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Effects of nitrogen levels on growth, photosynthesis, and nutrient interactions in sour orange seedlings grown in nutrient solution

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ABSTRACT

Purpose: Nitrogen (N) plays a crucial role in citrus growth, but its deficiency or excess can disrupt nutrient balance and physiological functions in plant. This study investigated how varying N levels (2, 4, 8, 16, and 32 mM) affect growth, photosynthesis, root morphology and nutrient interactions in sour orange (*Citrus aurantium* L.) seedlings. **Research Method:** Seedlings were grown in nutrient solutions with different N concentrations. Biomass, photosynthetic efficiency (Fv/Fm, PI), chlorophyll content, nutrient uptake, and nitrate reductase activity were analyzed. **Findings:** Maximum and significant plant dry weight (4.95 g), root length (51.5 cm), chlorophyll content (8.6 mg g^{-1} FW), and photosynthetic efficiency (Fv/Fm = 0.73; PI = 4.2) occurred at 16 mM N compared to 2 mM N. In contrast, N deficiency (2–8 mM) reduced growth and photosynthetic performance, while N toxicity (32 mM) decreased plant biomass by 50%, impaired chlorophyll synthesis, and disrupted photosystem II efficiency (Fv/Fm = 0.55). Excessive N (32 mM) altered nutrient homeostasis, increasing root Ca and K concentration by 0.82% and 2.49% respectively but reducing their translocation to shoots, elevating the Ca/K ratio a key indicator of K deficiency risk in calcareous soils. Nitrate reductase activity declined under toxicity, reflecting suppressed N assimilation. Root N/Ca ratio decreased with increasing in N concentration and Ca/K ratio of root in N_{16} and N_{32} treatments was higher than that in other N treatments. Phenolic compounds accumulated in roots under high N, suggesting oxidative stress mitigation. **Research limitations:** No limitations were found. **Originality/Value:** Excessive N disrupts nutrient balance, photosynthetic inhibition, and growth suppression while 16 mM N optimizes sour orange growth.

Keywords:
Nitrogen deficiency, Nitrogen toxicity, Nutrient imbalance, Photosynthetic efficiency

INTRODUCTION

Nitrogen (N) is one of the most critical nutrients for plant growth, constituting approximately 1 to 5% of a plant's dry weight, and plants have the highest need for this element after carbon. It is a fundamental component of essential biomolecules such as proteins, nucleic acids, chlorophyll, amino acids, plant hormones, and secondary metabolites and playing a pivotal role in plant development and metabolism (Marschner, 2011). Nitrogen plays a key role in plant nutrition, and its deficiency not only reduces the productivity of the plant, but also leading to stunted growth, chlorosis, reduced leaf size, and diminished yield (Huang et al., 2021). To meet the high demand for nitrogen in agriculture, substantial amounts of nitrogen fertilizers are applied globally, with an estimated 108,000 tons used in 2022 alone (FaoStat, 2022). Despite this, only 40–50% of applied nitrogen is typically utilized by plants, with the remainder lost through leaching, volatilization, or runoff, contributing to environmental pollution and economic inefficiency (Sylvester-Bradley & Kindred, 2009; Marschner, 2011). Consequently, optimizing nitrogen management is crucial for both sustainable agriculture and environmental protection.

Nitrogen uptake and utilization in plants are regulated by complex physiological mechanisms that respond to internal plant requirements rather than solely to external nitrogen availability (Akhtar et al., 2024; Cerezo et al., 2007). For instance, studies on citrus seedlings have demonstrated that low nitrogen concentrations can be more effective in promoting vegetative growth than high concentrations in Hamlin orange grafted on Cleopatra mandarin rootstock (Maust & Williamson, 1994). They showed that the optimal N concentration in the nutrient solution for the growth of orange is about 10 mg L^{-1}, and at a concentration of 7 mg L^{-1}, the growth rate of the plant is greatly reduced (Maust & Williamson, 1994). Although the limit of N deficiency in the leaves of citrus trees is below 2.2%, its optimal range is 2.5 to 2.7 and a concentration above 3% is considered the limit of N toxicity in these trees, but the optimal concentration of N for vegetative growth and performance of citrus trees is determined based on the yield of the previous year and leaf analysis (Albrigo et al., 2019). Sorgonà et al., (2006) reported that N deficiency and excess conditions in pear seedlings not only affect vegetative growth and changes in N concentration in roots, stems and leaves, but also affect the concentration of other mineral elements of the plant (Sorgonà et al., 2006). In addition to the synergistic and antagonistic effects on other mineral elements, the N content of the plant can also affect their absorption, distribution and consumption (Feil & Bänziger, 1993). The results of a research on pear showed that under the conditions of N deficiency and toxicity root activity change and this can affect the absorption of other elements, so that under the influence of N concentration, the concentration of micro and macronutrients changes (Chen et al., 2018; Deng et al., 2019), on the other hand, N deficiency can affect the balance and homeostasis of other elements and therefore some of its adverse effects on growth intensify, the biosynthesis of photosynthetic pigments and the electron transport chain in the leaf (Huang et al., 2021). Such findings underscore the need for a comprehensive understanding of nitrogen's role in plant physiology and its interactions with other nutrients.

Sour orange (*Citrus aurantium* L.) is the most widely used citrus rootstock in the world but susceptibility to Tristeza virous has limited its use in many countries and under many conditions. Trees on this rootstock have medium growth vigor, large shoots and high total soluble solids and total acid content in the juice. This rootstock has a wide and deep root system and is relatively drought tolerant, tolerates heavy soils with poor drainage and Phytophthera infection, shows good tolerance to high pH and high soil salinity, but is sensitive to citrus nematode and blight (Albrigo et al., 2019). On the other hand, the behavior of this plant in different soil conditions, such as pH, is different from other common citrus

rootstocks (Mirsoleimani et al., 2023). Having a correct understanding of the plant's physiological responses to deficiency, excess and optimal N as well as the interaction of this element with other mico and macronutrients in the plant can help us in managing the use of nitrogenous fertilizers and increase their efficiency of use, thus avoiding risks and reduce the impact on the environment.

This study aimed to investigate the effects of varying nitrogen (N) concentration in the nutrient solution for maximizing the growth, photosynthetic efficiency, nutrient uptake and balance of macro- and micronutrients in sour orange seedlings.

It is hypothesized that an intermediate nitrogen concentration will enhance macro/micronutrient uptake, maximizes sour orange seedling growth and improve nutrient balance, while lower concentrations restrict development and higher concentrations cause toxicity, altering nutrient partitioning and photosynthetic performance. By elucidating these responses, this research seeks to provide insights into optimizing nitrogen fertilization strategies for sour orange, enhancing nutrient use efficiency, and minimizing environmental impacts.

MATERIALS AND METHODS

Plant materials and treatments

Sour orange (*Citrus aurantium* L.) seedlings were used in these experiments. Seeds were obtained from ripe fruits of "Common" sour orange trees located in the College of Agriculture and Natural Resources of Darab. Seeds were soaked with water for 24 h and surface sterilized for 10 min in a 5% sodium hypochlorite solution and 1.5 min in 70% ethanol solution then rinsed repeatedly by deionized water. Then the sterilized seeds were placed on a wet cotton cloth and placed in an incubator with a temperature of 30°C and a relative humidity of 75% (Albrigo et al., 2019). After 3 weeks, the germinated seeds were transferred to 5 L plastic pots containing sterilized perlite and kept at a temperature of 30°C and normal light intensity. The seedlings were first irrigated with distilled water, and at the 4-leaf stage, they were irrigated once with Hoagland nutrient solution of one-fourth concentration. After 45 days and at the stage of 6 to 8 leaves, when the height of the seedlings reached 10 to 12 cm, 80 uniform and equal sized seedlings were selected and four seedlings were transferred to each 7 L plastic pots. The experiment was carried out at 30/20°C, and 50% relative humidity, with a normal light intensity from September 2022 to January 2023 in the greenhouse of College of Agriculture and Natural Resources of Darab. The treatments consisted of five nitrogen (N) concentrations: 2, 4, 8, 16 and 32 mM N, supplied as $Ca(NO_3)_2$ and $NaNO_3$ in nutrient solution. These concentrations were selected to represent a gradient from deficiency (2–8 mM) to sufficiency (16 mM) and toxicity (32 mM). Full strength Hoagland's nutrient solution was used as the base for treatments N nutritive solutions, contained 14 mM $NaNO_3$, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1 mM KH_2PO_4, 2 mM $MgSO_4 \cdot 7H_2O$, 0.83 mg L^{-1} KCl, 6.2 mg L^{-1} H_3BO_3, 22.3 mg L^{-1} $MnSO_4$, H_2O, 8.6 mg L^{-1} $ZnSO_4$, $7H_2O$, 0.025 mg L^{-1} $CuSO_4$, $5H_2O$ 0.025 mg L^{-1} $CaCl_2$, 0.25 mg L^{-1} Na_2MoO_4, and 50 mM Fe-EDTA (Hoagland & Arnon, 1950). The solution was ventilated for 20 min every 3 h by ventilation pump, and replaced every two weeks. The pH of all nutrient solutions was adjusted to 6 with 0.1 M KOH.

Leaf pigments and chlorophyll fluorescence measurement

At the end of the experiment, the greenness of fully expanded young leaves (third and fourth from the tip of the shoot) (Six-month leaves) was measured by SPAD-502 (502, Minolta, Japan) and chlorophyll fluorescence parameters were determined by manual chlorophyll meter (OS30P, OPTI-Sciences Co., USA) as described by Warren, (2008). To determine

fluorescence parameters, leaves (four leaves of each plant) (n=4) were dark-adapted for 25 minutes using leaf clips and then chlorophyll fluorescence parameters including photosynthetic performance index (PI) and variable fluorescence ($F_v = F_m - F_o$) to maximal fluorescence (F_v/F_m), were recorded. F_v/F_m (maximum quantum yield of PSII) representing the photochemical efficiency of photosystem II (PSII) in unstressed conditions. Values around 0.80 indicate optimal photosynthetic function, while lower values suggest photoinhibition or stress. PI is a multi-parametric indicator (combining F_v/F_m, light absorption, and electron transport efficiency) that reflects overall photosynthetic performance. Higher PI values correlate with greater stress tolerance (Murchie & Lawson, 2013). For chlorophyll extraction 100 mg fresh leaves (n=4) were ground for 5 min in 2 mL of 80% acetone with a mortar and pestle. The homogenate was centrifuged at 6,000 rpm for 10 min, transferred into a micro-tube, and adjusted to a set volume with 80% acetone. The absorbance of the extract was measured at 480, 510, 663 and 645 nm with spectrophotometer (UV-1800, Shimadzu, Japan) (Lichtenthaler & Wellburn, 1983).

Plant growth parameter measurement

At the end of the experiment (after 10 weeks of treatment), plants of each treatment (n=4) were harvested and divided into leaf, stem, and root. Leaf area (cm^2) was measured by a leaf area meter (Li-3100C; LI-COR Biosciences Inc., Lincoln, NB, United States). The fresh weight of shoots and roots were measured by an electronic analytical balance, and the sample oven-dried for 3 days at 70°C and their dry weight were measured.

Root morphology assessment

Seedlings were sampled in each treatment (n=4), root and shoot separated and root samples were scanned using an Epson digital scanner (Expression 10000XL 1.0, Epson Inc., Japan), and the root images were analyzed with GiaRoot software (Galkovskyi et al., 2012). For each treatment, average root diameter and total root length were calculated.

Nutrient analysis

At the end of the experiment, the root, stem and leaves of the plants (n=4) were harvested separately and rinsed with deionized water, and oven-dried for 3 days at 70°C. Total N of roots and shoots was determined by micro-Kjeldahl method (Bremner, 1996). The dried root, stem and leaf were powdered using an electric mill, ashed at 550°C, acid dissolution and phosphorus (P) concentration in the extracts was determined by the yellow color method (Jackson, 2005), the concentrations of calcium (Ca) and magnesium (Mg) determined by complexometric titration using EDTA (ethylenediaminetetraacetic acid) (Wenzel et al., 2013), sodium (Na) were determined by a flame photometer (ELE, UK) and iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) were determined by an atomic absorption spectrophotometer (AAS; PG 990, PG Instruments Ltd. UK).

Nitrate reductase (NR) activity

The nitrate reductase (EC 1.7.1.1) activity was determined according to a method described by Li et al. (2017). Leaf or root samples (n=4) were prepared between 9 and 11 a.m. and 0.1 g of fresh root or leaf tissue was prepared in equal-sized pieces and mixed with 0.1 mol of sodium phosphate buffer (pH=7.5) and 0.2 mol of potassium nitrate in a test tube. Then the mixture was placed in a hot water bath with a temperature of 37 °C for one hour. To inactivate the mixed enzymes, they were placed at 100 °C for 5 min and vortexed again. For colorimetry, 1 ml of 0.2% 1-naphthylamine and 2 ml of 1% sulfanilamide were added to the

samples and after 15 minutes in the dark, the absorbance of each sample was read by a spectrophotometer at a wavelength of 540 nm.

Determination of total phenolics

Extraction and quantification of phenolics were performed according to Misan et al. (2011) with some modifications. Total phenolics were determined using Folin-Ciocalteu reagent and a microplate reader (Bio Tek ELx808) at 750 nm and the results were expressed as gallic acid equivalents.

Statistical analysis

The experiment was conducted in a completely randomized design to investigate the impact of five different N treatments (2, 4, 8, 16 and 32 mM) on a specific rootstock, with each treatment having four replicates and four plants per replicate. Statistical analyses of the data were performed using the SAS statistical software, data are presented as means ± SD (n=4) and the differences were statistically compared by employing the Tukey HSD post hoc test with a significance level of $P < 0.05$. All graphs were provided in Microsoft Office Excel 2013. Pearson correlation coefficient between different characteristics was obtained by SPSS 20.0 software

RESULTS

Growth parameters and plant appearance

Results indicated that increasing N in the nutrient solution from 2 to 8 mM had no effect on shoot dry weight of sour orange but N_{16} increased (46%) and N_{32} decreased it (37%) significantly and highest (2.67 g) and lowest (1.46 g) root dry weight were observed in N_{16} and N_{32}, respectively (Table 1). Plant dry weight was 3.27 g in N_2 and increased to 4.95 g in N_{16} and decreased to 2.44 g in N_{32}. In addition, root to shoot ratio was not affected by N treatments from 2 to 16, but significantly increased to 1.49 in N_{32}. N_{32} treatment also decreased leaf number from 33 to 17, while leaf area was increased with N_4, N_8, N_{16} and N_{32} treatments. N_{16} increased plant root length to 51.5 (27% increase), while N_{32} had no effect on it. The highest root diameter (1.34 mm) was observed in N_{32} (Table 1). Leaf greenness index (SPAD) for N_2 was 51.8 and increased with N_4, N_8 and N_{16}, however, N_{32} had no significant effect on it (48.3).

Table 1. Some plant morphological properties as influenced by different levels of nitrogen in nutrient solution.

Treatment	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Plant dry weight (g plant^{-1})	Root/Shoot	Leaf No.	Leaf Area (cm^2 plant^{-1})	Root length (cm plant^{-1})	Root diameter (mm)	SPAD
N_2	1.56±0.05^b	1.71±0.04^c	3.27±0.06^c	1.10±0.04^b	33±0.75^a	1.58±0.04^c	40.47±3.70^{ab}	1.26±0.03^c	51.8±1.6^b
N_4	1.66±0.01^b	2.19±0.04^b	3.85±0.04^b	1.32±0.02^{ab}	37±1.28^a	1.69±0.04^{bc}	37.21±1.38^c	1.27±.020^{bc}	60.6±0.3^a
N_8	1.56±0.03^b	1.89±0.06^c	3.45±0.06^c	1.21±0.05^{ab}	33±2.17^a	1.81±0.03^{ab}	38.62±2.45^c	1.32±0.01^{ab}	58.3±0.3^a
N_{16}	2.28±0.04^a	2.67±0.06^a	4.95±0.10^a	1.17±0.01^{ab}	33±1.44^a	1.96±0.04^a	51.50±1.45^a	1.28±0.03^{bc}	57.0±2.2^a
N_{32}	0.98±0.06^c	1.46±0.06^d	2.44±0.11^d	1.49±0.08^a	17±2.10^b	1.79±0.07^{ab}	47.56±4.42^{ab}	1.34±0.01^a	50.2±2.6^b

Data are presented as means ± SE of four replicates (n=4) and means followed by different small letters in each column are significantly different at $p < 0.05$ by Tukey HSD. Plants were supplied with 2, 4, 8, 16 and 32 mM N in nutrient solution (N_2, N_4, N_8, N_{16} and N_{32}).

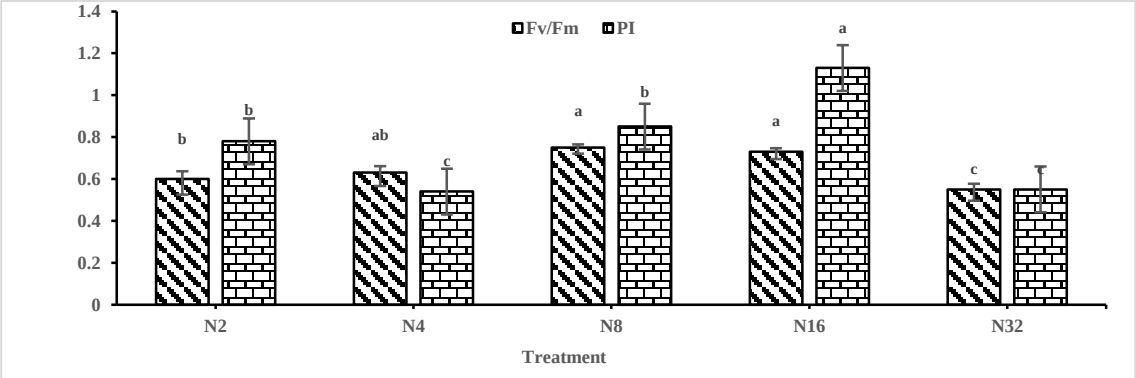

Fig. 1. Fv/Fm and PI parameters for sour orange seedling leaves as influenced by 2, 4, 8 ,16 and 32 mM N in nutrient solution (N_2, N_4, N_8, N_{16} and N_{32}) (n=4). Significant differences ($P < 0.05$) were determined by different lowercase letters according to Tukey HSD test.

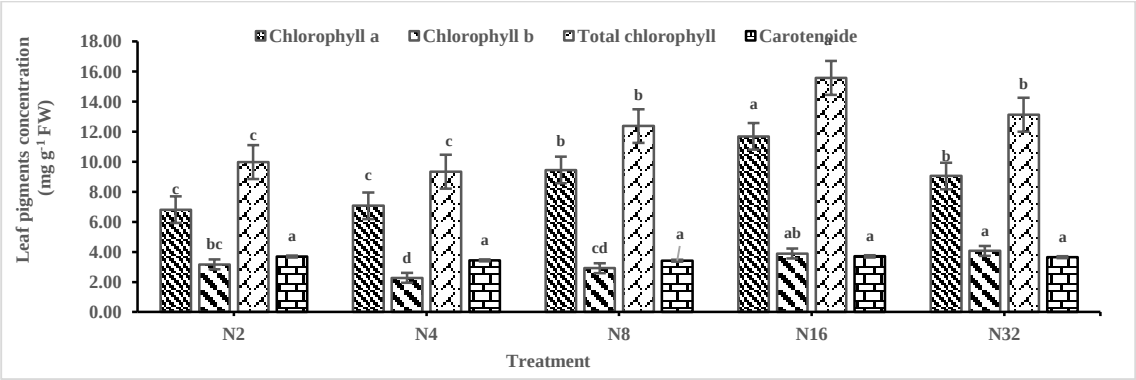

Fig. 2. Leaf chlorophyll and cartenoid concentration in sour orange seedling as influenced by 2, 4, 8 ,16 and 32 mM N in nutrient solution (N_2, N_4, N_8, N_{16} and N_{32}) (n=4). Significant differences ($P < 0.05$) were determined by different lowercase letters according to Tukey HSD test.

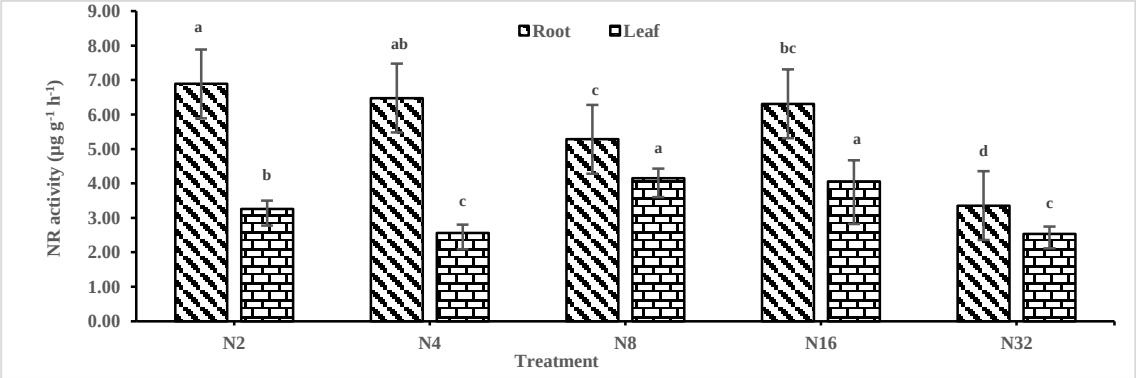

Fig. 3. Root and leaf nitrate reductase (NR) activity as influenced by 2, 4, 8 ,16 and 32 mM N in nutrient solution (N_2, N_4, N_8, N_{16} and N_{32}) (n=4). Significant differences ($P < 0.05$) were determined by different lowercase letters according to Tukey HSD test.

Fig.1 indicated the influence of N on PSΠ efficiency index (Fv/Fm) and PI parameters in leaves. The Fv/Fm was 0.60 in N_2 and significantly increased to 0.74 and 0.73 in N_8 and N_{16}, respectively, but decreased in N_{32} to 0.55. The PI value was increased in N_{16} and decreased in N_4 and N_{32}. The content of chlorophyll *a* in leaves increased with N addition and reached to the highest value in N_{16} and then decreased in N_{32} (Fig. 2). Leaf chlorophyll *b* content was also increased with N addition, but it showed no change in N_{32}. In addition, total chlorophyll in N_2 was 3.0 mg g^{-1} FW and increased to 8.6 mg g^{-1} FW in N_{16} and then decreased to 6.2 mg g^{-1} FW in N_{32}. No change was observed in leaf carotenoid content with N application (Fig. 2).

Nitrate reductase (NR) enzyme activity and total phenolic compounds

Nitrate reductase enzyme activity in the root was lowest (0.32 µg $g^{-1}h^{-1}$) at N_{32} and increased more than two-fold with other treatments (Fig. 3). In the shoot, enzyme activity was highest in N_8 and N_{16} (0.42 and 0.41 µg $g^{-1}h^{-1}$) and decreased significantly to 0.27-0.33 µg $g^{-1}h^{-1}$ in other treatments. Although the total root phenol concentration did not change significantly with the increase of the N concentration of the nutrient solution from 2 to 4 and 16 mM, the concentration of total root phenol in N_{16} and N_{32} increased and reached to 1.8 and 2.4 mg g^{-1} FW, respectively (Fig. 4).

Nutrient concentration and its ratio in root and shoot

By increasing the N concentration from 2 to 4 and 8, the shoot N concentration showed no change and it significantly increased to 2.80 and 3.15% in N_{16} and N_{32}, respectively. But in the roots, the N concentration decreased significantly with the increase of N concentration from 2 to 4 and 8, and increased again in N_{16} and N_{32}, reaching the highest value (2.43%) in N_{32}, so the lowest value was observed in N_4 (1.67%). The ratio of N in shoot to root was 1.11 in N_2 treatment and increased to 1.45 in N_4 and N_8 and decreased to 1.30 in N_{16} and N_{32} (Fig. 5).

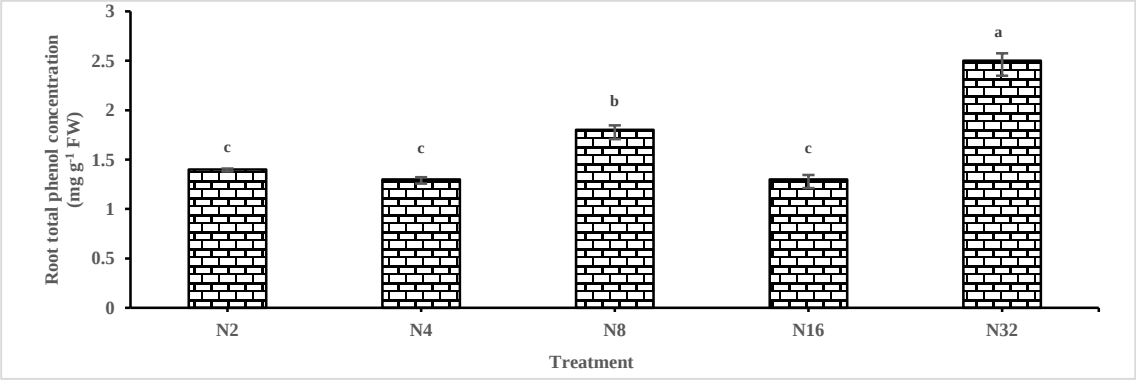

Fig. 4. Root total phenol concentration as influenced by 2, 4, 8 ,16 and 32 mM N in nutrient solution (N_2, N_4, N_8, N_{16} and N_{32}) (n=4). Significant differences ($P < 0.05$) were determined by different lowercase letters according to Tukey HSD test.

Table 2. Shoot macronutrients concentration of sour orange seedlings as influenced by different levels of nitrogen in nutrient solution.

	Shoot macronutrient concentration (% DW)					
Treatment	N	P	K	Ca	Mg	Na
N_2	2.42±0.06^c	0.25±0.01^a	3.25±0.10^a	1.70±0.04^b	0.30±0.01^b	0.39±0.02^c
N_4	2.43±0.03^c	0.24±0.01^a	3.24±0.07^a	1.72±0.03^{ab}	0.38±0.03^a	0.37±0.01^c
N_8	2.55±0.01^c	0.24±0.01^a	3.05±0.09^a	1.59±0.05^{bc}	0.38±0.01^a	0.40±0.01^c
N_{16}	2.80±0.06^b	0.24±0.01^a	2.63±0.07^b	1.41±0.05^c	0.22±0.01^c	1.10±0.09^b
N_{32}	3.15±0.01^a	0.26±0.01^a	2.57±0.04^b	1.89±0.04^a	0.30±0.02^b	2.54±0.05^a

Table 3. Root macrnutrients concentration of sour orange seedlings as influenced by different levels of nitrogen in nutrient solution.

	Root macronutrient concentration (% DW)					
Treatment	N	P	K	Ca	Mg	Na
N_2	2.18±0.06^{b}	0.27±0.01^{a}	2.16±0.05^{b}	0.42±0.01^{c}	0.33±0.01^{bc}	0.29±0.02^{b}
N_4	1.67±0.01^{c}	0.27±0.01^{a}	2.20±0.06^{b}	0.44±0.01^{c}	0.37±0.01^{ab}	0.28±0.01^{b}
N_8	1.75±0.03^{c}	0.27±0.01^{a}	2.22±0.05^{b}	0.44±0.02^{c}	0.40±0.02^{a}	0.27±0.02^{b}
N_{16}	2.19±0.04^{b}	0.26±0.01^{a}	2.20±0.05^{b}	0.67±0.02^{b}	0.30±0.01^{c}	0.28±0.02^{b}
N_{32}	2.43±0.02^{a}	0.28±0.01^{a}	2.49±0.04^{a}	0.82±0.02^{a}	0.40±0.02^{a}	0.63±0.02^{a}

Data are presented as means ± SE of four replicates (n=4) and means followed by different small letters in each column are significantly different at $p < 0.05$ by Tukey HSD. Plants were supplied with 2, 4, 8, 16 and 32 mM N in nutrient solution (N_2, N_4, N_8, N_{16} and N_{32}).

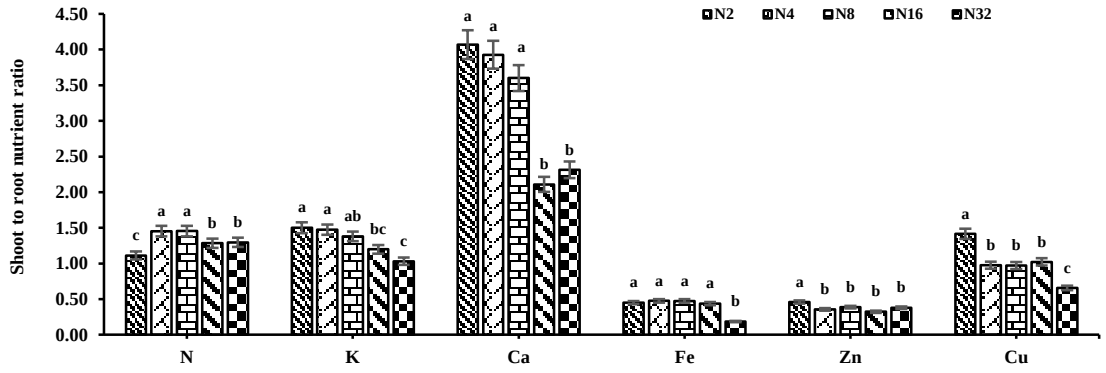

Fig. 5. Shoot to root nutrient ratio in sour orange as affected by 2, 4, 8 ,16 and 32 mM N in nutrient solution (N_2, N_4, N_8, N_{16} and N_{32}) (n=4). Significant differences ($P < 0.05$) were determined by different lowercase letters according to Tukey HSD test.

Phosphorus concentration in shoot and root was 0.24-0.26% and 0.26-0.28%, respectively and showed no change with N treatment (Table 2, 3). In addition, shoot to root P ratio was not affected by N treatments. Potassium shoot concentration was 3.05-3.25%, with no significant difference in N_2, N_4 and N_8, but its value decreased to 2.63 and 2.57% in N_{16} and N_{32} treatments, respectively (Table 2). Nitrogen treatments from 2 to 16 mM had no effect on root K concentration, but it increased to 2.49% in N_{32} (Table 3). Nitrogen treatments decreased shoot to root K ratio from 1.50 in N_2 to 1.03 in N_{32} treatment (Fig. 5). Shoot Ca concentration was 1.70% in N_2 and decreased to 1.41% in N_{16}, increased again in N_{32} and reached to the highest value (1.89%) (Table 2). Root Ca concentration was 0.42-0.44% in N_2, N_4 and N_8 with no significant difference, but it significantly increased in N_{16} and N_{32} to 0.67 and 0.82%, respectively (Table 3). On the other hand, shoot to root Ca ratio in N_2, N_4 and N_8 was significantly higher than N_{16} and N_{32} treatments (Fig. 5). Shoot Mg concentration was the highest in N_4 and N_8 (0.38%) and decreased to N_2 and N_{32} (0.30%); the lowest value was observed in N_{16} treatment (0.22%) (Table 2). N_8 and N_{32} treatments increased the Mg concentration in plant root to 0.40%; while the lowest value was observed in N_{16} (0.30%). Ratio of Mg in shoot to root was not affected by N treatments. N_{16} and N_{32} treatments decreased significantly Fe, Mn and Cu concentration in plant shoot to 68-69 mg kg^{-1}, 432-495 mg kg^{-1} and 9.5 mg kg^{-1}, respectively (Table 4). In addition, the lowest significant value of root Zn concentration was observed for N_{16} treatment (42 mg kg^{-1}); while the N_2 and N_{32} had the highest values (197 mg kg^{-1}) (Table 4). The N_{16} treatment decreased the concentration of Fe, Mn, Zn and Cu in plant root to 156, 1037, 132 and 9.3 mg kg^{-1}, respectively (Table 4). In addition, the highest values were observed in N_{32}. Shoot to root Mn ratio was not affected by N treatments, while for Fe and Cu, N_{32} treatment significantly decreased their shoot to root

ratios (Fig. 5). Shoot to root Zn ratio in N_2 treatment was 0.46 and significantly decreased in other N treatments. As shown in Figure 5, nutrient ratio in shoot and root of sour orange was affected by N concentration in the growth media. Nitrogen treatment from 2 to 8 mM had no effect on the N/P and N/Ca and N/Mg ratios in the shoots, while these ratios increase in N_{16} and N_{32} (Fig. 6A). The ratio of Ca to K in N_{32} treatment was significantly higher than other N treatments (0.7 versus 0.5). The ratios of N/P, N/K and N/Mg of plant roots in N_4 and N_8 were significantly lower than those in N_2, N_{16} and N_{32}. Root N/Ca ratio decreased with increasing in N concentration in the growth media. Ca/K ratio of root in N_{16} and N_{32} treatments was significantly higher than that in other N treatments (Fig. 6B).

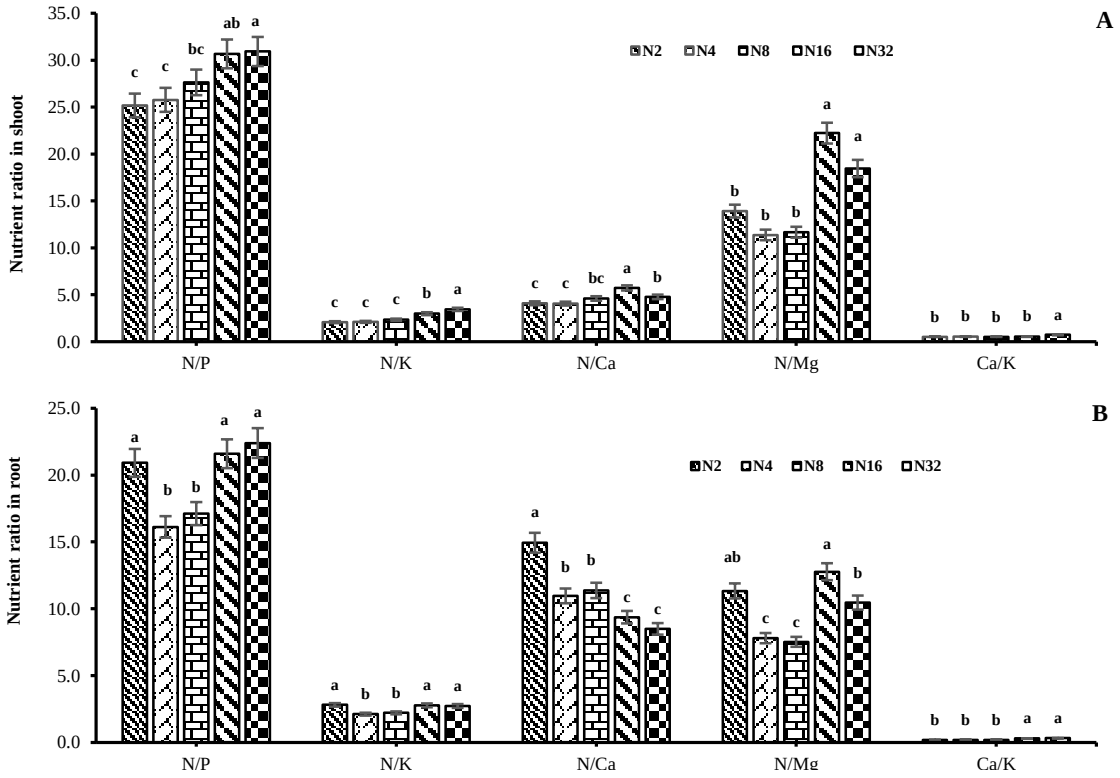

Fig. 6. Nurient ratio in shoots (A) and roots (B) of sour orange seedlings as affected by 2, 4, 8 ,16 and 32 mM N in nutrient solution (N_2, N_4, N_8, N_{16} and N_{32}) (n=4). Significant differences ($P < 0.05$) were determined by different lowercase letters according to Tukey HSD test.

Table 4. Shoot and root micronutrients concentration of sour orange seedlings as influenced by different levels of nitrogen in nutrient solution.

	Shoot micronutrient concentration (mg kg^{-1} DW)				Root micronutrient concentration (mg kg^{-1} DW)			
Treatment	Fe	Mn	Zn	Cu	Fe	Mn	Zn	Cu
N_2	80.4±0.57^{b}	576±11.99^{a}	197±2.13^{a}	14.0±0.30^{a}	178±3.35^{bc}	1372±23.73^{a}	430±6.28^{c}	9.9±0.14^{cd}
N_4	87.8±1.03^{a}	600±10.46^{a}	161±1.51^{c}	10.9±0.26^{b}	185±7.19^{c}	1367±15.80^{a}	451±5.81^{bc}	11.1±0.26^{b}
N_8	80.5±1.33^{b}	643±20.93^{a}	186±0.64^{b}	10.7±0.29^{bc}	170±3.57^{bc}	1370±23.23^{a}	479±7.44^{b}	11.1±0.17^{b}
N_{16}	68.3±1.31^{c}	432±19.45^{b}	42±2.42^{d}	9.5±0.29^{c}	156±3.60^{c}	1037±17.80^{c}	132±10.87^{d}	9.3±0.30^{d}
N_{32}	69.4±0.22^{c}	495±17.45^{b}	197±1.75^{a}	9.5±0.25^{cd}	376±10.29^{a}	1255±39.44^{b}	521±8.51^{a}	14.6±0.42^{a}

Data are presented as means ± SE of four replicates (n=4) and means followed by different small letters in each column are significantly different at $p < 0.05$ by Tukey HSD. Plants were supplied with 2, 4, 8, 16 and 32 mM N in nutrient solution (N_2, N_4, N_8, N_{16} and N_{32}).

DISCUSSION

Nitrogen (N) concentration in the growth medium significantly influences nutrient uptake, translocation, and physiological responses in sour orange, with effects varying across N levels. The optimal leaf N range for citrus (2.3–2.8%) (Liu et al., 2021) aligns with classic thresholds (Smith, 1966), where deficiency (<2.2%) and toxicity (>3.0%) impair growth and the leaf chlorosis appears when the leaf N concentration is in the range of 1.25 to 1.75% (Chapman, 1968). In this study, leaf N concentrations reflected these ranges: N_2 and N_4 were deficient, N_8 and N_{16} were optimal, and N_{32} was toxic.

Under low N (N_2–N_8), shoot N remained stable, root N was decreased in N_4 and N_8 compared to N_2 (Table 2). Generally, these N treatments do not significantly impact the concentration of other nutrients except Mg, Zn and Cu that increased in shoots and roots (Table 2, 3 & 4). In optimal N conditions (N_{16} with maximum plant dry weight), there is a substantial increase in N absorption in the root and shoot, resulting in decreased concentrations of nutrients including K, Ca, Mg, Fe, Mn, Zn, and Cu in shoots and Mg, Mn, Zn and Cu in roots. Toxicity condition (N_{32} treatment) associated with an increase in roots and shoots N, a relative increase in shoot Ca, Mg and Zn and root K, Ca, Mg, Fe, Mn, Zn and Cu concentration (Table 2, 3 & 4). Mg, Zn, and Cu concentration increased in shoots and roots at low N (Tables 2, 3), possibly due to dilution effect, ion competition for absorption, translocation of nutrients from roots to shoots, accumulation of nutrients in roots, and plant toxicity (concentration effect) (Dieter Jeschke & Hartung, 2000; Jarrell & Beverly, 1981; Marschner, 2011).

By increasing the N level in the nutrient solution from 2 mM (N_2) to 8 mM (N_8), the plant dry weight (both shoot and root) and shoot N showed no change (Table 1) but root N decreased in N_4 and N_8 treatments compared to N_2. At low N level (N_2), there was a considerable decrease in the N translocation from the root to shoot, as concluded from root to shoot nutrient ratio in Fig. 5, leading to N accumulation in the root. Nitrogen accumulation in the root tissue in the N deficiency conditions may be due to the plant adaptation mechanisms. Reducing the growth of the branches and leaves and continuing the growth of the roots causes the redistribution of N from the shoots to the roots. On the other hand, in the N deficiency condition and in order to compensate it and reduce its adverse effects, the growth and activity of the root increases and all these factors will cause N accumulation in the root (Lopez et al., 2023). Qin et al. (2019) concluded that under N deficiency, rapeseed roots become longer and softer, with denser cells in the meristematic zone.

The increase in N levels does not alter the concentration of other nutrients (excluding Zn and Cu) in the roots and shoots (Table 4). The absorption and translocation of these nutrients remain unaffected by the increase in N levels. The findings suggest that increasing N levels minimally affects Zn and Cu absorption but influences their translocation, resulting in their accumulation in the roots (78% and 56%, respectively). Garcia-Gomez et al. (2023) also concluded that increasing of N from 4 to 8 mM in the culture medium resulted to lower Zn and Cu concentration in the mature leaves and roots of *Citrus macrophylla*. A decrease in Zn and Cu concentration in plant with increase in N from 2 to 8 mM may be due to the increase in the phenolic compounds in root and its exudation to solution media that react with soluble Cu and Zn (Chen et al., 2022; Chen et al., 2020) and inhibits its uptake by plant roots and consequently translocation to the aerial parts. In confirmation of this, the results of our research (Fig. 3) also showed that the highest concentration of total phenol in the root was in N_{32} treatment.

By elevating N level in the nutrient solution, the plant dry weight (both shoot and root), particularly the aerial part, shows a significant increase (Table 1). Similar result was obtained by Garcia-Gomez et al. (2023) for *Citrus macrophylla*, by addition of 4, 8, and 16 mM N to

the culture medium; they concluded that after 28 days of N application, the stem length, the number of new leaves and the relative growth of the *Citrus macrophylla* showed a significant increase in the 16 mM treatment. Chen et al. (2020) also observed an increase in the dry weight of *Citrus macrophylla* after nitrate addition up to 4 mM and no significant change with 8 mM nitrate addition. These contradictory results may be due to the difference in plant variety and N source. It seems that citrus is sensitive to ammonia compared to nitrate (Chen et al., 2020). It can be said that the relatively lower N increase in the aerial parts can be attributed to the dilution effect caused by the higher dry weight. Furthermore, reaching the optimal N concentration level leads to a reduction in the concentration of other nutrients in the shoot due to the dilution effect. It is important to also consider the adverse impacts of nitrate absorption on the uptake of certain nutrients. A decrease in P, Ca, and Mg concentrations and an increase in Mn, Cu and Zn concentrations in roots, stems, and leaves of sweet orange (*Citrus sinensis*) by increasing in N supply from 0 to 20 mM have been observed by Huang et al. (2021). In addition, Garcia Gomez et al. (2023) observed that, 8 mM N nutrition decreased the Ca, K, Mg, Fe and Zn concentration in the leaves of *Citrus macrophylla*, as compared with the 4 and 16 mM. They concluded that any changes in N content may alter the accumulation of other nutrients in leaves by affecting various mechanisms, including alteration in root system architecture, decreased expression of nutrient transporters, or changes in hormone production and signaling that ultimately leads to reduced water uptake (Nawaz et al., 2016). However, no changes in nutrients concentration have been observed by Huang et al. (2021) who concluded that in leaves of *Citrus sinensis*, N supply did not affect P, K, Mg, S, Fe, or B concentrations; however, they found an increase in the concentrations of Ca and Mg.

Investigating the status of nitrate reduction in roots through the measurement of nitrate reductase levels indicates that elevating N concentration up to optimal level (16 mM) decreases nitrate reduction in roots significantly (Fig. 3). Conversely, it appears that the increase N concentration in culture media has a more pronounced effect on nitrate reduction in leaves. Garcia- Gómez et al. (2023) for *Citrus macrophylla* indicated that with increasing in N level in culture media from 4 to 16 mM, a decrease in nitrate reductase was observed in leaves while N level had no effect on root nitrate reductase content.

Generally, in the 2-16 mM N levels, there was significant relationships between leaf N and plant dry weight (0.79**), leaf area (0.76**), root length (0.77**), Fv/Fm (0.57*), PI (0.76**) and total chlorophyll (0.84**). This suggests that leaf area may be considered as index of N status in sour orange leaves; because it may be determined by non-destructive (Sorgonà et al., 2006) and cheap techniques. Lawlor (2002) also concluded that leaf area is a parameter that is highly sensitive to variations in nitrate levels; it increases with the availability of nitrate until it reaches a saturation point. Under the 32 mM N in the culture media, a 50% reduction in plant dry weight leads to a high N concentration in plant (Table 1 & 2), potentially reaching toxic levels. This situation results in a concentration effect, causing an increase in the concentration of other nutrients in the plant. However, this increase is not proportionate to the 50% decrease in dry weight, indicating a reduction in the absorption of various nutrients under N toxicity conditions. Additionally, nutrients translocation reveals a disruption, leading to nutrients accumulation in the roots, particularly evident for K, Fe and Zn.

When plants are exposed to high nitrate levels, the accumulation of NO_3^- in the plant cells can lead to an abrupt electrochemical balance and increased osmotic pressure within the cells. To counteract this, plants often increase the uptake of K ions, an important osmotically active solute (Raddatz et al., 2020) and also helps balance the negative charge of nitrate ions (Raddatz et al., 2020). On the other hand, K is an essential cofactor for many enzymes

involved in various metabolic processes in plants, including those related to N assimilation and energy production (Raddatz et al., 2020). The increased K uptake during nitrate toxicity may help support the increased metabolic activity required to manage high nitrate levels (Liu et al., 2022). Potassium is also important for protein synthesis, and the increased K uptake may be a response to the need for more proteins to cope with the stress of nitrate toxicity (Liu et al., 2022; Raddatz et al., 2020).

Nitrogen toxicity has the greatest effect on the increase of Ca concentration in roots and shoots. The increase in the concentration of Ca at high N levels shows that the entry of large amounts of nitrate ions into the cells disrupted the electrochemical gradient of the root and shoot cells and to compensate for the accumulated negative charge inside the cells inevitably involves the absorption of Ca cation (Marschner, 2011). However, Ca accumulation in roots is more concrete than shoots and this leads the low translocation of Ca from root to shoot in the presence of high nitrate level.

The ratio of Ca to K is one of the important indicators used to evaluate the K nutrition status in citrus, particularly in calcareous soils (Najafi-Ghiri et al., 2022). This ratio determines the root's ability to absorb these two elements in balance in the presence of Ca and K, as well as the plant's ability to transport these elements in equilibrium. Different citrus cultivars vary in their capacity to accumulate these elements in roots and leaves. Najafi-Ghiri et al. (2022) demonstrated that in calcareous soils, sour oranges accumulate Ca in the roots, while Mexican lime accumulates K in both leaves and roots. Researchers have reported that despite K being sufficient in calcareous soils, the competition between Ca and K often leads to K deficiency in most citrus trees (Najafi-Ghiri et al., 2017). Observing the increase in the Ca to K ratio in the roots and leaves of sour oranges, and the decrease in the K ratio in shoots to roots with higher N levels in the growth medium, it is crucial to carefully manage nitrate application in citrus orchards. Excessive nitrate application can exacerbate K deficiency issues in these trees.

Nitrogen toxicity causes a decrease in the number of leaves (Table 1), PI and Fv/Fm (Fig. 1) and leaf chlorophyll concentration (Fig. 2). Excess N interferes with the uptake of other essential nutrients like Mg, K, and Fe, which are critical for chlorophyll synthesis and ultimately leading to lower chlorophyll concentration and chlorosis (Taiz & Zeiger, 2015). In this study, excessive N reduced the Mg, K and Fe content of seedling shoots by 42%, 58.3% and 56.6%, respectively in compared with 16 mM. Excess nitrate can disrupt the electron transport chain in the photosynthetic apparatus. It can also be reduced to nitrite in the plants and the accumulation of nitrite and other nitrogen-containing compounds can generate reactive oxygen species (ROS), which cause oxidative stress and damage to the photosynthetic apparatus, including PSII and both effects leads to a reduction of the PI and Fv/Fm and a decrease in the efficiency of PSII (Jia et al., 2021; Noor et al., 2022; Swoczyna et al., 2022). In the same direction, the results showed that phenolic compounds accumulate in the roots when there is an excess of nitrate (Fig. 4). The accumulation of ROS in the state of nitrate toxicity, which can cause oxidative stress in plants, can stimulate the biosynthesis of phenolic compounds. These compounds, such as flavonoids and phenolic acids, can act as free radical scavengers and metal chelators, helping plants deal with nitrate-related oxidative stress (Gill & Tuteja, 2010; Pratyusha, 2022; Taiz & Zeiger, 2015).

The nitrate reductase enzyme concentration decreases drastically in N toxicity condition in roots and shoots (Fig. 1), and this may be an indication of the reduced ability for N regeneration in all parts of the plant. Nitrate reductase is a multi-subunit enzyme that catalyzes the first step in the nitrate assimilation pathway, converting nitrate to nitrite. Nitrate itself is a major regulator of nitrate reductase activity. In response to nitrate toxicity, the activity of nitrate reductase may decrease in both the root and shoot systems. This can be a

protective mechanism to limit the uptake and assimilation of excess nitrate (Chen et al., 2018; Marschner, 2011).

CONCLUSION

This study demonstrates that N availability plays a critical role in the growth, nutrient dynamics and physiological responses of sour orange seedlings. The optimal nitrogen concentration for growth was 16 mM, which maximized plant dry weight, root length, chlorophyll content and photosynthetic efficiency. However, deviations from this optimal range - either deficiency or toxicity - disrupted nutrient uptake and translocation, particularly for K, Ca, Fe, Zn, and Cu. Nitrogen toxicity at 32 mM severely impaired growth, reduced photosynthetic efficiency (Fv/Fm, PI), nitrate reductase activity (nitrogen metabolism) and increased the Ca/K ratio, exacerbating K deficiency risks in calcareous soils. Despite high N availability, chlorophyll a, b, and total chlorophyll decreased in N_{32} treatment, likely due to Mg, K, and Fe deficiency, oxidative stress from reactive oxygen species (ROS), degrading chlorophyll and impairing PSII efficiency. These findings underscore the importance of carefully managing N application in citrus cultivation to avoid nutrient imbalances. Excessive N fertilization should be avoided, especially in calcareous soils as it leads to nutrient imbalances (particularly K and Mg deficiency), chlorophyll degradation, and reduced photosynthetic efficiency. Monitoring leaf area and nutrient ratios, particularly Ca/K, can provide valuable insights into N status and nutrient balance in sour orange seedlings. Future research should focus on optimizing nitrogen management strategies to ensure sustainable citrus production in calcareous soil environments.

Conflict of interest

The authors declare that they have no conflict of interest.

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