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Nitrogen fertilizer use by *citrus* trees is affected by varying nitrate and ammonium ratios

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Abstract: Nitrogen (N) fertilization in citrus orchards is prone to significant environmental losses. Managing nutrients for sustainable development and optimizing nitrogen use efficiency (NUE) are key tasks in modern agriculture. NUE depends on N uptake and assimilation. In addition to intrinsic fertilizer use efficiency, we evaluated plant growth, nitrate reductase (NRase) activity, and the nutritional status of citrus plants fertilized with different N sources [calcium nitrate (CN), ammonium nitrate (AN), and ammonium sulfate (AS)]. After 240 days, plants supplied with either CN or AN exhibited higher biomass production and N accumulation in the leaves than those fertilized with AS. The supply of ammonium-containing sources (AN and AS) reduced the substrate pH, in which those with AS exhibited lower N partitioning to the leaves, as well as reduced the absorption and accumulation of magnesium and phosphorus. NRase activity was higher in plants supplied with CN or AN compared to AS, and CN fertilization provided the greatest NUE. The data demonstrated that citrus plants fertilized with sources containing nitrate increased the NUE from 10 to 25% and consequently had greater growth compared to those fed with AS.

Index terms: Fertilizer sources, N assimilation, nutrient use efficiency, sweet orange.

O uso de fertilizantes nitrogenados por plantas de citrus é afetado por diferentes proporções de nitrato e amônio

Resumo: A fertilização com nitrogênio (N), em pomares de citros, está sujeita a perdas ambientais significativas. O manejo do nutriente para o desenvolvimento sustentável, otimizando a eficiência de uso de N (EUN), é uma tarefa fundamental na agricultura moderna. A EUN depende da absorção e da assimilação de N, além da eficiência intrínseca do uso de fertilizantes; então, avaliamos o crescimento das plantas, a atividade da nitrato redutase (NRase) e o estado nutricional de plantas de laranjeiras fertilizadas com diferentes fontes de N [nitrato de cálcio (NC), nitrato de amônio (NA) e sulfato de amônio (SA)]. Após 240 dias, as plantas supridas com NC ou NA apresentaram maior

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produção de biomassa e acúmulo de N nas folhas, em comparação àquelas fertilizadas com SA. O fornecimento das fontes contendo amônio (NA e SA) reduziu o pH do substrato, sendo que aquelas supridas com SA apresentaram menor partição de N para as folhas, bem como redução na absorção e no acúmulo de magnésio (Mg) e de fósforo (P). A atividade da NRase foi maior nas plantas supridas com NC ou NA, em comparação com SA, e nas quais a adubação com NC proporcionou maior EUN. Os dados demonstraram que plantas cítricas adubadas com fontes contendo nitrato aumentaram a eficiência de uso de N de 10% para 25% e, consequentemente, maior crescimento em comparação àquelas fertilizadas com SA.

Termos para indexação: Eficiência no uso de nutrientes, fontes de fertilizantes, assimilação de N, laranja-doce.

An adequate nitrogen (N) supply is essential for improving the fruit yield of citrus trees, one of the most cultivated fruit crops in the world (BOARETTO et al., 2007; SPREEN et al., 2020). In this context, the efficient use of nitrogen fertilizer has become a major concern in agricultural production, mainly because of the need to reduce environmental contamination of surface and underground water, reduce production costs and improve crop profitability (MATTOS-JR et al., 2020).

Among the N fertilizer forms available, urea is the most widespread form, followed by the inorganic forms nitrate (NO_3^-) and ammonium (NH_4^+). However, plants preferentially meet their N needs through the acquisition of the latter two forms (BUOSO et al., 2021). The preference for the N form to be absorbed is also related to plant species, soil pH, and other environmental conditions (CHEN et al., 2021). Among the N fertilizers used in agricultural production, in addition to urea, those in which N is present in the nitrate and/or ammonium form are common, such as potassium nitrate, calcium nitrate (CN), magnesium nitrate, ammonium nitrate (AN), and ammonium sulfate (AS) (MATTOS-JR et al., 2020; SAUDY; MOHAMED EL-METWALLY, 2023).

In citrus, NO_3^- is the most common inorganic N form absorbed by roots, favoring cation absorption and the maintenance of soil pH in acidic tropical soils. However, the isolated NO_3^- presence is not sufficient for the proper pH maintenance, as this variable is influenced by multiple factors, including the fertilizers application and the cations absorption by plants (MATTOS-JR et al., 2020). The NO_3^-:NH_4^+ ratio is crucial for soil pH buffering, as NO_3^- and NH_4^+ have antagonistic effects on soil pH. The absorption of NO_3^- can increase soil pH, while the absorption of NH_4^+ can lead to its decrease (XIONG et al., 2021; YE et al., 2022).

Furthermore, the application of fertilizers through fertigation intensifies rhizosphere acidification and changes the availability of nutrients to plants (SOUZA et al., 2014). Excessive acidification limits orchard productivity, demanding studies that optimize best nutrient management practices, especially for N. In fertigated orchards, AN is commonly used due to its high N content and low price compared to other N sources. However, a CN supply via fertigation contributes to an increase in fruit production and improves the ionic balance in the plant, in addition to supporting the maintenance of a higher soil pH (QUAGGIO et al., 2014).

Ammonium is absorbed from the soil and translocated in plants via ammonium transporters, while NO_3^- is absorbed via nitrate transporters, a process that occurs against the electrochemical gradient and thus requires more energy compared to the NH_4^+ absorption (RENGEL et al., 2022). Although, the accumulation of NH_4^+ can cause toxicity to plants, such as inhibition of root respiration, depletion of carbon supply, stimulation of photorespiration, damage to chloroplast ultrastructure, disruption of hormonal homeostasis and photosynthesis (CHEN et al., 2023). While NH_4^+ absorbed by plants is assimilated mainly in the roots to avoid the large accumulation of free ions in the cytosol (CARR et al., 2020), NO_3^- is reduced to NH_4^+ by the enzyme nitrate reductase (NRase) (CHEN et al., 2021). NH_4^+ resulting from nitrate reduction or absorption by the roots is subsequently incorporated into amino acids by the enzymes glutamine synthase and glutamate synthase (BITTSÁNSZKY et al., 2015).

In this context, several studies have sought to identify better nitrogen fertilizer management practices that contribute to nitrogen use efficiency (NUE), optimize tree growth, and increase fruit production (QUAGGIO et al., 2014; HIPPLER et al., 2017). However, a more comprehensive understanding of the effects of fertilizer-N sources, variations in the proportions of NO_3^- and NH_4^+ on plant growth, and N assimilation by woody plants is still needed (ZHANG et al., 2015).

Therefore, our objective was to evaluate the plant growth, NRase activity, and nutritional status of citrus plants fertilized with different sources of N [calcium nitrate (CN), ammonium nitrate (AN), and ammonium sulfate (AS)]. We hypothesized that the use of different N sources would affect NUE in citrus trees. Therefore, our study evaluated plant growth and NRase activity in young citrus trees supplied with CN, AN, or AS grown in greenhouse.

The experiment was set up with the treatments in a completely randomized design. The treatments consisted of application via fertigation of three N sources in different N-NO_3^-:N-NH_4^+ ratios [NC (100% N-NO_3^-); NA (50% N-NO_3^- and 50% N-NH_4^+); and SA (100% N-NH_4^+)], with five replications.

One-year-old sweet orange [*Citrus sinensis* (L.) Osbeck cv. Pera] grafted onto Rangpur lime (*C. limonia Osbeck*) were grown in a screen house in pots containing 12 dm^3 of organic substrate (80% *Pinus* bark, 15% vermiculite, and 5% carbonized material). The plants were fertilized with N sources biweekly for 240 days at a rate of 1.5 g of N per pot per application (totaling 24 g of N per plant). Nutrient fertilization with other macronutrients and irrigation was performed following Hippler et al. (2015), except for the metal micronutrients that were applied monthly with sulfate via foliar sprays (0.8 g L^{-1} Cu, 0.3 g L^{-1} Fe, 0.5 g L^{-1} Mn, and 0.75 g L^{-1} Zn).

After 210 days of treatment, when the second vegetative growth flush was physiologically mature, the *in vivo* activity of the NRase enzyme was measured in leaves according to Dovis et al. (2014a) at 5, 11, 14, 18, 21, and 31 days after the last application of the N fertilizer source. After the last NRase assay, at 240 days of treatment application, the trees were destructively harvested and separated into leaves, twigs, trunk, and roots. The total leaf area was measured using a leaf area integrator (LI 3100C, LI-COR, Lincoln, Nebraska, USA), and the plant parts were dried at 58–60°C until a constant weight to quantify the dry mass produced. The concentrations of N and other nutrients were determined according to Bataglia et al. (1983). Nutrient accumulation and N partitioning in the trees were calculated directly. The NUE was calculated according to Siddiqi and Glass (1981) as follows: NUE (g^2 mg) = (total dry mass of the plant, $g)^2$/(accumulation of the nutrient, mg). The means were subjected to analysis of variance (ANOVA) and in significant cases the Tukey test was used at 5% statistical probability.

Plants fertilized with CN or AN exhibited approximately 20% more total dry mass production than those supplied with AS, which was mostly associated with an increased shoot dry mass (leaves and twigs) since no differences were verified for root dry mass among the N fertilizer sources (Figure 1). Furthermore, plants supplied with CN exhibited a higher leaf area of 1900 cm^2 per plant compared to AS with 1400 cm^2 per plant (Figure 1). However, plants supplied with AS exhibited a lower specific leaf area (Figure 1), as verified for plants supplied either with CN or AN. AS-fed plants did not grow a second vegetative flush; thus, it was reasonable to expect a thicker leaf mesophyll and higher carbohydrate accumulation because of the absence of new sink growth (DOVIS et al., 2014b). Indeed, highest NUE in citrus trees results from the increased biomass and N partition to leaf production, as well the plasticity in specific leaf area under N supply (DOVIS et al., 2021).

N concentrations in the leaves (29.4±1.8 g kg^{-1}) and twigs+trunk (10.1±1.1 g kg^{-1}) did not differ among fertilizer sources (Table 1). Differences in N concentration according to fertilizer sources were found only in roots, with levels higher in plants fertilized with AS (29.4±1.8 g kg^{-1}) than in those fertilized with CN (29.4±1.8 g kg^{-1}) or AN (29.4±1.8 g kg^{-1}) (Table 1). This response is likely explained by nutrient dilution in biomass since plants supplied with either CN or AN exhibited higher dry mass production of leaves and twigs+trunk than those supplied with AS (Figure 1) and, consequently, accumulated a greater amount of N in leaves and twigs+trunk (Figure 2).

Table 1. Concentrations of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S) in different parts of sweet orange plants supplied with calcium nitrate, ammonium nitrate or ammonium sulfate.

Plant part / N source		N	P	K	Ca	Mg	S
		------	------	------	g kg^{-1}	------	------
Leaves	Calcium nitrate	28.7 ns	1.7 a	5.3 ns	28.0 a	2.3 b	2.4 b
	Ammonium nitrate	30.8	1.7 a	5.0	18.0 b	2.6 a	2.1 b
	Ammonium sulfate	28.6	1.4 b	5.0	15.2 b	1.1 c	3.5 a
Twigs + Trunk	Calcium nitrate	9.1 ns	1.3 ns	2.5 ns	10.5 a	1.0 ab	0.7 b
	Ammonium nitrate	10.5	1.3	2.0	7.2 b	1.1 a	0.6 b
	Ammonium sulfate	10.5	1.4	1.5	6.6 b	0.9 b	1.0 a
Roots	Calcium nitrate	11.6 b	1.2 a	2.0 b	7.4 a	1.5 a	0.6 b
	Ammonium nitrate	10.8 b	1.1 ab	2.0 b	4.7 b	1.4 ab	0.7 b
	Ammonium sulfate	13.6 a	1.0 b	3.8 a	3.8 b	1.1 b	1.5 a

ns not significant; means followed by different letters differ significantly among N treatments (Tukey, p<0.05).

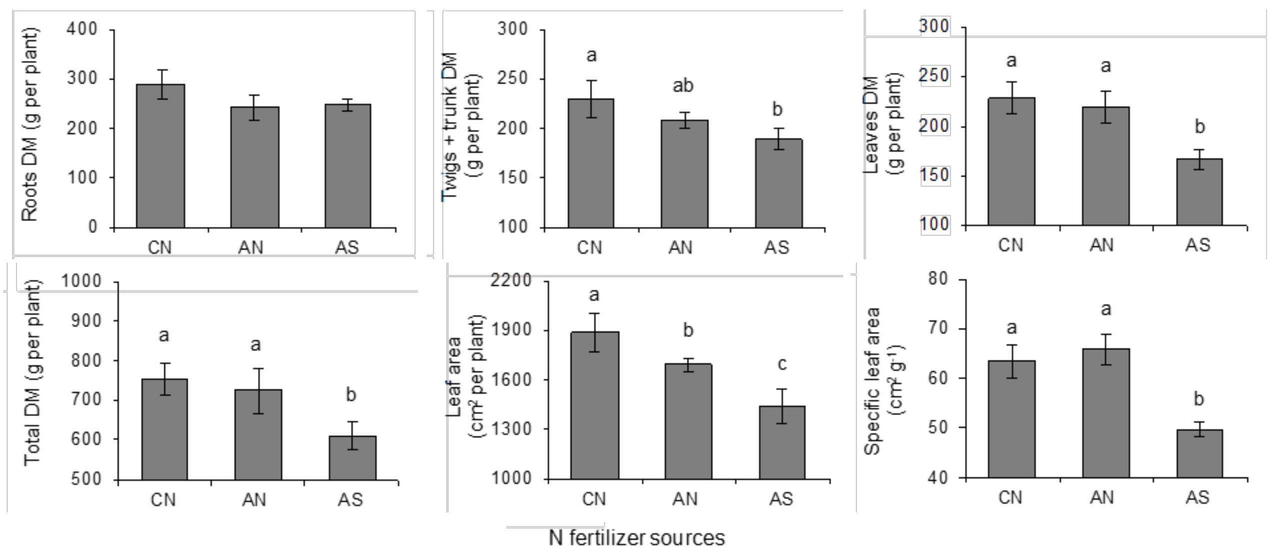

Figure 1. Dry mass (DM), leaf area, and specific leaf area of sweet orange plants supplied with varying nitrogen fertilizer sources for 240 days. Legend: CN: calcium nitrate; AN: ammonium nitrate; AS: ammonium sulfate. Mean (± SEM) is shown (n=5). Different letters indicate significant differences among N treatments (Tukey's test, p<0.05).

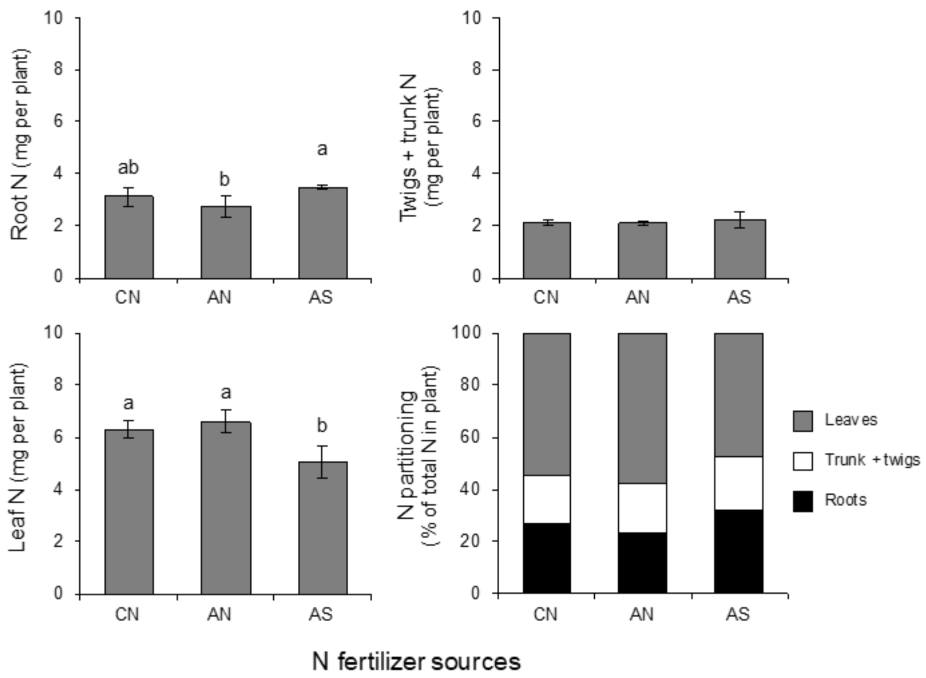

Figure 2. Accumulation and partitioning of nitrogen (N) in sweet orange supplied with varying N fertilizer sources for 240 days. Legend: CN: calcium nitrate; AN: ammonium nitrate; AS: ammonium sulfate. Mean (± SEM) is shown (n=5). Different letters indicate significant differences among N treatments (Tukey's test, p<0.05).

In citrus, the greatest amount of N is allocated to the leaves, mostly in organic pools, such as proteins, amino acids, and other compounds (XIONG et al., 2021). However, the growth of the sweet orange plants was the lowest when supplied with AS, and the N partitioning to leaves was also lower (47% of N) compared to the plants supplied with either CN (56% of N) or AN (58% of N; Figure 2). Conversely, plants supplied with AN exhibited greater N partitioned to roots (32% of N) than those supplied with CN or AN (25% of N; Figure 2).

The substrate pH decreased when using fertilizer containing NH_4^+, as follows: 4.1±0.2 with CN > 3.7±0.1 with AN ≥ 3.5±0.2 with AS (p<0.05). Ammonium-containing fertilizers favor soil acidification due to proton (H^+) extrusion to the root medium during NH_4^+ absorption (SOUZA et al., 2012), where the electroneutrality of the soil solution must be maintained by the release of H^+ or OH^- in stoichiometrically equal amounts to absorbed cations or anions (CARR et al., 2020). The H^+ release, in addition to promoting rhizosphere acidification, can contribute to intracellular alkalinization. This process can interfere with the absorption of cations, such as calcium (Ca^{2+}), potassium (K^+), and magnesium (Mg^{2+}), and favor the absorption of potentially toxic ions, such as manganese (Mn^{2+}). Therefore, these alterations can result in biochemical imbalances, affecting vital processes such as photosynthesis (QUAGGIO et al., 2014; ESTEBAN et al., 2016).

Furthermore, under fertigation conditions, the NH_4^+ nitrification process in the soil occurs to a lesser extent than under non-fertigation conditions, mainly due to the decrease in redox potential in the wet bulb region. Consequently, higher H^+ proton concentrations in the soil solution and a decrease in soil pH are expected (QUAGGIO et al., 2014). On the other hand, the N supply in the anionic form (NO_3^-) favors the cations, K^+, Mg^{2+}, and Ca^{2+}absorption, compared to the cationic supply (NH_4^+) in response to the charge balance during the uptake process (SARASKETA et al., 2016; RENGEL et al., 2022).

For instance, the application of N at 80 kg ha^{-1} as AN in a fertigated citrus orchard caused the soil pH to reach 4.0, in which orchard soil acidification was intensified with the increment of the N dose up to 240 kg ha^{-1} of N, causing the soil pH to decrease to 3.0. In contrast, CN application provided a soil pH of 6.6 at the same N doses (QUAGGIO et al., 2014). In the same study, QUAGGIO et al. (2014) observed that plants fertirrigated with CN showed higher cation uptake when compared to plants whose N source was AN.

Plants supplied with AS exhibited a reduction in the uptake and accumulation of Ca, Mg, and phosphorus (P), especially when compared to those supplied with CN. Plants supplied with AN did not exhibited differences in the accumulation of P and Mg when compared to plants supplied with CN, while Ca accumulation was lower in relation to plants supplied with CN and higher than in plants supplied with AS. This occurs as a consequence of the effects of soil pH on the availability of ions to plants and the electrochemical balance, such that cations have their availability decreased under more acidic pH conditions, while the P solubility can also be affected under these conditions (QUAGGIO et al., 2014).

Fertilization with CN improved the NUE by 27% compared to AS (Table 2), which resulted in an increment in the biomass production of the sweet orange plants supplied with CN (Figure 1). Furthermore, plants supplied with CN exhibited lower Ca use efficiency, whereas those supplied with AS presented lower sulfur (S) use efficiency when compared to AN (Table 2). However, plants fertilized with AS exhibited higher Mg use efficiency compared to CN and AN (Table 2).

Table 2. Nutrient accumulation and nutrient use efficiency (NUE) in sweet orange plants supplied with calcium nitrate, ammonium nitrate or ammonium sulfate.

N sources	N	P	K	Ca	Mg	S
	-------------- Accumulation (mg per plant) --------------					
Calcium nitrate	12.1 ^{ns}	1.0 a	2.4 a	11.3 a	1.3 a	0.9 b
Ammonium nitrate	11.4	0.9 a	2.0 ab	6.6 b	1.3 a	0.8 b
Ammonium sulfate	10.7	0.7 b	1.9 b	4.5 c	0.7 b	1.2 a
	---------------------- NUE* (g^2 mg^{-1}) ----------------------					
Calcium nitrate	46 a	555 ^{ns}	227 ^{ns}	47 b	410 b	568 a
Ammonium nitrate	40 ab	533	240	76 a	396 b	592 a
Ammonium sulfate	36 b	485	182	80 a	544 a	323 b

* NUE = (total dry mass of plant, g)^2/(accumulation of the nutrient, mg).
^{ns} not significant; means followed by different letters differ significantly among N treatments (Tukey, p<0.05

In addition to nutrient absorption by plant roots, the ratio of NO_3^-:NH_4^+ in the soil solution also affects other metabolic processes, such as enzyme activity and photosynthesis (RENGEL et al., 2022). The NRase activity in the leaves was higher in plants fertilized with either CN or AN than in those with AS (Figure 3). Nitrate is an important regulator of NRase activation, and the activity of this enzyme is used as an indicator of the potential for N assimilation by plants (DOVIS et al., 2014a; HIPPLER et al., 2017). After the 18th day of the last application of the N fertilizers, the activity of the NRase decreased in plants supplied with nitrate-containing fertilizers (CN and AN), and the activity of both N fertilizer sources did not differ (Figure 3).

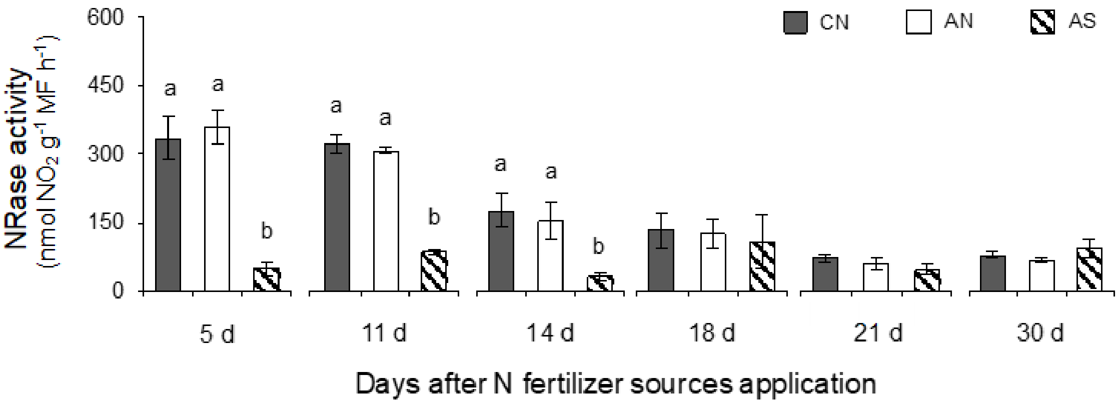

Figure 3. Nitrate reductase (NRase) activity (*in vivo*) in leaves of sweet orange plants at 5, 11, 14, 18, 21, and 30 days (d) after the last application of nitrogen (N) sources 210 days after treatment started. Legend: CN: calcium nitrate; AN: ammonium nitrate; AS: ammonium sulfate. Mean (± SEM) is shown (n=5). Different letters within each day indicate significant differences among N treatments (Tukey's test, p<0.05).

Although no nitrate was applied to the plants treated with AS, the activity of the NRase enzyme verified in the leaves could represent the assimilation of a small amount of nitrate stored in the cells once nitrate is the main inorganic N inside the plant (MATTOS-JR et al., 2003). Moreover, part of the ammonium from AS applied to the substrate is likely converted to nitrate by nitrifying bacteria and then absorbed by plants, which would explain the measured NRase activity (DELAGADO et al., 2006; CANTARELLA et al., 2008).

We concluded that citrus plants supplied with nitrate-containing fertilizers, such as CN and AN, achieved greater plant growth and NUE, which correlated with the nutrient assimilation expected with the increased NRase activity measured in leaves. An increased NUE was also correlated with increased uptake and accumulation of other nutrients, such as P, K, Ca, and Mg, while S showed an inverse relationship. However, fertilization with AS impaired N uptake and partitioning in the citrus plants, limiting plant growth, as the shoot to root ratio decreased when compared with CN- or AN-fed plants. The AS supply may also have caused substrate acidification, resulting from root proton extrusion, creating an unfavorable root activity environment.

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