SHORT COMMUNICATION

Micronutrients affecting leaf biochemical responses during pineapple development

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ABSTRACT: An adequate mineral nutrition is essential for the development and productivity of pineapple. However, little is known about the nutritional and metabolic changes that occur in this crop in response to micronutrient deficiency or excess, particularly on tropical conditions. Thus, the objective of this study was to evaluate the application effects of micronutrients in soil and in leaf on biochemical responses of leaves during the development cycle of the pineapple crop. Samples were collected at 3, 6, 9, 12, and 17 months after transplantation. Leaf soluble carbohydrates and N-aminosoluble compounds were determined, as well as variations in the titratable acidity and pH. The soil and leaf micronutrient application increased the concentrations of carbohydrates and N-aminosoluble and reduced the leaf pH, and the changes were more significant in the last sampling (17 months after transplantation). Reductions in concentrations of carbohydrates and increase in the titratable acidity of the pineapple leaves collected at the end of the night were also observed, a fact that reflects the metabolism of Crassulacean acid metabolism species. The strategy of micronutrient application contributes positively to alter the metabolism of plants of pineapple cv. Victoria, especially during flowering and fruit development.

KEYWORDS: *Ananas comosus*, carbohydrates, metabolism, mineral nutrition, titratable acidity.

The pineapple has always stood out in the horticulture, not only due to the qualities of its fruit, but also because of the high profitability and social importance, being an activity that requires labor-intensive in the field. Monitoring of the plant metabolism is important as vigorous plants with adequate mineral nutrition may produce a high-quality fruit (Soares et al. 2005, Agbangba et al. 2011).

When analyzing the photosynthetic metabolism, notably the cycle of carbon reduction, it was found that higher plants and algae developed mechanisms of CO_2 concentration, which minimize losses related to photorespiration and enhance water use efficiency (Leegood 2002, Moroney and Ynalvez 2007). Crassulacean acid metabolism (CAM) is a striking example of convergent evolution that substantially improves plant water use efficiency, exceeding those of C4 and C3 plants by at least three and six times, respectively, enabling partial or predominant uptake of CO_2 at night. At least 343 genera in 35 plant families are known to engage this photosynthetic specialization (Borland et al. 2011).

Pineapple belongs to the group of CAM plants, whose main characteristic is to close their stomata during the day and open them at night in order to save water (Martín, Rius and Podestá, 2011). In semiarid and arid conditions, similar to those of Northeast Brazil, CAM crops present comparative advantages, which enable them to produce food, paper, beverages, and pharmaceutical extracts through consecutive seasons (Borland et al. 2011). Thus, pineapple is a CAM crop adapted

to maintain a positive carbon balance under a wide range of environmental stresses (Keller and Luttge 2005, José, Montes and Nikonova 2007).

The inadequate supply of a particular nutrient in the pineapple crop often results in metabolic and nutritional disorders that can compromise growth, yield, and fruit quality (Soares et al. 2005, Vieira et al. 2010). However, little is known about the nutritional and metabolic changes that happen in this crop in response to micronutrient deficiency or excess, particularly on tropical conditions (Siebeneichler et al. 2008, Feitosa et al. 2011, Maeda et al. 2011). The most important micronutrients in pineapple are iron, zinc, copper, and boron (Su 1975). The supply of micronutrients in pineapple crop can be done by solid or liquid applications, the latter being the most used (Reinhardt and Cunha 2010). However, information that relates these types of micronutrient application with changes in metabolism during the development of this crop are rarely reported (Bartholomew et al. 2003). Therefore, the aim of this study was to evaluate the effects of soil and leaf application of micronutrients on leaf biochemical responses during the pineapple crop cycle.

The experiment was conducted from December 2008 to October 2010 in an irrigated area located in Marco county, at the Northern region of State of Ceará, Brazil (3°07'13''S and 40°05'13''W). According to the Köppen's classification, the climate type is Aw' (tropical raining). The experimental area soil is classified as "Typic Quartzipsamment", with a sandy texture and density of 1.590 kg m^{-3}. The soil chemical characteristics at 0 to 20 cm depth are: pH=5.8; EC=0.15 dS m^{-1}; and 0.77, 0.30, 0.08, 0.02, and 0.75 $cmol_c$ kg^{-1} of Ca, Mg, K, Na, and Al, respectively.

The experimental design was in split plot with four levels of soil fertilization, four of leaf fertilization, and five sampling times with 90 days intervals, including five repetitions. Each plot consisted of four subplots, having four double rows with 11 plants in each, and the evaluations were taken at the two central lines of every subplot. The experimental areas were mulched with *bagana* (the straw resulting from the extraction of the carnauba wax sheet) of carnauba (*Copernicia prunifera*).

For the soil fertilization, the commercial micronutrient formulation FTE-12 (9, 1.8, 0.8, 3 and 3% of Zn, B, Cu, Fe and Mn, respectively) was used. It was applied in the pits of each plot before planting at doses of 0, 60, 120, and 180 kg ha^{-1}. The four levels of leaf fertilization were: LF0 (no fertilizer); LF1 (15 leaf fertilization applications, using 1158.8, 844.7, 391.5, 322.7, and 216.0 g ha^{-1} of Fe, Mn, Zn, Cu, and B, respectively); LF2 (15 leaf fertilization applications, using twice the quantities applied in LF1); and LF3 (15 leaf fertilization applications, using three times the amount in LF1).

The leaf fertilization with micronutrients was performed monthly, and the concentrations were defined having as reference the modified Murashige and Skoog (1962) nutrient solution. The concentrations of the salts used in the micronutrient solution formulation in the first two applications are shown in Table 1. These initial ones were doubled in the three following usages, tripled in three other ones and quadrupled in the last seven. To facilitate uptake of micronutrients, it was used urea 2%, which was added to all treatments from the third to the last application. The total volume of the solution in each application was 463 L ha^{-1}.

Macronutrients were applied to all plants via fertigation beginning two months after transplanting, following the same procedure done by producers. The total applied and the fertilizers used were as follows: 688, 797, 98, 80, 20 e 24 kg ha^{-1} of urea, K_2SO_4, H_3PO_4, $NH_4H_2PO_4$, $Ca(NO_3)_2$ and $MgSO_4$, respectively.

Ninety days-old pineapple seedlings (*Ananas comosus* L. Merril) cv. Vitória , which is a cultivar resistent to fusariosis (Ventura et al. 2009), were transferred from trays to black polyethylene plastic bags containing sand as substrate, with 800 g m^{-3} of simple superphosphate. They were acclimated under shade cloth with 50% of shading for six months and irrigated twice a week with water (electrical conductivity of 0.44 dS m^{-1}) during this period. Transplantation was performed in April 2009, when the plants reached about 150 mm; they were arranged in double rows, spaced 0.9 x 0.4 x 0.3 m, with an area of 19.2 m wide and 44.0 m long, totaling 7,040 plants in 0.174 ha.

The leaf samples were taken at 3, 6, 9, 12, and 17 months after transplantation (MAT), the first four coinciding with the period of vegetative growth and the fifth corresponded to the time of development of inflorescences, as it was done about a month before harvesting. In each period, two leaves "D" (the leaf of greater length among the youngest of pineapple) were taken randomly in two plants of each subplot. They were collected in the late afternoon (between 4 and 5 pm) and in the end of the night (between 4 and 5 am). The environmental conditions in each sampling time are shown in Table 2.

The leaves were wrapped in foil and stored in a refrigerator at -20°C for about 72 hours. The frozen ones were ground in a mortar and whole juice from leaf tissues obtained was placed in

Table 1. Salt concentrations (g L^{-1}) used in the formulation of micronutrients solution in the first two applications for different treatments

Salts	LF0	LF1	LF2	LF3
H_3BO_3	No application	0.062	0.124	0.186
$CuSO_4.5H_2O$	No application	0.062	0.124	0.186
$ZnSO_4.7H_2O$	No application	0.086	0.172	0.258
$MnSO_4.4H_2O$	No application	0.169	0.338	0.507
$FeSO_4.7H_2O$	No application	0.278	0.556	0.834

Eppendorf tubes and immediately frozen to be used for determinations of soluble carbohydrates (Dubois et al. 1956) and N-aminosoluble compounds (Yemm and Cocking 1955). The rest of the juice and the residue resulting from grinding were used for the measurement of pH and titratable acidity (TA). For these determinations, samples of 1.0 g were weighted and diluted with deionized water at a ratio of 1:50. The pH was determined by a pH meter. Then, three drops of a 1% phenolphthalein solution were added for determination of TA by titration with 0.1 N NaOH solution (IAL 1985).

The data were submitted to analysis of variance (ANOVA), and the regression one was performed for data in which significant ($p<0.05$) effects occurred.

The two forms of micronutrients application caused increases in the concentration of carbohydrates in both late afternoon and in the end of the night (Figure 1). Regarding the sampling times, it was found that the highest responses were obtained at 12 and 17 MAT. Furthermore, it was concluded that the concentrations of soluble carbohydrates at the late afternoon were higher than those at the end of the night, indicating the consumption of carbohydrate in plant metabolism.

From the first to the last sampling time, the concentrations of carbohydrates in the late afternoon, in relation to

Table 2. Air temperature (T) and relative humidity (RH) at 5 and 16 hours; the average temperature (TL) and relative humidity (RHL) of the light period; and the average temperature (TN) and relative humidity (RHN) at night.

Months	T16	T5	TL	TN	RH16	RH5	RHL	RHN
	(°C)				(%)			
3	26.7	21.7	23.8	22.1	77	95	91.5	94.2
6	33.6	22.9	28.8	23.5	41	88	61.4	86.3
9	28.8	24.2	26.9	24.4	65	90	78.9	89.7
12	28.2	24.5	26.8	24.4	80	95	87.0	94.3
17	34.1	23.8	29.4	24.5	35	83	56.5	81.3

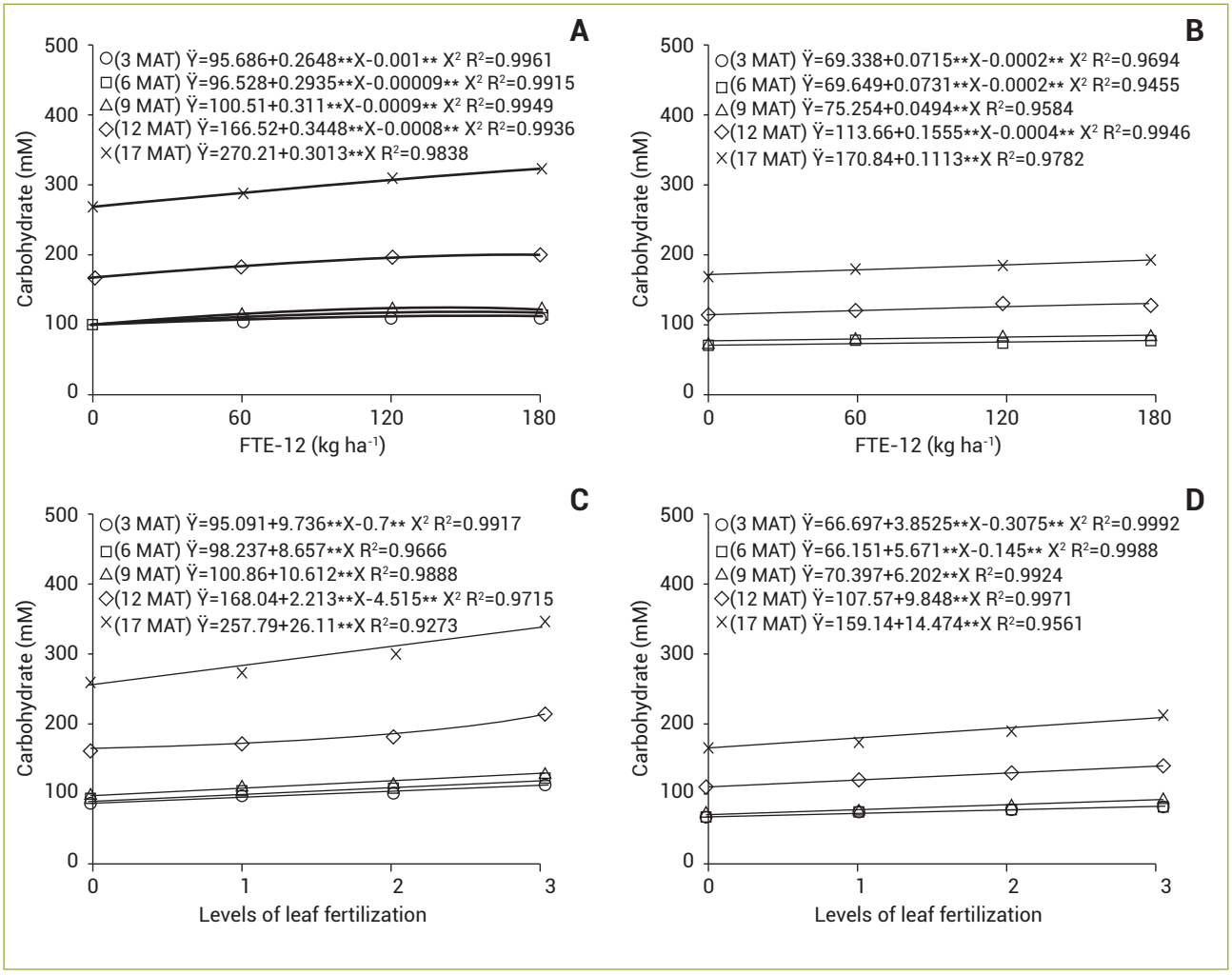

Figure 1. Concentrations of soluble carbohydrates in the leaves of pineapple cv. Vitória as a function of FTE-12 **(A** and **B)** doses and levels of foliar fertilization **(C** and **D)** at five sampling times (3, 6, 9, 12, and 17 months after transplantation), analyzed at late afternoon **(A** and **C)** and at the end of the night **(B** and **D)**. *p<0.05; **p<0.01.

FTE-12 doses, ranged from 95.69 to 270.21 mM (1.8 times increase) at the dose of 0 and from 110.95 to 324.44 mM (1.9 times increase) for the treatment containing 180 kg ha^{-1} FTE-12 (Figure 1A). At the end of the night, the variation was from 69.34 to 170.84 mM (1.5 times increase) for plants that were not receiving FTE-12, and from 75.73 to 190.87 mM (1.5 times increase) at the highest dose of this micronutrient formulation (Figure 1B). Similar results were observed when using increasing levels of leaf fertilization (Figures 1C and D).

Likewise the concentrations of soluble carbohydrates, leaf concentrations of N-aminosoluble compounds also showed increments provided by the two types of the micronutrient application (Figure 2), with the highest values observed at 12 and 17 MAT. The increase was linear for almost all plants, except for those subjected to treatment with FTE-12 at 12 and 17 MAT in those harvested at late afternoon (Figure 2A) and to those taken at 3 MAT at the end of the night (Figure 2B), which showed quadratic behavior.

Despite TA not be changed significantly throughout the crop cycle, there were increases in TA in both types of micronutrient application (Figure 3), and the values at the end of the night were higher than those at late afternoon. The leaf pH values found herein decreased with increasing doses of micronutrients applied (Figure 4). The average of leaf pH changed from 4.74 at late afternoon to 3.5 at the end of the night, noting that the oscillations in TA between nighttime and day time on the leaves of pineapple were consistent with the values of pH in each period, therefore there is an inverse relationship between these variables.

Making up a joint analysis of variations in the concentration of carbohydrates between the end of the day and night, we observed that the largest differences occurred at the 12 and 17

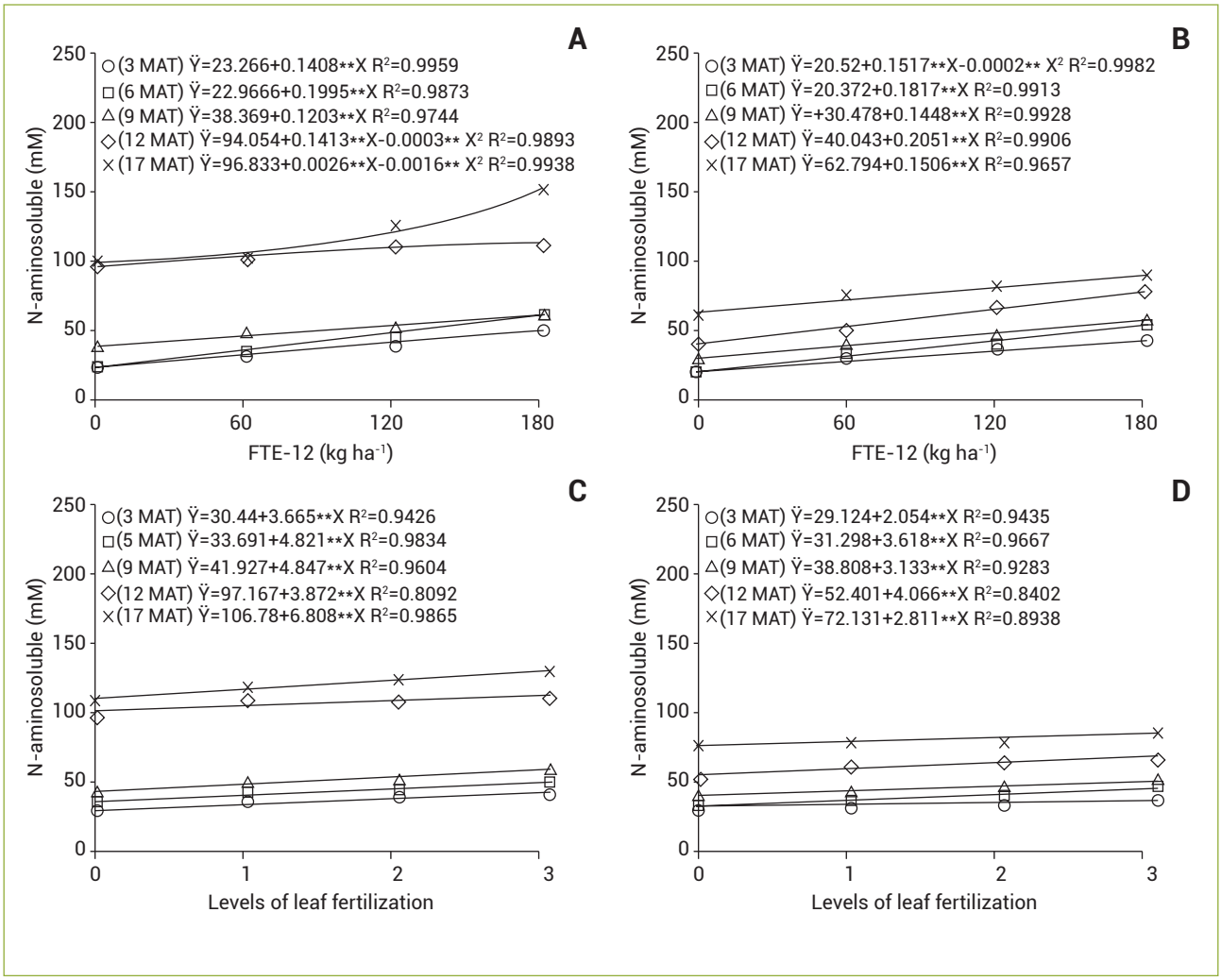

Figure 2. Concentrations of N-aminosoluble compounds in leaves of pineapple cv. Vitória as a function of FTE-12 **(A** and **B)** doses and levels of foliar fertilization **(C** and **D)** at five sampling times (3, 6, 9, 12 and 17 months after transplantation), analyzed at late afternoon **(A** and **C)** and at the end of the night **(B** and **D)**. *p<0.05; **p<0.01.

MAT (Figure 5). While at the first sampling time this variation was 33.6 mM, it reached 116.5 mM at the last (Figure 5A).

Analyzing the relative reductions in the concentrations of soluble carbohydrates (late night towards the end of the day), it was also observed that the largest reductions occurred at the last sampling time (Figure 5B); however, the differences were less pronounced than the changes in absolute terms (Figure 5A). While in the first sampling time there is a reduction in carbohydrates concentrations of 30.9%, in the last sampling it reached a 38.7% value. On the other hand, variations in TA were relatively small throughout the crop cycle (Figures 5C and D). TA showed increases of over 70% during the night, indicating the occurrence of CAM metabolism.

The highest accumulation of carbohydrates observed at the last sampling (Figure 1) can be explained in part by the physiological stage of plant development. During the formation of pineapple inflorescences, there is an increased demand for carbohydrate and other organic solutes. According to Carvalho et al. (1991), concentrations of total sugars in leaves of Smooth Cayenne pineapple were higher at the harvest of inflorescences.

The lowest leaf concentrations of soluble carbohydrates at night compared to day time (Figure 1) can be explained considering the nocturnal acidification of CAM plants (Ceuters et al. 2009). According to Borland and Taybi (2004), the circadian clock plays a central role in controlling many of the metabolic, transport, and physiological components of CAM. The level of control exerted by the clock can range from transcriptional to post-translational regulation, depending on genes, proteins, and even plant species. Further control is provided by metabolites, including organic acids and carbohydrates, which show substantial reciprocal fluctuations in content over the daily cycle.

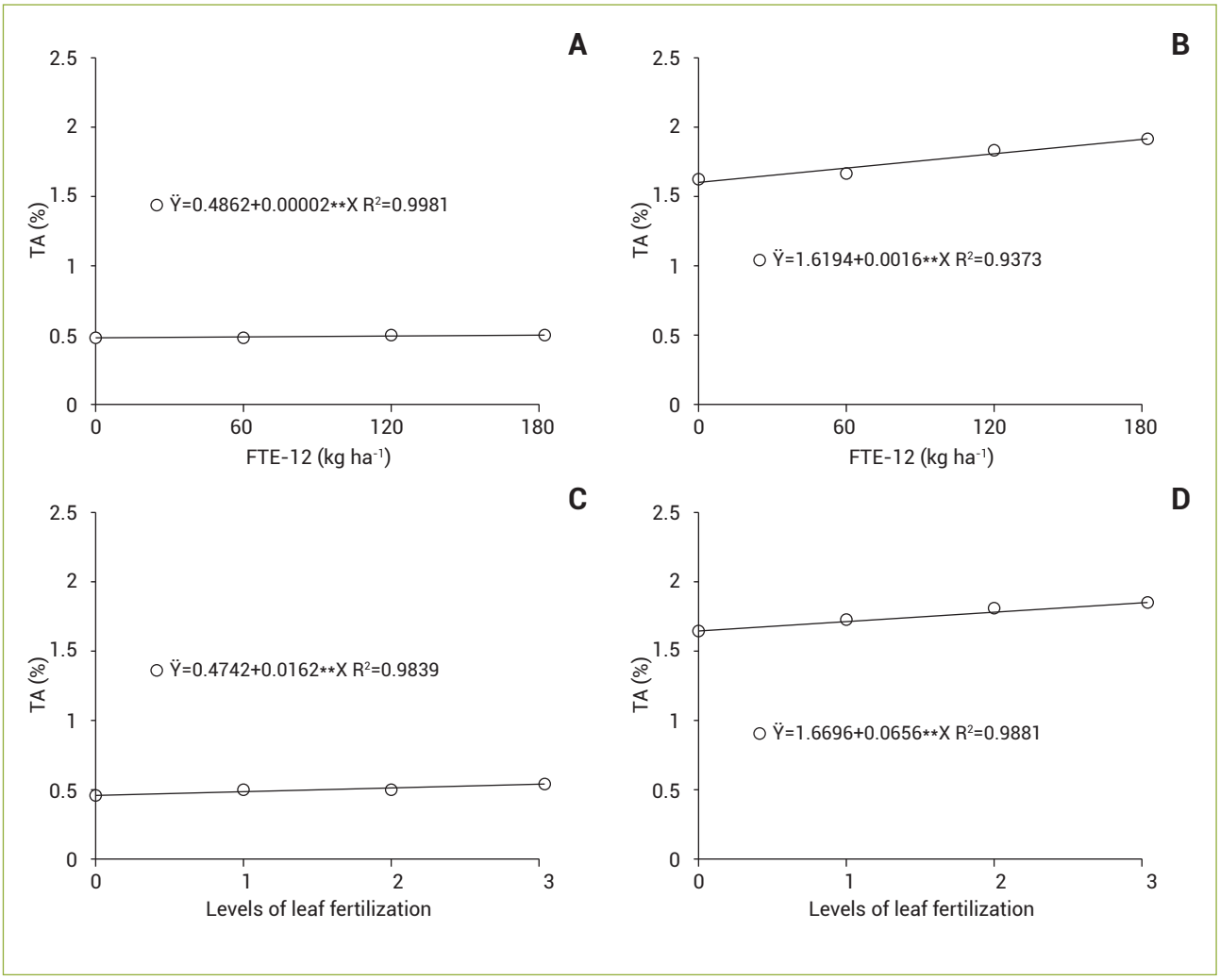

Figure 3. Titratable acidity (TA) in the leaves of pineapple cv. Vitória as a function of FTE-12 **(A** and **B)** doses and levels of foliar fertilization **(C** and **D)** at five sampling times (3, 6, 9, 12 and 17 months after transplantation), analyzed at late afternoon **(A** and **C)** and at the end of the night **(B** and **D)**. *p<0.05; **p<0.01.

The carbohydrate used for the synthesis of organic acids can vary between CAM species. While pineapple uses soluble carbohydrates, in most plants the starch is the source of hexoses for malate synthesis (Carnal and Black 1989, Cushman et al. 2008). Study conducted by Chen, Lin and Nose (2002) demonstrated in CAM plants the occurrence of increases in concentrations of glucose-6-phosphate, fructose-6-phosphate and glucose-1-phosphate at the early hours of the night and decreases at the end of this period. The results suggest that hexoses-P produced in glycolysis may be in more excess than that required to malate accumulation during the first part of dark period, while the opposite may be the case during its latter part. These authors also found that the concentrations of the abovementioned three hexoses were higher in leaves of pineapple than in two other CAM species: *Kalanchoe daigremontiana* and *K. pinnata*.

The highest leaf concentrations of N-aminosoluble compounds at the last two sampling periods (Figure 2) may be associated with reproductive stage of plants, which demand a larger amount of these solutes for flowering and forming inflorescences. Independent of the fertilizer type, the concentrations of N-aminosoluble compounds observed at the late afternoon were higher than those seen at the end of the night, mainly the ones of the two last samples (12 and 17 MAT). This indicates that amino acids can also have contributed to the production of organic acids during the night, using reversible reaction of Krebs cycle. N-aminosoluble compounds represent an important fraction of the pool of soluble nitrogen from leaf tissues and they are also important for maintaining the pH of the cell, protecting macromolecules, and eliminating reactive oxygen species (Mansour 2000). In the present study, it was found that leaf concentrations of N-aminosoluble compounds (Figure 2) in pineapple plants cv. Vitória showed trends similar to those for concentrations of carbohydrates (Figure 1), which is an indication of the strong interaction between the metabolism of carbon and nitrogen (Huppe and Turpin 1994).

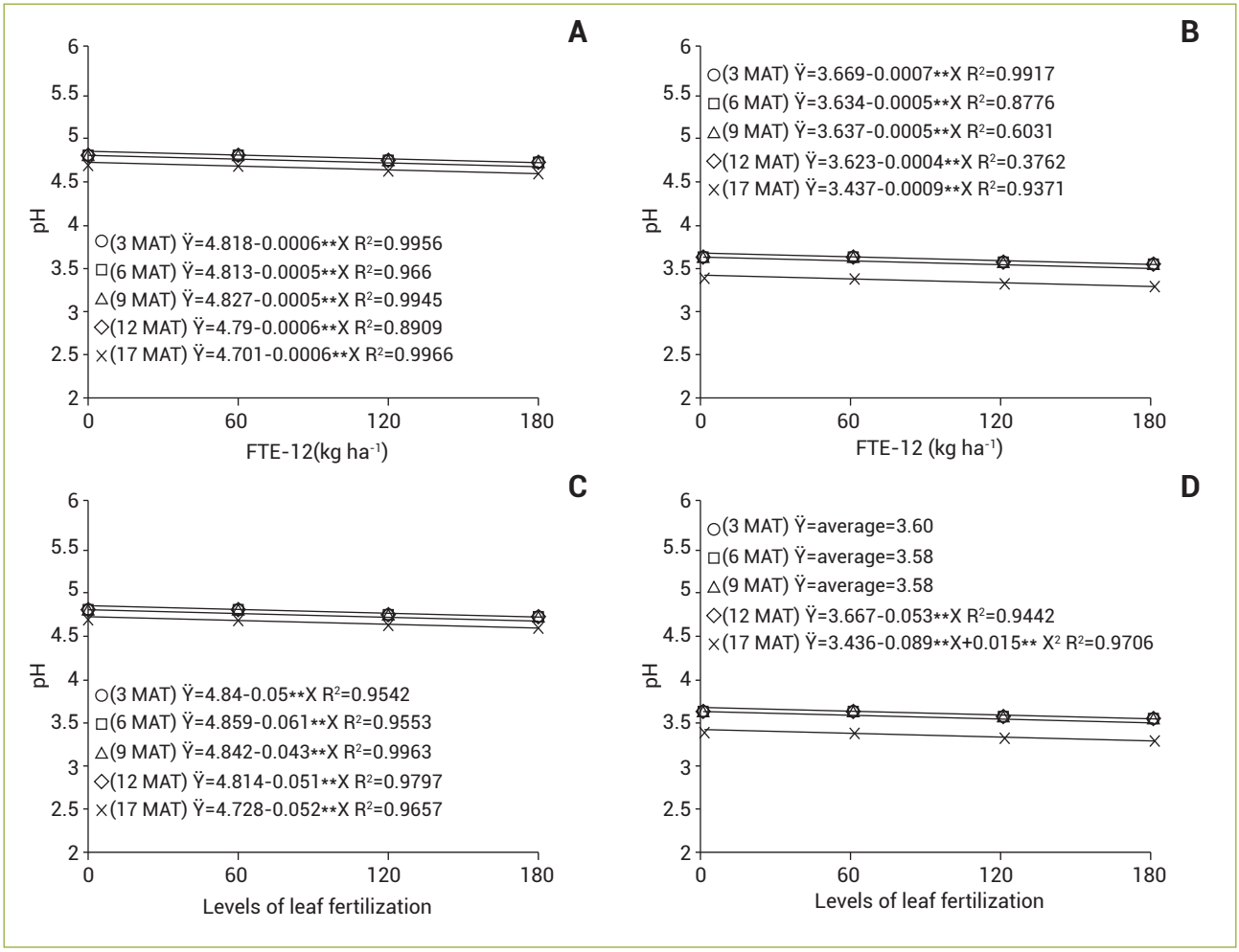

Figure 4. pH in leaves of pineapple cv. Vitória as a function of FTE-12 **(A** and **B)** doses and levels of foliar fertilization **(C** and **D)** at five sampling times (3, 6, 9, 12 and 17 months after transplantation), analyzed at late afternoon **(A** and **C)** and at the end of the night **(B** and **D)**. *p<0.05; **p<0.01.

Changes in pH and TA between day and night (Figures 3 and 4) reflect variations in acidity due to foliar CAM metabolism, with malate being the primary organic acid associated with increased acidity in pineapple plants (Medina et al. 1993, Chen et al. 2002). Nievola et al. (2005) conducted a study with Smooth Cayenne pineapple seedlings under two temperature regimes, 28°C for 24 hours and 28/15°C day/night, and found an increase in TA only when there was a reduction in night temperature. These authors concluded that pineapple plants acted as C3 ones when night temperatures remained high.

Differences in air temperature of about 5ºC between day and night were recorded throughout the crop cycle (Table 2), which may explain the increase in TA associated with CAM metabolism. However, it is possible the occurrence of C3 metabolism, especially at the late afternoon, when the supply of CO_2 generated by decarboxylation of organic acids, does not follow the demand of carbon dioxide for Calvin's cycle (Borland et al. 2011). According to Drennan and Nobel (2000) and Cushman (2001), when some CAM plants are under adequate soil moisture, the stomata can be opened during the day and closed at night; therefore, it presents metabolism similar to that of C3 plants. However, work conducted with three genotypes of pineapple plants demonstrated that they remained with the stomata closed most of the day, even with good water supply, and stomatal conductance equals to zero between 9 and 3 pm (Barreiro Neto et al. 2009). According to this study, the stomata began to open around 5 pm, stabilizing between 8 and 11:45 pm, with values of stomatal conductance from 2.8 to 2.7 mm s^{-1} in Perola cultivar, 4.2 to 4.8 mm s^{-1} and 3.5 to 3.9 mm s^{-1} in the hybrid Purple of Smooth Cayenne.

The intensity of the CAM metabolism may vary with environmental conditions and the developmental stage of the plant, including low night temperatures and favoring CO_2 absorption and organic acids production during the night (Nievola et al. 2005, Borland et al. 2011). In the present study, there were no major changes in metabolism during the crop cycle, based on small variations in TA and pH between different samples (Figures 3 to 5). This constancy in acidification during the night could be related, at least in part, to small variations in the nighttime temperatures during the 17 months of observation (Table 2). It is noteworthy that only the TA and pH were evaluated and other biochemical variables, such as the activity of PEPCase, could help for better understanding the behavior of CAM metabolism over time (Nievola et al. 2005).

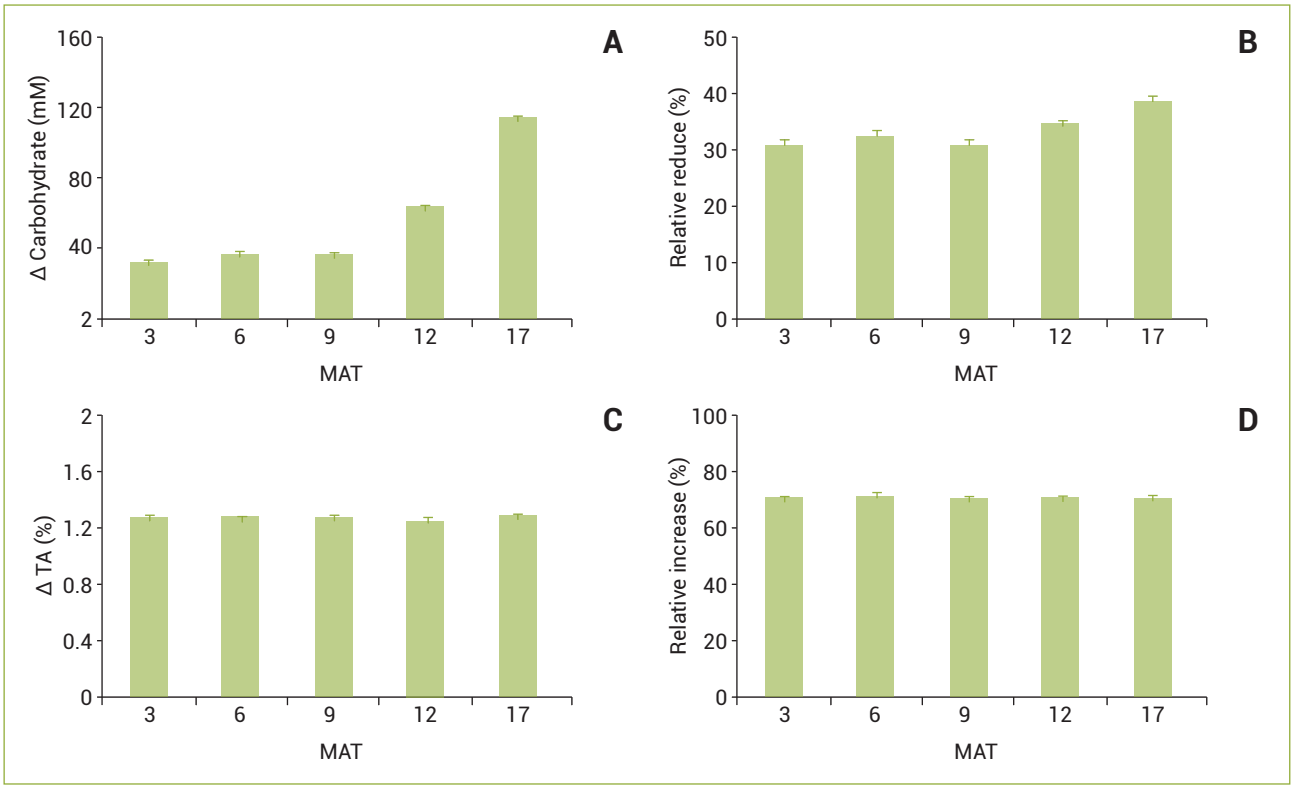

Figure 5. Diurnal and nocturnal changes in concentrations of soluble carbohydrates **(A)** and titratable acidity **(C)** with their respective percentage of decrease or increase **(B** and **D)**, in leaves of pineapple cv. Vitória at five sampling times (3, 6, 9, 12 and 17 months after transplantation).

Soil and leaf application of micronutrients provided increases in concentrations of carbohydrates and N-aminosoluble compounds and reduction in leaf pH of pineapple plants, in both diurnal and nocturnal evaluations. The effects provided by treatments with micronutrients in the plant metabolism studied were more significant at the last sampling (17 MAT), when plants required a greater amount of organic compounds for the final process of inflorescence formation. Reductions in leaf concentrations of carbohydrates and increases in the acidity of leaves collected at the end of the night confirm the CAM metabolism of pineapple plants. In relative terms, leaf carbohydrates and acidity were not affected either by application of micronutrients nor plant age. It could be concluded that the strategy of soil and leaf micronutrients application may help to alter positively the metabolism of pineapple plants, especially during flowering and fruit development.

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