-deficient conditions (used as a control), 2.5 mM ammonium + 2.5 mM nitrate, 2.5 mM ammonium, 2.5 mM nitrate, 5.0 mM ammonium and 5.0 mM nitrate (A–F , respectively). Proton transport was initiated by the addition of PPi (3.0 mM) at the start of the time-course. Results are from one experiment representative of a total of three.

143x129mm (300 x 300 DPI)



Appendix S4 PPi-dependent proton transport into isolated tonoplast vesilces monitored by quinacrine-fluorescence quenching (expressed as a percentage of the starting value) in control conditions (no added carboxylate anion), or in presence of 50 mM fumarate, 50 mM malate, or 50 mM citrate. Tonoplast vesicles were isolated as described in Materials and methods from leaves of Kalanchoë delagoensis plants in nitrogen-deficient conditions (used as a control), 2.5 mM ammonium + 2.5 mM nitrate, 2.5 mM ammonium, 2.5 mM nitrate, 5.0 mM ammonium and 5.0 mM nitrate (A–F , respectively). Proton transport was initiated by the addition of PPi (3.0 mM) at the start of the time-course. Results are from one experiment representative of a total of three.

143x129mm (300 x 300 DPI)