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Revisiting nutrient management for *Citrus* production: To what extent does molybdenum affect nitrogen assimilation of trees?

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ABSTRACT

Increasing the nitrogen (N) use efficiency of fruit trees to enhance fruit yield and decrease N rate and fertilization losses in the field is intensively discussed. Noteworthy, molybdenum (Mo) demand is likely to increase in high yielding citrus orchards. However, supply of this micronutrient through fertilization practices is not well-known. Thus, two experiments were carried out under greenhouse conditions to evaluate the nitrate reductase (NRase) activity and the Mo mobility in sweet orange plants (1-yr-old) after foliar application of Mo. For both experiments, the plants were supplied with two N levels via fertigation over 7-mo (totaling 2.8 and 17.5 g of N per plant), with Mo treatments applied in the final month. The first experiment consisted of leaf sprays to the whole plant canopy at 0 (control), 0.12, 0.60 and 1.20 g L^{-1} Mo. In the second experiment, the 0.60 g L^{-1} Mo spray was limited to one side of the canopy. The Mo supply enhanced the NRase activity either in leaves or roots and increased the nitrate uptake by roots. Consequently, the N content in the roots, twigs and leaves of plants increased. When the Mo was sprayed on one side of the canopy, the nutrient was translocated (30 − 40% from the absorbed) from the leaves to the roots, but at a lower percentage in plants grown with the highest N supply. Although the Mo concentration did not increase in leaves that did not directly receive the micronutrient spray, the NRase increased in both parts of the canopy, as well as in the roots, enhancing the N content in *Citrus*.

1. Introduction

Molybdenum (Mo) is a micronutrient that catalyzes a range of reactions, such as phytohormone synthesis, sulfite detoxification, purine degradation and nitrogen (N) assimilation by plants (Schwarz et al., 2009; Bittner, 2014). Molybdenum is a cofactor of nitrate reductase (NRase; E.C. 1.7.1.1), the first enzyme involved in the intracellular reduction of nitrate (NO_3^-) to ammonium (NH_4^+). The *in vivo* NRase activity is used as an indicator of the N assimilation potential of plants (Dovis et al., 2014).

Visual symptoms of Mo deficiency in field grown trees are unusual because the adequate and deficient values of the nutrient in plant tissue are constrained to a narrow range (Obreza and Morgan, 2008; Mattos Jr. et al., 2012). Stewart and Leonard (1952) reported on Mo deficiency of citrus plants in Florida (USA). However, no other reports of visual symptoms of Mo deficiency in tree crops are available in the literature. Furthermore, few experiments have evaluated the effects of Mo fertilization (Ezz and Kobbia, 1999; Srivastava and Singh, 2007), and consequent absorption and mobility of the foliar-applied nutrient. Yet, such information could contribute to practices that foster the maximum fruit yield capacity of plants.

The Mo demand by citrus trees is likely related to several agronomic factors, particularly in tropical soils, where the availability of the nutrient is limited by the high acidity potential of the soil and the low content of this micronutrient in the soil matrix (Kaiser et al., 2005). Among these factors, the increased vigor of the plant, due to intensive N fertilization (Alva et al., 2006; Boaretto et al., 2007; Quiñones et al., 2012) and the utilization of rootstocks with superior nutritional demand (Quaggio et al., 2004; Mattos Jr. et al., 2006) can lead to a greater need for micronutrients, such as Mo, involved in enzymatic processes (Bondada and Syvertsen, 2003; Schwarz and Mendel, 2006; Schwarz et al., 2009).

The recent increase in citrus areas grown under fertigation and the enhancement of NO_3^--containing fertilizer application (Quaggio et al., 2014), favors greater demand for Mo by citrus trees (Kaiser et al., 2005). Groves fertigated with calcium nitrate present improved soil

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chemical conditions for root development and provide a more balanced nutritional status than those fertilized with NH_4NO_3 (Quaggio et al., 2014) because high N doses and continuous application of NH_4^+ sources can cause soil acidification (Britto and Kronzucker, 2002).

Although fertigation with calcium nitrate enhances sweet orange fruit yields (Quaggio et al., 2014), Mo-deficient plants present low NRase activity, what could limit their ability to attain maximum production efficiency. In plants, NO_3^- is reduced to NH_4^+ before it is incorporated into organic forms by two distinct sequential reactions, catalyzed by different enzymes. The first reaction occurs in the cytosol of root and/or leaf cells and is mediated by NRase that reduces NO_3^- to nitrite (NO_2^-) (Lea et al., 1999; Mendel, 2011; Dovis et al., 2014). The NO_2^- formed is then transported to the plastids of roots or chloroplasts of leaves, where it is reduced to NH_4^+ by nitrite reductase (Lea and Azevedo, 2007). The assimilation of inorganic-N into an organic form impacts plant growth and the potential yield of crops (Lea et al., 1999). The supply of Mo improves the N assimilation by plants growing in acid soils (pH 4.5 − 5.0), resulting in improved growth and yield of wheat (Wang et al., 1999) and grapevines (Williams et al., 2004).

Despite the positive correlation between the NRase activity and the infiltration of Mo in leaf fragments (Shaked and Bar-Akiva, 1967), there is still a lack of information about how Mo supply and mobility characteristics in woody trees contribute to increase the enzyme activity of plants grown at different levels of N−NO_3 and this is the purpose of these experiments.

2. Material and methods

Two experiments were carried out sequentially in a greenhouse with similar management practices during the initial stages of plant growth. Homogeneous 1-yr-old sweet oranges plants [*Citrus sinensis* (L.) Osbeck *cv*. Valencia] grafted onto Rangpur lime (*C. limonia*) were grown in pots containing 20 dm^3 of an organic substrate (80% pinus bark, 15% vermiculite and 5% of carbonized material; pH 5.6).

2.1. Experiment 1: NRase activity in citrus plants after foliar application of Mo

The first experiment was set up in a 4 × 2 factorial design with four doses of Mo (nil application, 0.12, 0.60 and 1.20 g L^{-1}) and two levels of N, with four replicates. Plants were divided into two groups that received different levels of N to impose contrasting nutrient status based on the expected demand of the plants. Then a fertilization regime was conducted every 15 d via fertigation, which totaled 2.8 and 17.5 g of N per plant, as $Ca(NO_3)_2.4H_2O$. Plants that received the lowest N fertilization level were also supplied with $CaCl_2$ to standardize the amount of Ca against those with the highest N level.

During the experiment, the plant fertilization maintenance was conducted fortnightly according to Hippler et al. (2015), with the following modifications: 250 mg L^{-1} of KH_2PO_4, 493 mg L^{-1} of $MgSO_4{\cdot}7H_2O$, 3.0 mg L^{-1} of H_3BO_3, 2.0 mg L^{-1} of $CuSO_4{\cdot}5H_2O$, 4.0 mg L^{-1} of $ZnSO_4{\cdot}7H_2O$ and 2.0 mg L^{-1} of $MnSO_4{\cdot}3H_2O$. Overall, the experiment received a total of 7.5 g of P, 9.7 g of K, 29 g of Ca, 7.0 g of Mg, 9.4 g of S, 109 mg of B, 82 mg of Cu, 168 mg of Zn and 110 mg of Mn. Iron was applied separately every month, as Fe-EDDHA (iron salt of ethylenediamine-di-*o*-hydroxyphenylacetic acid), in a solution with 0.06 g L^{-1} of Fe.

Molybdenum doses were sprayed on the leaves in a single application 180 d after starting the different N levels, when trees presented the second vegetative flush already mature. The pots were covered with a plastic film to avoid contamination of the substrate with the micronutrient. The amounts of solution retained on the canopy ($Mo_{retained}$) were calculated according to Eq. (1). The volume of sprayed solution remaining on every plant canopy was 40 ± 2.6 mL, which totaled 7.8 ± 2.0 mg of Mo per plant when sprayed with 0.12 g L^{-1} of Mo solution; 39 ± 2.9 mg of Mo per plant with 0.60 g L^{-1} of Mo; and 78 ± 4.5 mg of Mo per plant with 1.20 g L^{-1} of Mo.

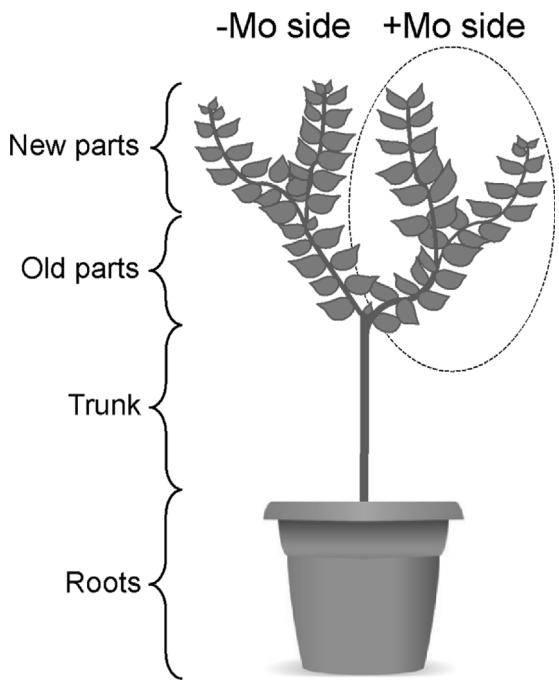

Fig. 1. Diagram of foliar spray of molybdenum (Mo) in Experiment 2. Plants conducted to grow two scaffold branches to which 0.60 g L^{-1} of Mo was sprayed to one side of the canopy (+Mo side; as outlined) or not (-Mo side).

$$Mo_{retained} = [(\text{plant weight after Mo spray}) - (\text{plant weight before Mo spray})] \times (\text{Mo solution concentration}) \quad (1)$$

The *in vivo* NRase activity assay (Dovis et al., 2014) was performed on leaves from the second vegetative flush (new parts; Fig. 1) and fine roots (< 3.0 mm ø) at 1, 4, 9, 15 and 30 d after the Mo application. Briefly, 200 mg fresh weight (FW) of leaves or 1000 mg of FW of fine roots were incubated in 100 mM sodium phosphate buffer solution (pH 7.5) with 200 mM KNO_3 and 1% *n*-propanol (w/v). The samples were vacuum filtered and kept in the dark at 40 °C for 30 min. The NO_2^- was quantified by absorbance at 540 nm, with the addition of 1% sulfanilamide solution in 2.4 N HCl + 0.02% *N*-[1-naphthyl] ethylenediamine dihydrochloride (NED; w/v). The NRase activity was transformed to relative activity considering the treatment with the lowest N supply and without Mo application as the control (100%).

Before NRase activity measurements were determined, the chlorophyll index (SPAD value) was indirectly measured using an SPAD-502 portable meter (Konica Minolta Holding Inc., Tokyo, Japan).

At 30 d after the Mo application, the plants of both experiments were destructively harvested and separated into old and new leaves (Fig. 1), twigs, trunk and roots. The plant parts were washed in detergent at 0.01% (v/v) and deionized water, and dried at 58 − 60 °C till constant weight to quantify the dry mass (DM) production.

The Mo concentration in the plant tissue was determined according to Bataglia et al. (1983), with modifications. One gram of tissue DM was mixed in 6 mL of perchloric acid and maintained for 14 h. Then, 3 mL of perchloric acid was added to the mixture and heated to 220 °C in a digestion block. The sample readings were acquired by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Perkin-Elmer, 5100 PC, Norwalk, CT, USA). A reference material was used as a standard in all sets of analyses. The plant tissue N concentration was quantified according to Bataglia et al. (1983), while NO_3^- and NH_4^+ were determined by steam distillation according to Tedesco et al. (1995).

2.2. Experiment 2: Mo mobility in citrus plants

Both experiments were conducted under equivalent conditions. Nevertheless, in the second experiment, the foliar application of Mo was performed on half of the plant canopy (+Mo side; Fig. 1). Treatments were set up in a 2 × 2 factorial design with two levels of Mo [trees without the application (control) and at 0.60 g L^{-1} of Mo] and two levels of N (similar to the first experiment), with five replicates. A plastic film cover was used to prevent Mo deposition on the without Mo (-Mo) plant side, trunk and substrate during the spray application. The $Mo_{retained}$, calculated using Eq. (1), on the with Mo (+Mo) side of the canopy was 15 ± 2.2 mg of Mo per plant.

After 30 d of the Mo supply, the NRase activity, SPAD values, biomass production and concentrations of Mo, N, NO_3^- and NH_4^+ were determined as described in the first experiment, but separately in the new parts (leaves and twigs) of both sides (-Mo and +Mo; Fig. 1) in those plants that received Mo.

The content of Mo and N in each plant part were calculated from the DM production and nutrient concentration values. Furthermore, using an indirect approach, the percentage of Mo taken up by the leaves (% Mo_{uptake}) and translocated to the roots and to the woody parts (% $Mo_{translocation}$) were estimated by Eqs. (2) and (3), respectively:

$$\%Mo_{uptake} = [(Mo_{treatment}) - (Mo_{control})] \times 100/(Mo_{retained}) \quad (2)$$

$$\%Mo_{translocation} = (Mo_{root/trunk}) \times 100/[(Mo_{treatment}) - (Mo_{control})] \quad (3)$$

where, $Mo_{treatment}$ (mg of Mo per plant) is the total Mo content in those plants that received the micronutrient application; $Mo_{control}$ (mg of Mo per plant) is the total Mo content in those plants without the micronutrient application; $Mo_{retained}$ (mg of Mo per plant) is the Mo amount retained in the plant canopy as described in the first experiment; and $Mo_{root/trunk}$ (mg of Mo per plant) is the Mo content in the roots or in the trunk of plants that received the micronutrient application.

2.3. Statistical analyses

A two-way analysis of variance (ANOVA) was performed to evaluate the effects of Mo rates and N levels on the evaluated variables, with a significance level of $\alpha = 0.05$. When the interaction between Mo rates and N levels (Mo*N) was significant, means were compared using Tukey's test at a 5% significance level.

3. Results

3.1. Experiment 1: NRase activity in citrus plants after foliar application of Mo

Increasing the Mo doses enhanced its concentration in leaves up to 12 mg kg^{-1} of Mo, independently of N supply (Fig. 2A). The Mo concentration in the roots also increased according to the increment of the nutrient dose applied but to a lesser extent (< 0.6 mg kg^{-1} of Mo) compared to the leaves (Fig. 2B). Furthermore, Mo concentration in roots was higher in plants supplied with low N, indicating that elevated levels of N decrease the micronutrient translocation from the canopy to the roots (Fig. 2B).

The NRase activity in leaves increased compared to the control plants 1 d after the Mo application what pattern was maintained after and up to 30 d (Fig. 3A–F). Based on the mean NRase activity of pooled data during the period evaluated (Fig. 3F), the maximum activity of this enzyme was verified at 0.75 g L^{-1} of Mo in plants supplied with the highest N level and, 0.69 g L^{-1} of Mo for those plants with the lowest N level (Fig. 3F). Furthermore, NRase activity in roots also increased proportionally to Mo doses applied in the leaves (Fig. 4).

Plants grown with high N supply exhibited the highest concentrations of total N, NO_3^- and NH_4^+ in leaves and roots (Fig. 5), and despite greater NRase activity was observed in both parts, Mo supply did not increase the concentration of total N or NH_4^+ in new leaves and roots of plants (Fig. 5). However, Mo sprayed at 0.60 or 1.20 g L^{-1} increased the NO_3^- concentrations in leaves and roots of trees supplied with the highest N level and, in roots of those with the lowest N level (Fig. 5).

3.2. Experiment 2: Mo mobility in citrus plants

Plants supplied with the highest level of N exhibited the greatest concentration and accumulation of Mo in the leaves (Tables 1 and 2), which corresponds to the maximum percentage of the micronutrient absorbed (%Mo_{uptake}), namely 9.4% of the total applied, compared to 7.3% in those with low N supply (Fig. 6A).

Besides leaves, the Mo concentration also increased in trunk and root tissues, even though neither part received the micronutrient application (Table 1). At 40% redistribution from the canopy to the roots, the Mo content in trunk and roots was higher in plants with low N supply (Table 2), compared to 28% in those with the highest N level (Fig. 6B). This later confirms that Mo presents moderate to high translocation into the plant when this micronutrient is supplied to the leaves. The most significant differences in Mo translocation according to the N supply was observed in the roots with 167 versus 98 μg of Mo in plants with low and high N supply, respectively (Fig. 6D).

As observed in the first experiment, the NRase activity in leaves increased one day after the Mo sprays (Fig. 7), demonstrating the rapid absorption and participation of this micronutrient in the plant metabolism, particularly in leaves that received the Mo application. However, leaves of the −Mo side (Fig. 1) presented intermediate NRase activity between leaves of the +Mo side and those from the control trees (Fig. 7). Important to note that, trees with the highest N supply presented a delayed activation of NRase activity in the roots, occurring after 15 d (Fig. 8), while those with the lowest N supply had an increased NRase activity after 9 d of the Mo application (Fig. 8).

3.3. Biomass production and indirect chlorophyll values

Plant biomass did not vary with either Mo or N supply in both

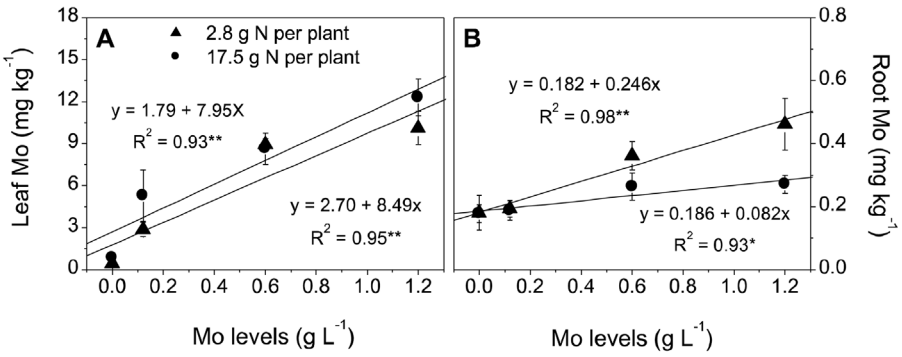

Fig. 2. Molybdenum (Mo) concentration in new leaves (A) and roots (B) of young sweet orange trees grown with low and high nitrogen (N) supply, at 30 days after foliar application of different levels of Mo (Experiment 1). Legend: ns: not significant ($p > 0.05$); **$p < 0.01$.

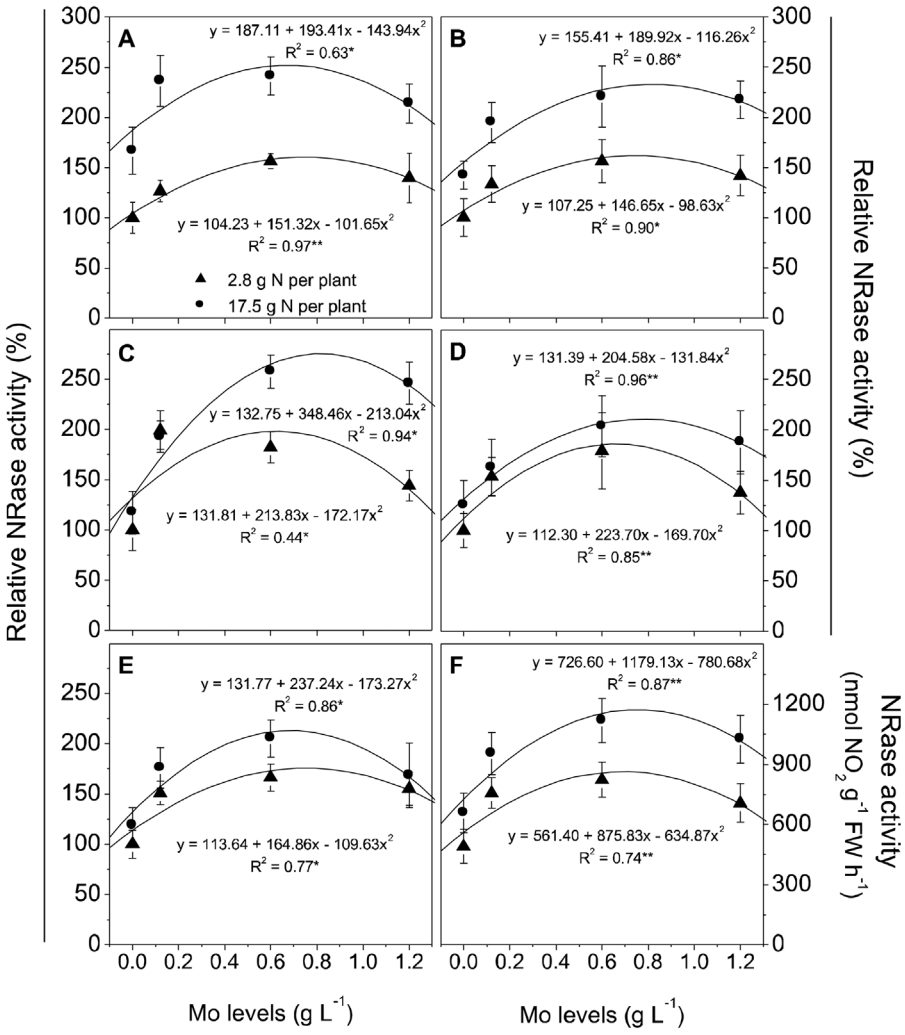

Fig. 3. Relative nitrate reductase (NRase) activity in new leaves of young sweet orange trees grown with low and high nitrogen (N) supply, at 1 (A), 4 (B), 9 (C), 15 (D) and 30 days (E) and mean NRase activity for pooled data (F) after foliar application of molybdenum (Mo) (Experiment 1). Legend: Vertical lines represent standard mean of the deviation (n = 5); $*p < 0.05$; $**p < 0.01$.

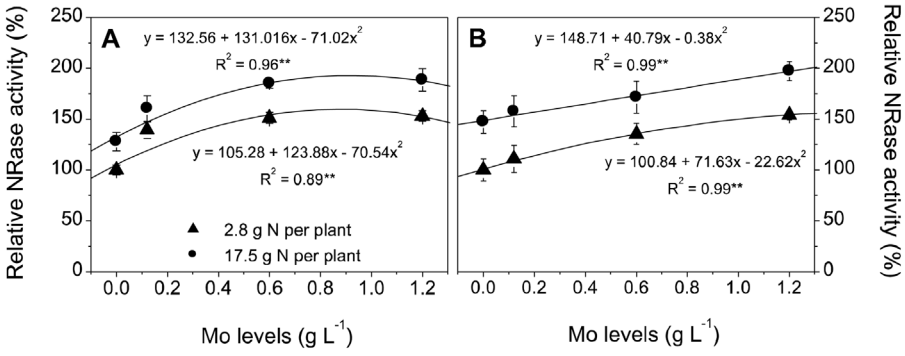

Fig. 4. Relative nitrate reductase (NRase) activity in roots of young sweet orange trees grown with low and high nitrogen (N) supply, at 15 (A) and 30 days (B) after foliar application of molybdenum (Mo) (Experiment 1). Legend: Vertical lines represent standard mean of the deviation (n = 5); $**p < 0.01$.

experiments (data not shown). Despite no differences in SPAD values were verified after Mo application in both experiments (data not shown), plants supplied with 2.8 versus 17.5 g per plant of N presented values of 55.4 ± 2.0 and 74.4 ± 4.5 SPAD, respectively ($p < 0.05$).

4. Discussion

The supply of Mo increased its concentration into plant parts according to the dose supplied (Fig. 1). Plants with high N level exhibited a higher concentration of Mo in leaves (Table 1), as well as the % Mo_{uptake} (Fig. 6), in which might be affected by different anatomical cell arrangement of plant leaves, such as increased leaf thickness and integrity of membranes and cell walls of epidermal cells (Smoleń, 2012), compared to those with low N level.

Boron, manganese and zinc foliar sprayed on citrus, have restricted translocation to new vegetative flushes due to their low mobility into the phloem vessels (Boaretto et al., 2003; Hippler et al., 2015). Although Mo is considered moderately mobile in fruit trees (Marschner, 2012), the redistribution of this micronutrient is not well characterized but suggested to occur as molybdate (MoO_4^{2-}) or as a Mo-S amino acid complex (Gupta, 1997). In the present study, the Mo translocation towards the roots (Tables 1 and 2) likely occurred as a MoO_4^{2-}-sugar-complex (Gupta, 1997; Williams et al., 2004). Furthermore, the Mo translocation (%$Mo_{translocation}$; Fig. 6D) was dependent on the N level supplied, which plays a main role in production and transport of carbohydrates from leaves to the roots (Bloom et al., 2012). Some proteins

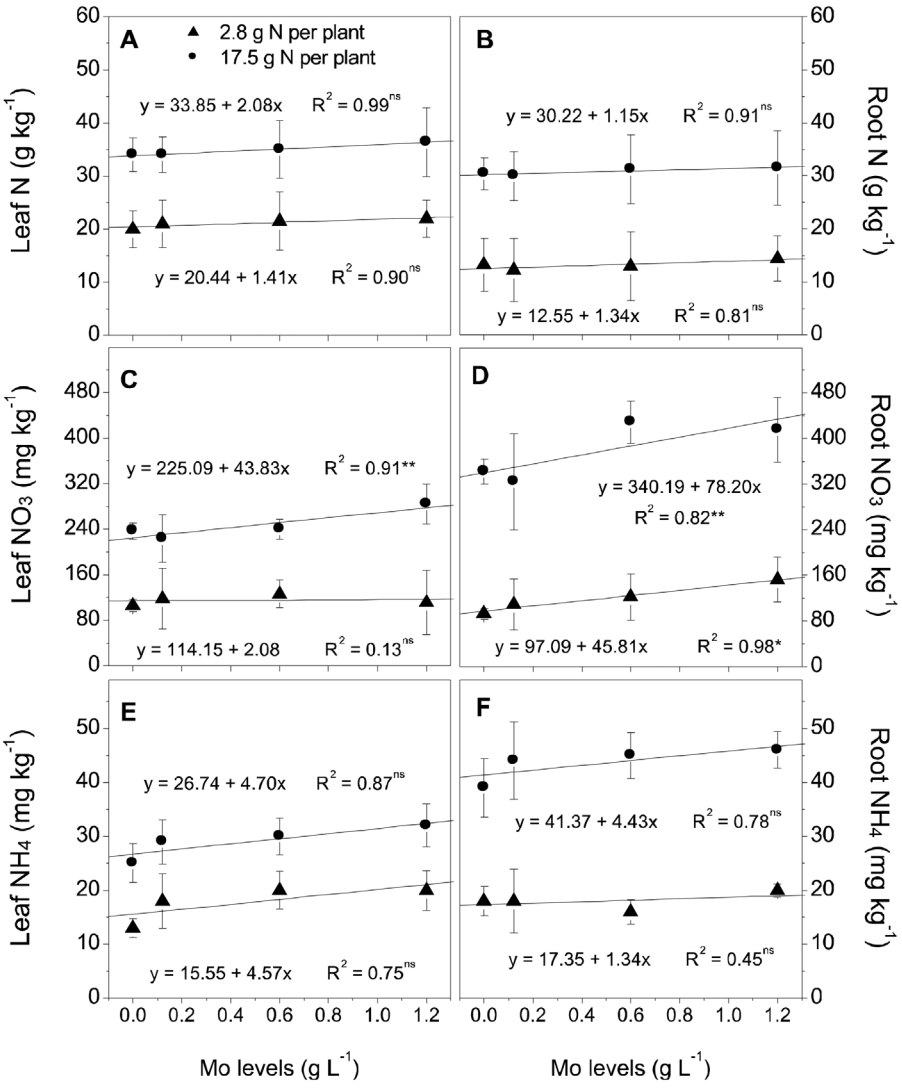

Fig. 5. Concentration of nitrogen (N), nitrate (NO_3) and ammonium (NH_4) in new leaves (A, C and E) and roots (B, D and F) of young sweet orange trees grown with low and high nitrogen (N) supply, at 30 days after foliar application of molybdenum (Mo) (Experiment 1). Legend: Vertical lines represent standard mean of the deviation (n = 5); ns: not significant ($p > 0.05$); *$p < 0.05$.

Table 1
Concentration of molybdenum (Mo) in young sweet orange trees grown with low and high nitrogen supply, 30 days after receiving foliar sprays with Mo (Experiment 2).

Molybdenum (Mo) g L^{-1}	Nitrogen (N) g per plant	Roots mg kg^{-1}	Trunk	Old twigs	Old leaves	New twigs	New leaves
0.0 (Control plants)	2.8	0.61 ± 0.37[a] Ab[b]	0.26 ± 0.07	0.22 ± 0.06	4.1 ± 0.8 Ab	0.26 ± 0.03	3.7 ± 0.7 Ab
	17.5	0.72 ± 0.32 Ab	0.34 ± 0.03	0.23 ± 0.03	3.4 ± 0.5 Ab	0.23 ± 0.03	2.0 ± 0.7 Bb
	Mean values	0.66	0.3 b	0.22 b	3.8 b	0.25 b	2.8
0.60 (side −Mo)	2.8	2.42 ± 0.47 Aa	2.45 ± 0.10	0.22 ± 0.07	3.8 ± 0.9 Ab	0.26 ± 0.04	3.6 ± 1.0 Ab
	17.5	1.62 ± 0.26 Ba	2.49 ± 0.29	0.33 ± 0.05	3.8 ± 1.7 Ab	0.25 ± 0.03	3.7 ± 1.3 Ab
	Mean values	2.01	2.47 a	0.28 b	3.8 b	0.26 b	3.6
0.60 (side +Mo)[c]	2.8	–	–	12.50 ± 3.4	7.8 ± 1.2 Ba	5.4 ± 1.1	26.7 ± 3.4 Ba
	17.5	–	–	8.70 ± 1.6	13.6 ± 2.3 Aa	4.7 ± 1.3	37.5 ± 6.1 Aa
	Mean values	–	–	10.6 a	10.6 a	6.4 a	32.1
F test[d]							
Mo		**	**	***	***	**	***
N		ns	ns	ns	ns	ns	ns
Mo*N		*	ns	ns	ns	ns	*

[a] Standard deviation of the mean.
[b] N levels: means (n = 5 or 15) followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$). Mo treatments: means (n = 5 or 10) followed by different lowercase letters are significantly different by Tukey's test ($p < 0.05$). *: significant at 5% for between the N levels.
[c] Plant parts that received Mo application.
[d] F test in the ANOVA of Mo vs. N for each parameter evaluated. ns: not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 2
Content of molybdenum (Mo) in young sweet orange trees grown with low and high nitrogen supply, 30 days after receiving foliar sprays with Mo (Experiment 2).

Molybdenum (Mo) g L^{-1}	Nitrogen (N) g per plant	Roots µg of Mo per plant	Trunk	Twigs	Leaves	Total
0.0 (Control)	2.8	57 ± 34[a] Ab[b]	22 ± 5	13 ± 2	157 ± 17 Ab	249 ± 46 Ab
	17.5	62 ± 16 Ab	28 ± 3	12 ± 2	108 ± 28 Ab	210 ± 28 Ab
	Mean values	59	25 b	12 b	133	229
0.60[c]	2.8	226 ± 33 Aa	215 ± 17	271 ± 30	611 ± 72 Ba	1323 ± 25 Ba
	17.5	158 ± 24 Ba	228 ± 37	264 ± 22	991 ± 135 Aa	1706 ± 156 Aa
	Mean values	192	222 a	267 a	801	1515
F test[d]						
Mo		***	***	***	***	***
N		ns	ns	ns	ns	ns
Mo*N		*	ns	ns	**	**

[a] Standard deviation of the mean.
[b] N levels: means (n = 5 or 15) followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$).Mo treatments: means (n = 5 or 10) followed by different lowercase letters are significantly different by Tukey's test ($p < 0.05$). *: significant at 5% for between the N levels.
[c] Sum of "–Mo side" and " + Mo side".
[d] F test in the ANOVA of Mo vs. N for each parameter evaluated. ns: not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

related to uptake and transport of Mo in the plants have been identified, such as the *MOT* family (molybdenum transporters) (Bittner, 2014) and the sulfate transporters (*SHST1*) (Fitzpatrick et al., 2008), but their mechanisms are not yet fully understood.

The NRase is the first enzyme in the reduction of NO_3^- to organic molecules within the N assimilation process in plants (Lea et al., 1999). In this study, the NRase activity in leaves increased the day after the Mo sprays (Fig. 7), demonstrating the rapid absorption and participation of this micronutrient in the plant metabolism, particularly in leaves that received the Mo application. When foliar Mo is applied as MoO_4^{2-}, the nutrient absorption and translocation into the plant generally occurs quickly, and thus, it is rapidly assimilated into the plant metabolism and then incorporated into NRase (Kaiser et al., 2005).

The foliar Mo spray also increased the NRase activity in roots (Figs. 4 and 8), in which trees with the highest N supply presented a delayed activation of NRase activity in the roots, occurring after 15 d (Fig. 8), while those with the lowest N supply had an increased NRase activity after 9 d of the Mo application (Fig. 8). Furthermore, the NRase activity in roots of young citrus plants supplied with 50% as N—NH_4 and 50% as N—NO_3 was reported to be only 17% of the activity level estimated in leaves (Dovis et al., 2014). In the present study, the NRase activity in roots was 66% of the same observed in leaves (Figs. 3 and 4), probably because of plants were supplied with 100% as N—NO_3.

Despite the Mo concentration in the leaves of the −Mo side (Fig. 1) was the same compared to the control ones (Table 1), NRase activity in leaves of the −Mo side was intermediate between the leaves of the +Mo side and those from the control trees (Fig. 7). It suggests the Mo application on half of the canopy can also increase the NRase activity in leaves on the other side of the plant that does not receive the direct application of the micronutrient (Fig. 7). In this instance, either the increased Mo concentration or the NRase activity activate the enzyme in other plant parts what likely results from activation of genes of transcription factors, such as the nodule-inception-like proteins (*NLPs*), responsible for controlling the expression of genes encoding NRase enzyme and transporters of NO_3^- and NO_2^- (Yanagisawa, 2014). Thus, the modulation by transcription factors might also explain the increased NO_3^- concentration in the roots of plants foliar sprayed with Mo (160 mg kg^{-1} of NO_3^-), compared to those without Mo application (138 mg kg^{-1} of NO_3) ($p < 0.05$; data not shown), similarly to that verified in the first experiment (Fig. 5). However, new studies might be developed to identify and confirm candidate genes as activator transcription factors of the NRase activity and the other genes evolved, such as the NO_3^- transporter family.

The highest level of N supply was the primary factor that induced the enzyme activity in both leaves (Fig. 7) and roots (Fig. 8), once the fertilizer source was only N—NO_3. Among the environmental factors

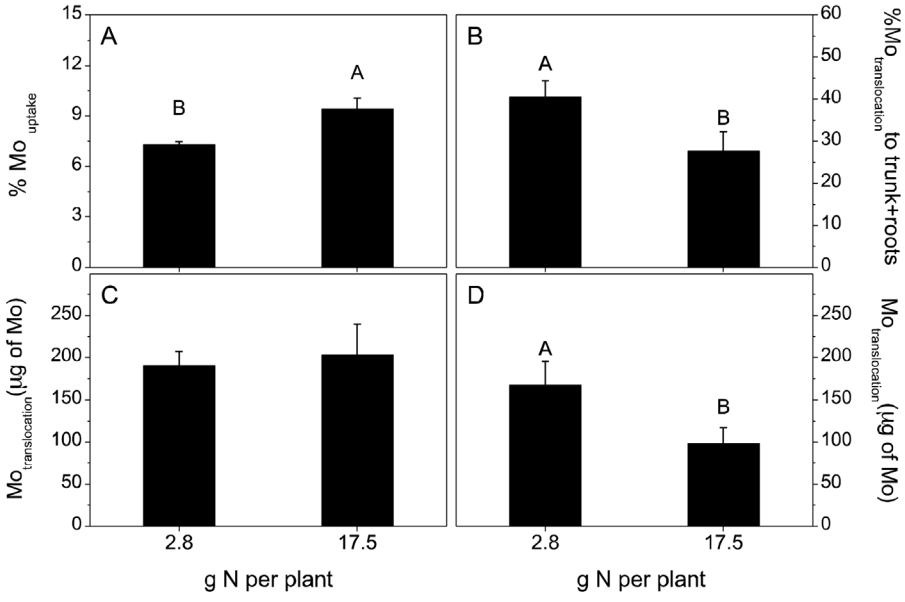

Fig. 6. Percentage of molybdenum (Mo) absorbed by leaves in relation to the total applied (%Mo_{uptake}; A), and the percentage (B; %$Mo_{translocation}$) and amount ($Mo_{translocation}$) of Mo translocated to trunk (C) and roots (D) from the absorbed in young sweet orange trees grown with low and high nitrogen (N) supply, 30 days after foliar application of Mo (Experiment 2). Vertical lines represent standard mean of the deviation (n = 5); N levels: means (n = 5 or 15) followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$).

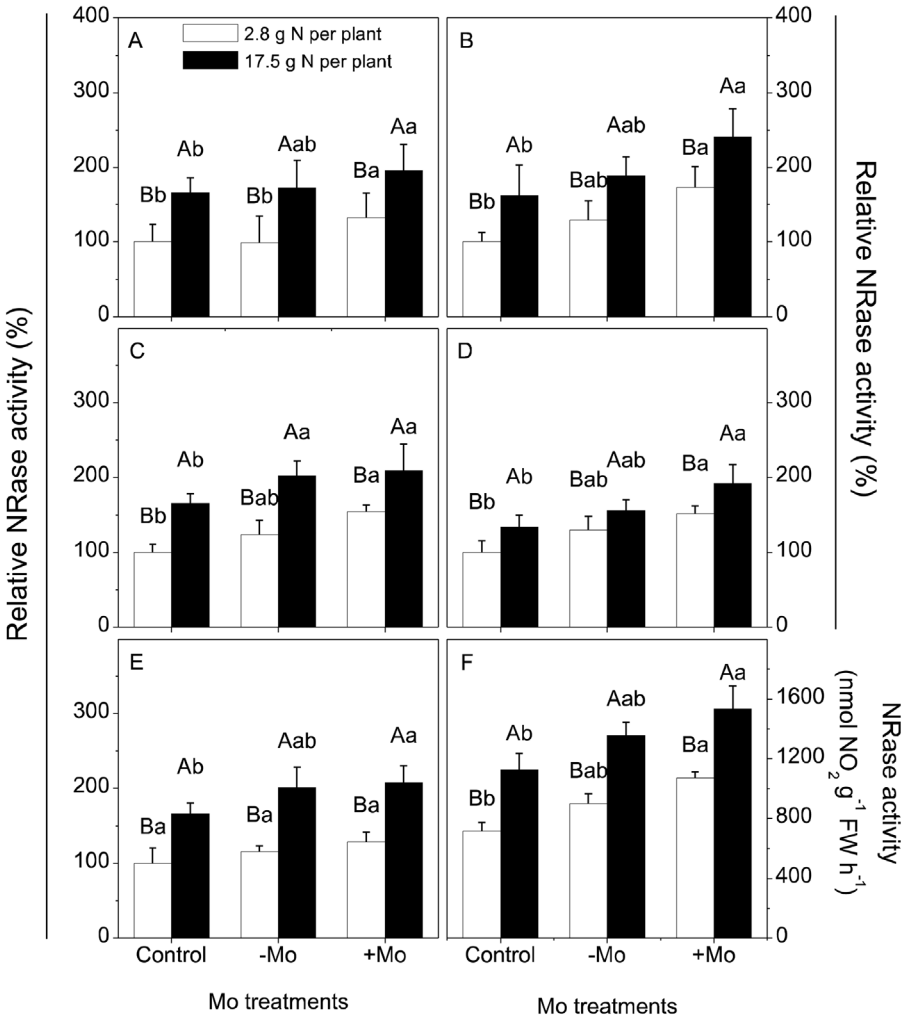

Fig. 7. Relative nitrate reductase (NRase) activity in new leaves of young sweet orange trees grown with low and high nitrogen (N) supply, at 1 (A), 4 (B), 9 (C), 15 (D) and 30 days (E) and mean NRase activity for pooled data (F) after foliar application of molybdenum (Mo) (Experiment 2). Vertical lines represent standard mean of the deviation (n = 5); N levels: means (n = 5 or 15) followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$).Mo treatments: means (n = 5 or 10) followed by different lowercase letters are significantly different by Tukey's test ($p < 0.05$).

that trigger the NRase activity in plants, the NO_3^- accumulation is considered the most valuable (Lea et al., 1999; Lea and Azevedo, 2007). This supports the hypothesis that Mo demand is enhanced in plants fertilized with NO_3^--containing fertilizers. Furthermore, plants with high N supply exhibited greater N contents in roots, twigs and leaves when Mo was sprayed to the leaves compared to other treatments with no Mo (Table 3). In this instance, the Mo application in plants with high N supply could increase the uptake and utilization efficiency of the applied N (Lea et al., 1999). Increasing the N utilization efficiency results in improved plant metabolism performance (Mattos Jr. et al., 2003; Alva et al., 2008; Martínez-Alcántara et al., 2012), mostly associated with photosynthesis that further allows plants to reach their highest yield potential. Despite the increased NRase activity, no differences in NH_4^+ concentration were verified in plants with Mo supply. Indeed, after being absorbed by roots or formed in the assimilation process, the NH_4^+ is quickly reduced to glutamine and glutamate (Lea and Azevedo, 2007).

Based on the increment of NO_3 concentrations in the plant parts, but not for N or NH_4^+, the increased NRase activity by the Mo supply (Figs. 3 and 4) supports the idea that NO_3^- uptake and accumulation by plants is favored by up-regulation of the enzyme and specific NO_3^- transporter genes expression at the plasma membrane level (Yanagisawa, 2014). Due to this regulatory role, the NRase activity correlates with plant yield (Martínez-Alcántara et al., 2012; Dovis et al., 2014). The increase in NO_3^- uptake also increased the NO_3^-/NH_4^+ ratio of leaves from 6.8 in the control plants to 9.0 and 8.3 in those that received 0.60 and 1.20 g L^{-1} of Mo, respectively ($p < 0.05$; data not shown). This increase could improve fruit yield, given the enhanced NO_3^-/NH_4^+ ratio of up to 8.0-8.5 in sap extracts, is proportional to the increased fruit yield in citrus trees (Quaggio et al., 2014).

Due to the short evaluation time, plant biomass did not vary with either Mo or N supply in both experiments. Longer-term studies are still necessary to corroborate if the greatest NRase activity will increase plant growth. In a long-term study, the Mo supplies in mandarin trees increased the NRase activity and consequently the plant yield (Ezz and Kobbia, 1999; Xu et al., 2012).

The SPAD index is an indirect indicator of chlorophyll content in leaves, which has been correlated with N content in leaves of citrus plants (Jifon et al., 2005; Souza et al., 2011). Such as observed in this study, plants with high N supply exhibited the highest SPAD indexes values, which represents the increased N accumulation, in part, as chlorophylls.

5. Conclusions

Molybdenum application via foliar spray increases assimilation efficiency and utilization of NO_3^- applied via fertigation as a result of the NRase activity increase in leaves and roots of sweet orange trees. The enhanced NRase activity increases NO_3^- uptake and assimilation. The Mo presents medium to high mobility into the plants after absorption by leaves because a significant proportion is translocated towards the roots. Plants with high N supply show higher Mo uptake than those with low N supply but reduced translocation into the plant. Therefore, the supply of Mo positively affects plant metabolism of trees supplied with NO_3^--containing fertilizers.

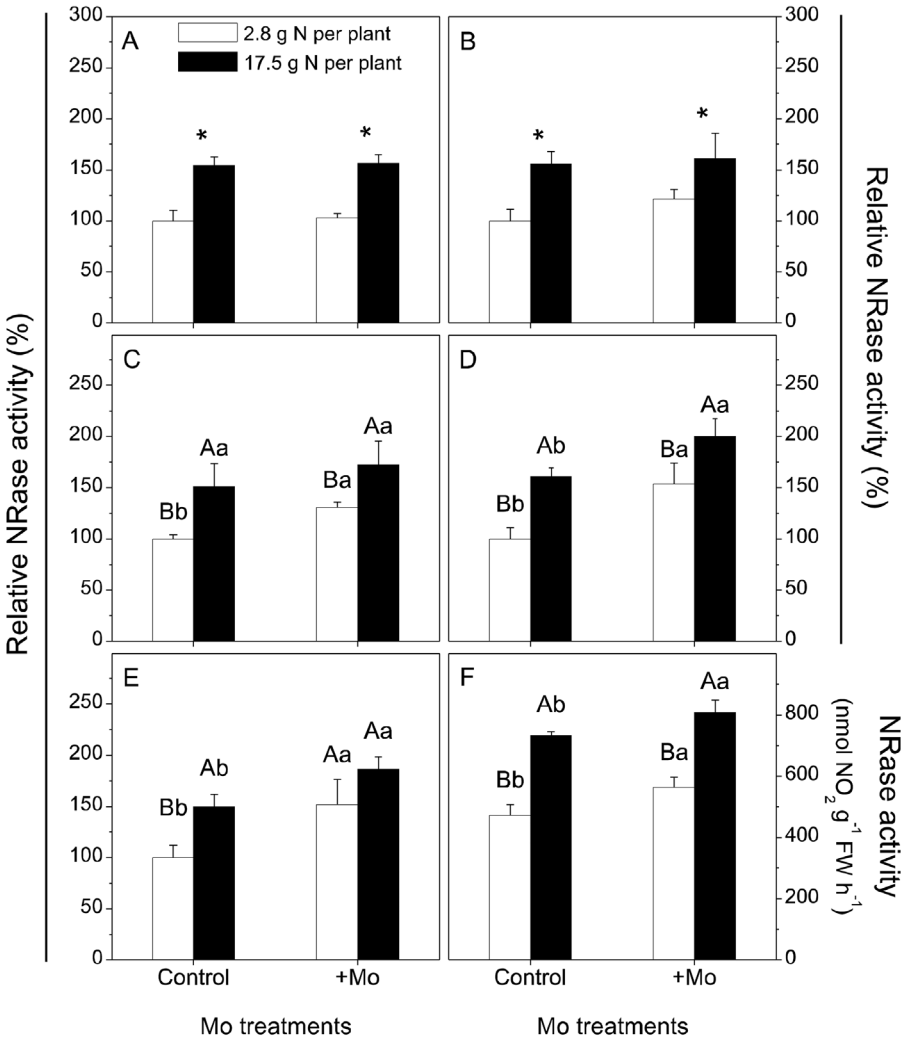

Fig. 8. Relative nitrate reductase (NRase) activity in roots of young sweet orange trees grown with low and high nitrogen (N) supply, at 1 (A), 4 (B), 9 (C), 15 (D) and 30 days (E) and mean NRase activity for pooled data (F) after foliar application of molybdenum (Mo) (Experiment 2). Vertical lines represent standard mean of the deviation (n = 5); N levels: means (n = 5 or 15) followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$). *: significant at 5% for between the N levels.Mo treatments: means (n = 5 or 10) followed by different lowercase letters are significantly different by Tukey's test ($p < 0.05$).

Table 3
Content of nitrogen (N) in young sweet orange trees grown with low and high nitrogen supply, 30 days after receiving foliar sprays with molybdenum (Mo) (Experiment 2).

Molybdenum (Mo) g L^{-1}	Nitrogen (N) g per plant	Roots g of N per plant	Trunk	Twigs	Leaves	Total
0.0 (Control)	2.8	0.53 ± 0.03[a] Ba[b]	0.38 ± 0.06	0.30 ± 0.02 Ba	0.83 ± 0.05 Ba	2.03 ± 0.06 Ba
	17.5	1.42 ± 0.21 Ab	0.76 ± 0.12	0.74 ± 0.05 Ab	1.65 ± 0.20 Ab	4.58 ± 0.38 Ab
	Mean values	0.98	0.57	0.52	1.24	3.30
0.60[c]	2.8	0.66 ± 0.09 Ba	0.35 ± 0.03	0.32 ± 0.05 Ba	0.80 ± 0.07 Ba	2.13 ± 0.16 Ba
	17.5	1.83 ± 0.12 Aa	0.84 ± 0.13	0.92 ± 0.06 Aa	2.05 ± 0.11 Aa	5.63 ± 0.24 Aa
	Mean values	1.24	0.59	0.62	1.24	3.88
F test[d]						
Mo		ns	ns	ns	ns	ns
N		**	**	**	**	**
Mo*N		*	ns	*	*	*

[a] Standard deviation of the mean.
[b] N levels: means (n = 5 or 15) followed by different uppercase letters are significantly different by Tukey's test ($p < 0.05$). Mo treatments: means (n = 5 or 10) followed by different lowercase letters are significantly different by Tukey's test ($p < 0.05$). *: significant at 5% for between the N levels.
[c] Sum of "–Mo side" and " + Mo side".
[d] F test in the ANOVA of Mo vs. N for each parameter evaluated. ns: not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$.

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