RESEARCH ARTICLE

Evaluation of acidifying nitrogen fertilizers in avocado trees with iron deficiency symptoms

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Abstract:

The control of iron chlorosis by synthetic Fe chelates is costly, and their application can have adverse environmental impacts. We investigated the effectiveness of alternative strategies to prevent Fe chlorosis in avocado based on different acidifying nitrogen fertilizers. The experiment was conducted on a commercial orchard of avocado *cv.* Hass cultivated in a calcareous soil. The application of $(NH_4)_2SO_4$ was highly effective at reducing the soil pH and, when applied with a nitrification inhibitor, at increasing the leaf chlorophyll concentration in trees. Fertilization with urea was also effective at increasing leaf chlorophyll, but only when applied without a nitrification inhibitor. However, the acidifying effect of urea on the soil was significantly lower than $(NH_4)_2SO_4$. The different N sources also modified the concentration of $N-NO_3^-$, $N-NH_4^+$ and total N within the leaves, but did not influence the length of the shoots or the leaf gaseous exchange. These results suggest that the fertilization strategy based on $(NH_4)_2SO_4$ + DMPP or urea can be an effective and sustainable alternative to counteract Fe chlorosis symptoms in avocado trees. Our results provide evidence of the effectiveness of agronomic strategies, alternative to synthetic Fe chelates, for preventing Fe deficiency in avocado.

Keywords: Ammonium sulfate, urea, DMPP, iron chlorosis, *Persea americana* Mill.

1. Introduction

Neutral to alkaline pH in the soils of many avocado-growing areas results in frequent micronutrient deficiencies in avocado (*Persea americana* Mill.) orchards (Manthey and Crowley, 1997). Avocado trees grown under these conditions are frequently affected by more than one micronutrient scarcity, and in particular by iron (Fe) deficiency. Some experiments into avocado cultivation in calcareous soils indicate that negative impacts may occur in fruit yield, tree growth, and disease resistance

even in trees with mild symptoms of Fe chlorosis (Manthey and Crowley, 1997). Yet, despite exhaustive attempts to develop effective management techniques focused on supplementing avocado trees with metal micronutrient fertilizers, the Fe chlorosis persists in many cases. Iron is the fourth most abundant element in the earth's crust, however, its deficit in plants occurs because only a small fraction of Fe is available to plants (Rombolà and Tagliavini, 2006). This is mainly due to its low solubility in the rhizosphere in soils characterized by neutral to alkaline pH (Bityutskii *et al.*, 2017). In this context, one of the main causes of Fe deficiency in fruit tree crops cultivated in such areas is the high calcium carbonate ($CaCO_3$) and bicarbonate (HCO_3^-) concentrations in the soil due to the effect of such ions on the soil solution pH (Bityutskii *et al.*, 2017; Rombolà and Tagliavini, 2006). In fact, higher pH favours carbonate or hydroxyl complexes (Rengel, 2015). In addition, high-pH soils range from calcareous (around pH 7) to alkaline saline and sodic soils (pH 8 and higher), have major nutritional constraints (Fe and P deficiency) and/or toxicity of Na coupled with excess HCO_3^-, water deficiency, mechanical impedance and poor aeration (Rengel, 2015).

Dicotyledonous plants, such as avocado, have evolved using Strategy I of Fe uptake, and consequently absorb Fe from the soil in its ferrous state. In soils with neutral to alkaline pH, Fe is oxidized to the less soluble ferric state, and is consequently less available for root uptake (Kim and Guerinot, 2007). In such conditions, Strategy I plants, characterized by a high tolerance to Fe chlorosis, can react by lowering the pH of the rhizosphere through proton release catalyzed by ATP-ase enzyme activity and by the root exudation of some organic acids and phenolic compounds, which promotes the solubility/availability of Fe in the soil (Kim and Guerinot, 2007). Also,

these plants can directly reduce Fe in roots by increasing the Fe^{3+}-reductase (FCR) enzyme activity (Kim and Guerinot, 2007). However, Fe chlorosis susceptibility is highly variable among plant species and even among genotypes of the same species (Rombolà and Tagliavini, 2006). On the South American continent, among the most important fruit tree species, avocado is one of the most susceptible to Fe deficiency, since Fe chlorosis in avocado begins to manifest from soil pH levels above 7.0 (Manthey and Crowley, 1997). In avocado orchards cultivated in calcareous soils, growers usually try to solve such nutritional deficiencies by acidifying the soil with the application of sulfuric acid via the irrigation system. However, this management technique is hazardous for the agricultural operator and repeated applications may have deleterious effects on the soil microflora. Another widely used strategy that is very effective at controlling Fe deficiency is the use of Fe synthetic chelates. The cost of such strategy, however, can reach 60% of the total allocated for fertilizers in these orchards (Rombolà and Tagliavini, 2006). In addition, treatments with Fe chelates require repeated applications, and due to their high stability and solubility in soils, the risks of leaching and groundwater contamination are often high (Rombolà and Tagliavini, 2006).

Such evidences strongly suggests the need to adopt management strategies to improve Fe nutrition in crops considering soil and plant variables integrally. In such a context, the application of fertilizers whose absorption and/or chemical reactions acidify the rhizosphere and/or the soil solution could constitute a valid and sustainable strategy for the Fe^{3+} reduction, enabling Fe root absorption by the crop. It is known that the application of ammoniacal and amide nitrogen (N) fertilizers may induce soil solution acidification, the intensity

of which is mainly regulated by the amount of fertilizer applied and the soil management (Guo *et al*., 2010, Tong and Xu, 2012). In agricultural soils, the ammonium (NH_4^+) concentration is in the range of 20-200 μM, and is frequently the preferred N source for some species. However, NH_4^+ is rapidly converted to nitrate (NO_3^-) through the nitrification process, which implies the ammonia (NH_3) oxidation to hydroxylamine (NH_2OH) and then to nitrite (NO_2^-) by edaphic *Nitrosomona* spp. bacteria (Zerulla *et al*., 2001). Subsequently, the NO_2^- is oxidized to NO_3^-, through the activity of *Nitrobacter* bacteria. The mineralization rate of NH_4^+ depends on soil environmental factors, mainly soil organic matter content, soil moisture and temperature, and chemical and physical factors, which determine the potential supply rate of mineralizable N (Ramírez *et al*., 2016). Owing to the rapid nitrification rates in most aerobic soils, nitrate (NO_3^-) is the primary N source for plants. For each NH_4^+ molecule oxidized to NO_2^-, two H^+ are released into the medium, decreasing the soil solution pH and promoting Fe^{3+} reduction.

Conversely, the absorption of some ions through the plasma membrane of root cells can induce rhizosphere acidification, favoring Fe solubilization and uptake by plants. Studies on grapevine conducted under controlled conditions showed that the presence of NH_4^+ ion in the substrate can counteract the negative effects of NO_3^- on Fe uptake by the roots (Jiménez *et al*. 2007; Covarrubias *et al*., 2014). Nitrate absorption is mediated by a two-proton co-transport, which can increase the pH in the rhizosphere, reducing Fe solubility (Nikolic and Römheld 2002). Consequently, the presence of NO_3^- in the soil contributes to inducing Fe chlorosis in plants cultivated in lime soils (Kosegarten *et al*., 2001), and such an effect has also been observed in avocado (Bar and Kafkafi, 1992). In contrast, the absorption of NH_4^+ by plants induces acidification in the rhizosphere due to the excretion of protons through the enzyme H^+-ATPase, favoring the reduction of Fe^{3+}. As a result, the presence of NH_4^+ in the rhizosphere could be beneficial to improving Fe nutrition in plants (Covarrubias *et al*., 2014).

In order to improve the effectiveness of the positive effects induced by NH_4^+ in Fe nutrition, it may be important to employ strategies focused on maintaining the NH_4^+ concentration in the soil at medium-to-low levels by slowing down the oxidation of NH_4^+ exerted by the activity of *Nitrosoma* bacteria (Zerulla *et al*., 2001). In such a context, the use of nitrification inhibitors applied to the soil may contribute to optimizing N and Fe nutrition simultaneously as well as to reducing NO_3^- leaching. However, in a tropical fruit species such as avocado there is no scientific evidence in this regard. The most frequently used synthetic nitrification inhibitors are DCD (dicyandiamide), nitrapyrin (2-chloro-6(trichloromethyl)pyridine) and the highly specific DMPP (3,4-dimethylpyrazol phosphate), which can inhibit nitrification at concentrations of 0.5-1.0 kg active compound ha^{-1}. In a controlled experiment conducted on wheat, the application of NH_4^+ with DMPP increased Fe concentration in grains (Selim and Mahmoud, 2015). Moreover, in a pot study conducted on grapevine cultivated in a calcareous soil, the application of NH_4^+ + DMPP increased leaf chlorophyll content and leaf stomata length compared to plants treated with NO_3^-, suggesting that this treatment is effective for preventing Fe chlorosis in vineyards located in calcareous soils (Covarrubias *et al*., 2014). Such scientific evidence points to the potential of using NH_4^+ as N fertilizer to control Fe deficiency in crops. The present investigation endeavors to evaluate the effectiveness of ammoniacal fertilizers application in avocado trees with Fe deficiency symptoms.

2. Material and Methods

2.1 Plant material, experimental conditions and treatments

The experiment was undertaken during the 2014-2015 season in a commercial orchard located in the Valparaiso Region, Chile (32º48´S and 71º09´W) in an alluvial soil, classified as Aeric Calciaquolls (Gleyic Kastanozems) and belonging to Los Pidenes soil Series (CIREN, 1997). The trial was conducted on avocado (*Persea americana* Mill.) trees *cv* Hass grafted on Mexicola rootstock. The trees were planted on October 2011 at the distance of 6 m between the rows and 3 m between trees along the row. In winter 2014, 36 avocado trees randomly located in the orchard, with evident and homogeneous Fe deficiency symptoms characterized by interveinal yellowing in young leaves (leaf chlorophyll concentration between 30 and 40 µg cm^{-2}), were selected for the experiment. The treatments tested were: (i) trees fertilized with a N-NO$_3^-$ source applied to the soil (control: Ca(NO$_3$)$_2$); (ii) trees fertilized with a N-NO$_3^-$ source + a synthetic Fe chelate (Ca(NO$_3$)$_2$ + Fe-EDDHA); (iii) trees fertilized with urea as N-NH$_4^+$ source applied to the soil (urea); (iv) trees fertilized with (NH$_4$)$_2$SO$_4$ as N-NH$_4^+$ source applied to the soil ((NH$_4$)$_2$SO$_4$); (v) trees fertilized with urea + a nitrification inhibitor applied to the soil (urea + DMPP); and (vi) trees fertilized with ((NH$_4$)$_2$SO$_4$) + a nitrification inhibitor applied to the soil ((NH$_4$)$_2$SO$_4$ + DMPP). Nitric nitrogen-fertilized trees received 1000 mL of Ca(NO$_3$)$_2$ solution (10 g N L^{-1}) every 15 days from October 1, 2014 to April 20, 2015, until the trees had received 140 g N. The Fe chelate used was ethylenediamine-N,N'-bis(2-hydroxyphenyl)acetic acid (Fe-EDDHA) (Basafer® PLUS, Compo®) 5% ortho-ortho, and it was applied according to the soil plant analysis development (SPAD) index in leaves, in order to maintain an intensive green color of leaves (SPAD index >45). Doses of 500 mL of 6% Fe-EDDHA solution (4 g Fe-EDDHA L^{-1}) were occasionally applied to the trees. During the season, Fe fertilized trees received 26 g Fe-EDDHA (1.56 g Fe per tree). The same amount of N supplied in the trees treated with Ca(NO$_3$)$_2$ was also applied in trees treated with urea or (NH$_4$)$_2$SO$_4$, through the application of 1000 mL solutions (10 g N L^{-1}) every 15 days. In treatments v and vi, the nitrification inhibition was maintained during the experiment with DMPP at doses of 1% of the N amount supplied. The orchard was regularly watered during the season with a drip irrigation system to recover the daily evapotranspiration rate. Weeds were frequently removed, and pest and disease protection was regularly carried out. The experimental design was completely randomized blocks, and each treatment was applied to six plants.

2.2 Soil chemical properties

In October 2014, before the treatments began, a soil sample composed by 15 sub-samples was collected from the avocado orchard at 40 cm depth (root zone), and some fertility parameters were determined. During the experiment, soil pH measurements were carried out in samples collected near the dripping bulb of each plant (at 30-40 cm depth). Chilean standard methods for soil analysis (Sadzawka *et al*., 2006) were used for soil pH$_{water}$ (1:2.5), determined using a portable pH meter (Elmetron model CP-4001). Samples were collected in early (March) and mid (May) autumn 2015, at 165 and 225 days of treatment, respectively.

2.3 Leaf chlorophyll concentration and plant growth

Leaf chlorophyll concentration was measured during the season in four shoots per tree every 30 days on

four points of the first completely expanded leaf with a Minolta SPAD 502 portable greenness meter (Konica Minolta, Inc., Osaka, Japan). The SPAD value was previously calibrated with leaf chlorophyll concentration (R^2=0.97, p <0.0001) in 16 leaves with different degrees of chlorosis according to Wellburn's method (1994). On each leaf, 2 discs of 12 mm^2 diameter were ground with a pestle, and submitted to an extraction solution with cold acetone 80% v/v. The resulted extracts were vortexed and centrifuged at 4000 g for 10 min at 4 °C, and the supernatants were collected and submitted to a new extraction and centrifugation. The supernatants were used measure the absorbance at 663 and 646 nm using a spectrophotometer (Jasco V-630, Tokyo, Japan). During the season, the length of the same shoots selected for the chlorophyll determinations was determined every 30 days.

2.4 Leaf gas exchange

Leaf gas exchange was measured on each tree using an infrared gas analyzer (IRGA, LCi-ADC, London, United Kingdom). During the experiment, measurements were performed on the first mature leaf inserted in shoots of the middle third of the tree. Net photosynthesis was measured when foliar CO_2 uptake was steady. Gas-exchange measurements including net photosynthesis (μmol CO_2 m^{-2} s^{-1}), stomatal conductance (mmol CO_2 m^{-2} s^{-1}) and transpiration (μmol H_2O m^{-2} s^{-1}) were taken at 12:00 pm and 4:00 pm on January 12, 2015 (72 days after treatment imposition). During the measurement performed at 12:00 pm, the photosynthetically active radiation (PAR) and the environmental temperature were around 1700-1800 μmol photons m^{-2} s^{-1} and 28 °C respectively, whereas at 4:00 pm, the PAR was around 1200-1300 μmol photons m^{-2} s^{-1} and the temperature was 32 °C.

2.5 Leaf N-NO_3^-, N-NH_4^+ and total N concentrations

In April 2015, at 155 days of treatment, samples of 20 healthy and fully expanded leaves were collected from each tree and analyzed for N-NO_3^-, N-NH_4^+ and total N concentrations. The samples were oven-dried at 75 °C and ground to pass a 40-mesh screen. The leaf N-NO_3^- concentration was determined by the extraction with an aluminum sulfate ($Al_2(SO_4)_3$) solution and subsequent determination by ion-selective electrode (ISE) (Miller, 1998). The leaf N-NH_4^+ concentration was determined colorimetrically using a wavelength of 650 nm, according to Beathgen and Alley (1989). Total leaf N concentrations were determined by Kjeldahl method (Schuman *et al*., 1973).

2.6. Statistics

Comparison of means and analysis of variance between treatments were done using the Infostat software according to a completely random block design. If significant differences were found with F-tests, means were compared using the Tukey test (P ≤ 0.05). Analyses were performed on raw data to maximize variance homogeneity.

3. Results

3.1 Soil chemical properties

The soil chemical properties determined in the composed soil sample collected before the trial establishment are described in the Table 1. The results obtained, show an initial soil pH value of 8.1 (Table 1). During the experiment, the soil pH showed significant differences between treatments (Figure 1). In the measurement taken at 165 days of treatment (March 2015), the results obtained in-dicate that the fertilization with $(NH_4)_2SO_4$, with or

without DMPP, decreased the soil pH compared to the other treatments (Figure 1). After two months, at 225 days of treatment (May 2015), the effect of $(NH_4)_2SO_4$ with or without DMPP kept the soil pH steady compared to the control and the other fertilization strategies assessed (Figure 1).

Table 1. Chemical properties of the soil utilized in the experiment.

Fertility	Unit	Value
pH (water, ratio 1:2,5)	1:2.5	8.1
Electric conductivity (in extract)	dS/m	1.3
Organic matter	%	4.4
Available nitrogen (N)	mg/kg	51
Available phosphorus (P)	mg/kg	18
Available potassium (K)	mg/kg	97
Exchangeable cations		
Calcium (Ca)	cmol+/kg	17.4
	% CEC	80
Magnesium (Mg)	cmol+/kg	3.5
	% CEC	16
Potassium (K)	cmol+/kg	0.25
	% CEC	1.1
Sodium (Na)	cmol+/kg	0.32
	% CEC	1.5
CEC (Cation Exchange Capacity)	cmol+/kg	21.7
Available microelements		
Iron (Fe)	mg/kg	14.4
Manganese (Mn)	mg/kg	19.0
Zinc (Zn)	mg/kg	2.0
Copper (Cu)	mg/kg	8.5
Boron (B)	mg/kg	3.5
Carbonates		
Total carbonates	$CaCO_3$%	4.3
Active lime	$CaCO_3$%	2.5

*For available microelements and exchangeable cations determinations, DTPA and ammonium acetate were used as extractants, respectively (Sadzawka *et al*., 2006).
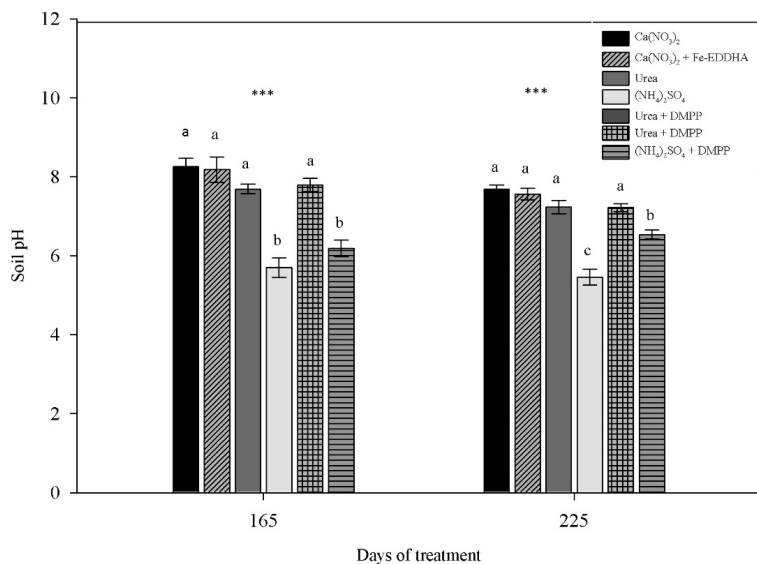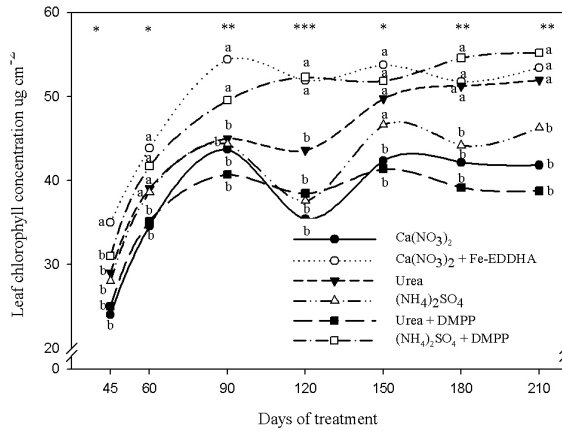
Figure 1. Soil pH for avocado trees *cv* Hass treated with different nitrogen fonts to prevent Fe deficiency. Bars with the same letter in each date were not significantly different according to the DGC test. Data are mean ± SE of six replicates. ***Significant at P ≤ 0.001 level. $Ca(NO_3)_2$ = calcium nitrate; EDDHA = ethylenediamine-N,N'-bis(2-hydroxyphenyl)acetic acid; $(NH_4)2SO_4$ = ammonium sulphate; DMPP = 3,4-dimethylpyrazole phosphate (nitrification inhibitor).

3.2 Leaf chlorophyll concentration and plant growth

During the vegetative season, higher chlorophyll concentrations were recorded in the first expanded leaf from trees treated with $Ca(NO_3)_2$ + Fe-EDDHA and $(NH_4)_2SO_4$ + DMPP than trees supplied with $(NH_4)_2SO_4$, urea + DMPP and $Ca(NO_3)_2$ (Figure 2). Trees treated with urea showed a low leaf chlorophyll concentration from 60 to 120 days after treatments, and the chlorophyll level gradually increased until values similar to $Ca(NO_3)_2$ + Fe-EDDHA and $(NH_4)_2SO_4$ + DMPP treatments were reached (Figure 2). At the end of the season, data obtained revealed a significant shoot growth (10-20 cm) on the trees, however, no differences between treatments have been recorded in this parameter (Figure 3).

Figure 2. Leaf chlorophyll concentration in avocado trees *cv* Hass treated with different nitrogen fonts to prevent Fe deficiency. Means with the same letter in each date were not significantly different according to the DGC test. Data are mean ± SE of six replicates. *Significant at P ≤ 0.05 level; **Significant at P ≤ 0.01 level; ***Significant at P ≤ 0.001 level. $Ca(NO_3)_2$ = calcium nitrate; EDDHA = ethylenediamine-N,N'-bis(2-hydroxyphenyl)acetic acid; $(NH_4)2SO_4$ = ammonium sulphate; DMPP = 3,4-dimethylpyrazole phosphate (nitrification inhibitor).
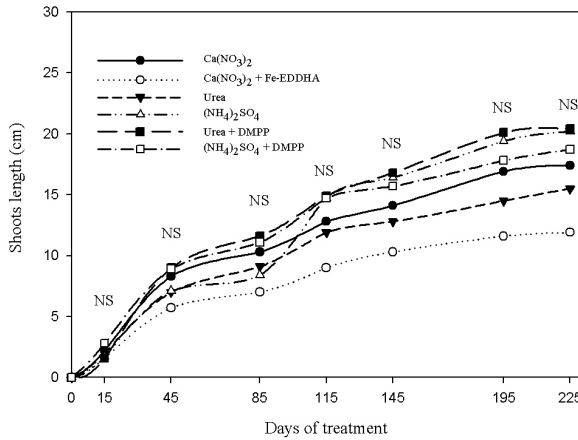
Figure 3. Shoots length (cm) during the season in avocado trees *cv* Hass treated with different nitrogen fonts to prevent Fe deficiency. Means with the same letter in each date were not significantly different according to the DGC test. Data are mean of six replicates. NS = not significant. $Ca(NO_3)_2$ = calcium nitrate; EDDHA = ethylene-diamine-N,N'-bis(2-hydroxyphenyl)acetic acid; $(NH_4)2SO_4$ = ammonium sulphate; DMPP = 3,4-dimethylpyr-azole phosphate (nitrification inhibitor).

3.3 Leaf gas exchange

In the present work, the infrared gas analyzer registered a higher net photosynthesis, stomatal conductance and transpiration in leaves at 12:00 pm than values recorded at 16:00 pm (Table 2). No significant differences were detected, with the methodology used, in the leaf gaseous exchange parameters evaluated in our experiment (Table 2).

Table 2. Net photosinthesys (A; μmol m^{-2} s^{-1}), stomatal conductance (g_s; mmol m^{-2} s^{-1}) and transpiration (E; mmol m^{-2} s^{-1}) in leaves of avocado trees *cv* Hass treated with different nitrogen fonts to prevent Fe deficiency. Measures were made 72 days after treatments, at 12:00 pm and 16:00 pm. Ca(NO$_3$)$_2$ = calcium nitrate; EDDHA = ethylenediamine-N,N'-bis(2-hydroxyphenyl)acetic acid; (NH$_4$)2SO$_4$ = ammonium sulphate; DMPP = 3,4-dimethylpyrazole phosphate (nitrification inhibitor).

Treatments	A (μmol m^{-2} s^{-1})		g_s (mmol m^{-2} s^{-1})		E (mmol m^{-2} s^{-1})	
	12:00	16:00	12:00	16:00	12:00	16:00
Ca(NO$_3$)$_2$	11.4	8.8	0.14	0.10	4.6	2.9
Ca(NO$_3$)$_2$ + Fe-EDDHA	9.8	8.9	0.13	0.12	4.4	3.1
Urea	11.2	8.9	0.16	0.10	4.9	3.3
(NH$_4$)$_2$SO$_4$	9.4	8.4	0.12	0.12	4.0	3.2
Urea + DMPP	10.8	9.7	0.14	0.14	4.9	3.8
(NH$_4$)$_2$SO$_4$ + DMPP	8.5	7.8	0.10	0.06	3.9	2.8
Significance	NS	NS	NS	NS	NS	NS

Data are mean of six replicates. NS = not significant.

3.4 Leaf N-NO$_3$-, N-NH$_4$$^+$ and total N concentrations

The results concerning the leaf N concentration indicate that the different fertilization strategies influenced the N-NO$_3$- and N-NH$_4$$^+$ content in leaves. In contrast, no statistical differences between treatments were registered in the leaf total N concentration (Table 3). The fertilization with (NH$_4$)$_2$SO$_4$ + DMPP significantly decreased the leaf N-NO$_3$- concentration compared to the other treatments (Table 4). On the other hand, the addition of Ca(NO$_3$)$_2$ increased the N-NH$_4$$^+$ concentration in leaves as compared with the other treatments (Table 3).

Table 3. Concentration of N-NO$_3^-$ (mg kg^{-1}), N-NH$_4^+$ (mg kg^{-1}) and N-total (%) in leaves of avocado trees *cv* Hass treated with different nitrogen fonts to prevent Fe deficiency. Ca(NO$_3$)$_2$ = calcium nitrate; EDDHA = ethylenediamine-N,N'-bis(2-hydroxyphenyl)acetic acid; (NH$_4$)2SO$_4$ = ammonium sulphate; DMPP = 3,4-dimethylpyrazole phosphate (nitrification inhibitor).

Treatments	N-NO$_3^-$ (mg kg^{-1})	N-NH$_4^+$ (mg kg^{-1})	Total N (%)
Ca(NO$_3$)$_2$	216.3 a	861 a	2.6
Ca(NO$_3$)$_2$ + Fe-EDDHA	239.5 a	552 b	2.4
Urea	246.3 a	516 b	2.4
(NH$_4$)$_2$SO$_4$	217.3 a	585 b	2.5
Urea + DMPP	212.1 a	622 b	2.5
(NH$_4$)$_2$SO$_4$ + DMPP	185.8 b	626 b	2.3
Significance	*	**	NS

Data are mean of six replicates. NS = not significant; *Significant at P ≤ 0.05 level; **Significant at P ≤ 0.01 level.

4. Discussion

Data concerning the soil pH after treatment imposition indicate a strong soil acidification induced by the fertilization with (NH$_4$)$_2$SO$_4$, with or without DMPP, whereas the application of urea did not significantly modify this soil property. Soil pH has a dominant effect on solubility and therefore availability of ions, whereas low pH shifts the equilibrium toward free metal cations and protonated anions, higher pH favours carbonate or hydroxyl complexes (Rengel, 2015). Reductions in the soil pH as a consequence of (NH$_4$)$_2$SO$_4$ supply in calcareous soils have been previously reported (Chien *et al*., 2011), and have been attributed to the descaling action exerted by the reaction of the sulfate group (SO$_4^{-2}$) contained in the fertilizer with the calcium carbonate (CaCO$_3$) present in the soil to form calcium sulfate (CaSO$_4$). Calcium sulfate may be highly susceptible to being carried down deep into the soil by rainwater or irrigation (Chien *et al*., 2011). In calcareous soils, Ca is the most abundant cation and its loss promotes H$^+$ fixation to counteract the complex charge, triggering medium acidification (Chien *et al*., 2011). In addition, the NH$_4^+$ ion present in the (NH$_4$)$_2$SO$_4$ molecule also contributes to decreasing the soil pH, since the conversion of 1 mol NH$_4^+$ ion to NO$_2^-$ (nitrification process) releases 2 mol H$^+$ into the soil solution. Some authors suggest that the nitrification of NH$_4^+$-based fertilizers contributes more to soil acidification than acid deposition and other factors (Guo *et al*., 2010, Tong and Xu, 2012), which could explain the lower acidifying capacity of (NH$_4$)$_2$SO$_4$ when applied with the nitrification inhibitor DMPP as recorded in our experiment. In contrast, the pH registered in the soil treated with urea was similar to that treated with Ca(NO$_3$)$_2$. Urea is an amide classified as an N-NH$_4^+$ source, and therefore its hydrolysis and the subsequent oxidation of the NH$_4^+$ group should release protons, thus increasing the medium acidification (Chien *et al*., 2011). In acid soils, the addition of urea may accelerate nitrification and even soil acidification,

since it increases the ammonia-oxidizing bacteria population (Tong and Xu, 2012). However, some studies into different N sources applied in calcareous soils have reported significant N loss when supplied as urea, due to NH_3 volatilization as a consequence of the high soil pH (Mahmood *et al*., 2011). Possibly, in our experiment, the NH_3 volatilization phenomena occurred in the soil treated with urea, with or without DMPP, preventing a fraction of the urea-derived NH_4^+ from being nitrified, and delaying soil acidification.

The leaf chlorophyll concentration in plants is often influenced by mineral nutrition and in particular by N and Fe status. Regarding Fe, different experimental experiences on Fe-deficient fruit tree species converge in that the interveinal yellowing in young leaves is the most common Fe deficiency symptom (Covarrubias *et al*., 2016). In fact, in several fruit tree species, the leaf chlorophyll concentration and Fe status of plants are significantly related (Rombolà and Tagliavini 2006). Taken together, this suggests that the leaf chlorophyll content is surely a reliable indicator of Fe nutrition in plants cultivated in calcareous soils. In this experiment, trees treated with Fe-EDDHA and $(NH_4)_2SO_4$ + DMPP showed a higher leaf chlorophyll concentration during the season, and from 120 days of treatment, the application of urea also increased this variable compared to plants treated with $Ca(NO_3)_2$. Beneficial effects of NH_4^+ on Fe (and other nutrients) availability and uptake that countered the adverse effects of NO_3^-, have been reported by several authors, and may be due to the effect of a N source on the soil acidification that increases the concentration of the most available Fe^{+3} in the medium (Houdusse *et al*., 2007; Jiménez *et al*., 2007; Covarrubias *et al*., 2014). However, the application of $(NH_4)_2SO_4$ without DMPP was not effective at increasing leaf chlorophyll concentration in trees, despite it significantly reducing

soil pH, even more than ammonium $(NH_4)_2SO_4$ + DMPP. To explain such results, it is important to resort on the acidification mechanisms related to the presence of NH_4^+ in the soil. One of these is related to the NH_4^+ nitrification process, which acidifies the soil solution (previously explained). The other acidification mechanism occurs in the rhizosphere, since the NH_4^+ absorption by root cells is mediated by an excretion of protons through the enzyme H^+-ATPase. A fraction of the NH_4^+ supplied as $(NH_4)_2SO_4$ (without DMPP) was probably submitted to nitrification, acidifying the soil solution, but the NO_3^- derived from such process has been absorbed by plant roots, inducing an alkalization in the rhizosphere. In contrast, it is highly likely that the DMPP supplied with $(NH_4)_2SO_4$ was effective at inhibiting NH_4^+ nitrification, reducing its acidification capacity in the soil solution, but increasing rhizosphere acidification through NH_4^+ absorption by roots. Such evidence suggests that the addition of $(NH_4)_2SO_4$ with a nitrification inhibitor seems to be a more efficient strategy than the addition of $(NH_4)_2SO_4$ without a nitrification inhibitor to reduce the Fe^{3+} present in the rhizosphere, and therefore to improve the Fe nutrition in trees. Otherwise, it is possible that the N source, regardless of its effects on Fe nutrition, may influence the leaf chlorophyll concentration since N is a crucial element in different processes related to chlorophyll synthesis and concentration in leaves. In fact, some genes linked to chloroplast and chlorophyll biosynthesis activated by N have been previously reported in the literature (Scheible *et al*., 2004). In addition, some NH_4^+ advantages for plant N nutrition may also have contributed to the increase in leaf chlorophyll concentration such as the higher N uptake efficiency, since NH_4^+ is more stable in the soil and less susceptible to leaching than NO_3^- (Zheng-Qin *et al*., 2010). It is also well

known that NH_4^+ requires less energy to be metabolized at cellular level than NO_3^- (Loulakakis *et al*., 2009). In fact, NH_4^+ assimilation consumes from 2 to 5% of the total energy production, whereas NO_3^- assimilation consumes nearly 15% (Loulakakis *et al*., 2009).

Our data showed that, from 150 days of treatment, the addition of urea increased the leaf chlorophyll concentration compared to the control trees. This result suggests that, despite the low effect of urea in reducing the soil pH, possibly due to the NH_3 volatilization, the trees probably managed to uptake a fraction of the NH_4^+ derived from it, promoting Fe absorption by roots. However, no effect was recorded on leaf chlorophyll concentration when urea was applied with DMPP. Apparently, in our experiment, the addition of DMPP substantially reduced the benefits of urea as $N-NH_4^+$ fertilizer to correct Fe chlorosis in avocados. In an experiment conducted by Liu *et al*. (2013), the application of urea with the nitrification inhibitors DMPP and DCD significantly increased the crop yield, plant biomass and nitrogen uptake in a wheat-maize cropping system due to a shift in the main soil inorganic N from NO_3^- to NH_4^+. However, in other investigation conducted on cotton, maize and wheat under greenhouse conditions using an alkaline calcareous soil, the addition of DCD significantly increased ^{15}N-labelled urea loss associated with a reduced fertilizer N uptake (Mahmood *et al*., 2011). Some authors have suggested that the increased fertilizer N loss, when urea is applied with a nitrification inhibitor, may be a result of a favored NH_3 volatilization derived from the complete urea hydrolysis and blocking of nitrification (Nastri *et al*. 2000; Gioacchini *et al*. 2002). In fact, urea hydrolysis may increase the pH in the soil solution, and consequently, the balance of NH_4^+ and NH_3 could be displaced, thereby promoting the

volatilization of NH_3 in the atmosphere (Schlegel *et al*., 1986). Moreover, in addition to increasing fertilizer N loss, the nitrification inhibitor DCD is known to increase the soil-derived N loss (Gioacchini *et al*. 2002). These results suggest that the use of urea with a nitrification inhibitor in avocado trees established in alkaline calcareous soils probably increases N losses compared to urea applied without the nitrification inhibitor, thus counteracting the possible benefits obtained from inhibiting nitrification.

Contrary to our expectations, the effect of treatments on the leaf chlorophyll concentration was not associated with the shoot length and leaf gaseous exchange of avocados, the data for which showed a high variability within and among treatments. In grapevine, some authors have reported the occurrence of leaf chlorosis strongly associated with a shoot growth reduction in Fe-deficient plants (Covarrubias *et al*., 2016,), whereas others did not observe any changes in shoot growth as a symptom of Fe-deficiency (Covarrubias *et al*., 2016). In an experiment conducted in hydroponic conditions, the grapevine genotype tolerant to Fe chlorosis 110 Richter reacted to Fe deficiency by reducing the main shoot length and biomass, without modifying the net photosynthesis or the leaf chlorophyll concentration, whereas opposite results were recorded in the susceptible genotype 3309-C (Covarrubias *et al*., 2016). The plant reaction to Fe deficiency linked to shoot growth and photosynthetic activity is likely related to the Fe chlorosis tolerance level, which is highly variable according to the species or genotype. Avocado is classified as a highly susceptible species to Fe deficiency, so it is probably not capable of counteracting the Fe deficit through physiological strategies based on shoot growth contraction, as do other Fe deficiency-tolerant

species. It is also possible that the Fe deficiency severity of the trees treated with NO_3^- was not so high as to modify the vegetative and physiological variables evaluated in the present experiment. Regarding photosynthetic activity, although a lower leaf chlorophyll concentration was recorded in trees treated with $Ca(NO_3)_2$, $(NH_4)_2SO_4$ and urea + DMPP, it is possible that no changes occurred in the chlorophyll molecule composition, in the lipid peroxidation or in the thylakoid organization (Bertrand and Poirier, 2005). Thus, the trees treated with $Ca(NO_3)_2$, $(NH_4)_2SO_4$ and urea + DMPP performed a similar photosynthetic activity in comparison with those treated with $Ca(NO_3)_2$ + Fe-EDDHA, urea and $(NH_4)_2SO_4$ + DMPP. Moreover, it should be mentioned that while it is known that Fe deficiency alters both chloroplast structure and photosynthesis rate in plants, the knowledge regarding the impact of Fe homeostasis on photosynthesis efficiency and therefore on biomass production is still limited in a tropical species as *Persea americana* Mill.

Data concerning the nutritional status of trees reveal that the use of $(NH_4)_2SO_4$ + DMPP reduced the leaf $N-NO_3^-$ concentration in avocados, indicating a lower $N-NO_3^-$ absorption in these trees compared to those subjected to the other treatments. Surprisingly, the application of the other $N-NH_4^+$ sources assessed did not reduce the leaf NO_3^- concentration, which could be related to the different processes that each $N-NH_4^+$ source suffered in the soil (nitrification in the case of $(NH_4)_2SO_4$ and volatilization in the case of urea). These results suggest that fertilization with $(NH_4)_2SO_4$ + DMPP is the most effective strategy for conserving NH_4^+ in soil, in which case the trees probably prefer this N source at the expense of NO_3^-. Conversely, the application of $Ca(NO_3)_2$ increased the $N-NH_4^+$ accumulation, suggesting a possible retarding effect on the activity of key enzymes (GS/GOGAT) linked

to the conversion of NH_4^+ to amino acids (Loulakakis *et al*., 2009). On the other hand, since the leaf total Fe concentration is not a reliable indicator of the Fe status in plants grown in calcareous soils because the total Fe concentration in chlorotic leaves are often similar or even greater than in green leaves ("chlorosis paradox" phenomena) (Römheld, 2000; Kosegarten *et al*., 2001), the concentration of this mineral in leaves was not considered in our investigation.

5. Conclusions

Data obtained indicate that the application of $(NH_4)_2SO_4$ is highly effective at reducing soil pH, and, when applied with a nitrification inhibitor, at increasing the leaf chlorophyll concentration in trees. Additionally, fertilization with urea is also effective at increasing leaf chlorophyll, but only when applied without a nitrification inhibitor. However, the acidifying effect of urea in the soil is significantly lower than $(NH_4)_2SO_4$. In this experiment, the different N sources also modified the concentration of $N-NO_3^-$, $N-NH_4^+$ and total N within the leaves of avocados, but did not influence the shoot length or leaf gaseous exchange. These results suggest that the fertilization strategy based on $(NH_4)_2SO_4$ + DMPP or urea can be an effective and sustainable alternative to counteract Fe chlorosis symptoms in avocado trees, with a similar effectiveness to that achieved with Fe-EDDHA.

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