biology and life sciences forum

Proceeding Paper

Determining the Response of Citrus Plants to Reduced Nitrogen Fertilization †

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† Presented at the 2nd International Electronic Conference on Plant Sciences—10th Anniversary of Journal Plants, 1–15 December 2021; Available online: https://iecps2021.sciforum.net/.

Abstract: It is well known that there is a greater demand for food, due to a larger global population. To cope with this situation, conventional agriculture uses various strategies, the most important of which being the use of nitrogen-based fertilizers. However, inappropriate and excessive use of these fertilizers leads to the appearance of serious environmental problems, such as the pollution of aquifers or the eutrophication of lakes and reservoirs. In order to solve this problem, several studies have been conducted on various crops, seeking to find the optimization point in the use of these fertilizers, in order to guarantee high crop yields in a sustainable way. In our work, we determine the optimal amount of nitrogen for rootstock citrus crops (*Citrus macrophylla*) grown in controlled temperature and light conditions. Reduction to 50% of nitrogen content was studied through the analysis of several phenotypic (number of leaves, leaf area and fresh weight of roots) and biochemical (total proteins and enzymatic activities of nitrogen-fixing enzymes, such as nitrate reductase and nitrite reductase) parameters. Data obtained showed that there were no significant differences between plants grown under conditions of 100% nitrogen content and plants grown with 50% nitrogen content in all the analyzed parameters, except for the fact that plants grown with 50% of nitrogen content showed less leaf area than plants grown with 100% nitrogen content. This suggests that *C. macrophylla* plants are able to develop, transport and assimilate nitrogen with half nitrogen fertilization, without any symptom of plant stress.

Keywords: nitrogen fertilization; *Citrus macrophylla*; nitrogen-fixing enzymes; growth and development

Citation: García-Gómez, P.; Carvajal, M. Determining the Response of Citrus Plants to Reduced Nitrogen Fertilization. *Biol. Life Sci. Forum* **2022**, *11*, 81. https://doi.org/10.3390/IECPS2021-11927

Academic Editor: Dimitris L. Bouranis

Published: 29 November 2021

1. Introduction

The population of the planet is increasing to such an extent that there will be over-population in the near future. Therefore, it is critical to augment quantity of food to avoid famines, without damaging the environment. Several strategies have been established in agriculture to cope with this problem. One of them is the use of nitrogen-based fertilizers in order to increase crop yields. Nitrogen (N) is a very important element for plant nutrition, due to two facts: (a) nitrogen is the main limiting nutrient for plant growth and (b) nitrogen is involved in many processes to improve crop yield. The main source of N for land plants are nitrate (NO_3^-) and ammonium (NH_4^+). However, overuse of N-based fertilizers is linked to important environmental problems and can even provoke reduction in the nutritional quality of crops, due to an excessive accumulation of nitrate [1], that can be harmful to human health [2].

Citrus fruits are among the most widely grown, and economically important, fruit tree crops in the world. In order to analyze the nitrogen use efficiency (NUE) of citrus crops, a meta-analysis performed by Qin et al., 2016 [3], showed that the median NUE (fruit yield/N input) in citrus production ranged from 150−350 kg kg^{-1}, based on yields of 30−60 t ha^{-1}, and NUE (plant N uptake/soil N input) was only 20-34%, with a N rate ranging from 354 to 534 kg ha^{-1} [4]. In accordance with these results, the N uptake of citrus trees was estimated as 30% N fertilizer [5]. These results were obtained in citrus plants grown in soil, but there is little information about optimal nitrogen fertilization in citrus plants grown in controlled conditions. Although some authors have used them as objects of study [6–8], nitrogen needs have not been studied. Most of the previous papers mentioned used Hoagland solution, or variants of it, in order to grow citrus plants. So, the aim of this study is to determine how citrus plants respond to reduced nitrogen fertilization, and the effects of this reduced nitrogen fertilization in several phenotypic (number of leaves, leaf area and fresh weight of roots) and biochemical (total proteins and enzymatic activities of nitrogen-fixing enzymes) parameters.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The citrus variety Macrophylla was selected to conduct the experiments. These experiments were performed in a growth chamber with the following conditions: 16 h photoperiod, a day/night relative humidity of 60/85% and a day/night temperature of 25/18 °C. Citrus plants were cultured in pots which contained a substrate made with 50% perlite and 50% vermiculite, and watered with 50 mL, using the following nutrient solution 3 times per week, over 2 weeks: 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 50 μM H_3BO_3, 4 μM $MnSO_4$,4 μM $ZnSO_4$, 1 μM $CuSO_4$, 0.13 μM $(NH_4)_6Mo_7O_{24}$ and 40 μM Fe_3^+-EDDHA. This solution was considered the control solution. Then, plants were divided in 2 groups: control and 50% nitrogen deficiency. Whereas control plants were watered with the control solution, plants exposed to 50% nitrogen deficiency were watered with the following solution: 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 50 μM H_3BO_3, 4 μM $MnSO_4$,4 μM $ZnSO_4$, 1 μM $CuSO_4$, 0.13 μM $(NH_4)_6Mo_7O_{24}$ and 40 μM Fe_3^+-EDDHA, compensating the lack of K, Ca and P with $CaCl_2$, K_2HPO_4 and KH_2PO_4. Both treatments were applied to the respective groups over 6 weeks.

At the end of the treatment, root weight, number of leaves and mean leaf area were determined. Also, leaves and roots of every plant were harvested individually and homogenised into a small powder with liquid nitrogen, using a pestle and mortar.

2.2. Total Protein Measurement

Total protein measures were done from 100 mg of fresh material, according to Bradford et al., 1976 [9].

2.3. Nitrate Reductase and Nitrite Reductase Activity Assay

Nitrate reductase activity in leaves was measured in vivo. For that, 100 mg of leaf discs were introduced in a mix containing 1 mL of phosphate buffer 100 mM ph 7.5, 2 mL of KNO_3 50 mM and 3 mL of distilled water. Samples were incubated at 30 °C for 2 h in darkness. After 2 h of incubation, 1 mL of 1% sulfanylamide (w/v), dissolved in 1.5 N HCl and 1 mL of 0.02% N-(1-naphthyl) ethylene diamine dihydrochloride. was added to 3 mL of the previous mix. The amount of formed nitrite was measured using a spectrophotometer at 540 nm and a standard curve, using $NaNO_2$.

Nitrite reductase activity in leaves was measured in vitro according to the method of dithionite assay, described in Joy and Hageman 1966 [10], with some modifications. Instead of benzyl viologen, methyl viologen was used. The amount of left nitrite was measured using a spectrophotometer at 540 nm and a standard curve, using $NaNO_2$.

2.4. Statistical Analyses

For each analysis, 5 plants of each treatment were selected. Statistical analyses were performed using the SPSS 25.0.0.1 software package. Significant differences between the values of all parameters were determined at $p \leq 0.05$, according to Tukey's test. The values presented are the means ± SE.

3. Results

3.1. Number of Leaves, Leaf Area and Root Growth

To determine the effect on growth of plants watered with 50% of nitrogen content, several phenotypic parameters, such as the number of leaves, leaf area and weight of roots, were analysed. As represented in Table 1, plants watered with 50% of nitrogen showed no significant differences with plants watered with the control solution, except in leaf area, where plants exposed to 50% nitrogen solution showed less mean leaf area than control plants.

Table 1. Root weight, number of leaves and mean leaf area of plants watered with control solution and half nitrogen-containing solution. Means were obtained from 5 plants of each condition.

	Root Weight (mg)	**Number of Leaves**	**Mean Leaf Area (cm^2)**
Control	1023 ± 45	15 ± 3	10.247 ± 0.791
50% nitrogen	968 ± 65	14 ± 5	6.222 ± 0.453

3.2. Total Protein Content

The fact that plants watered with the 50% nitrogen-containing solution showed little differences in growth parameters with plants watered with 100% nitrogen-containing solution, suggested that the amount of nitrogen in the case of half-nitrogen exposure was sufficient to permit plant growth. To explore this hypothesis, total protein content, both in control plants and plants exposed to 50% nitrogen, was analysed. As expected, the total protein content was similar with both treatments (Figure 1).

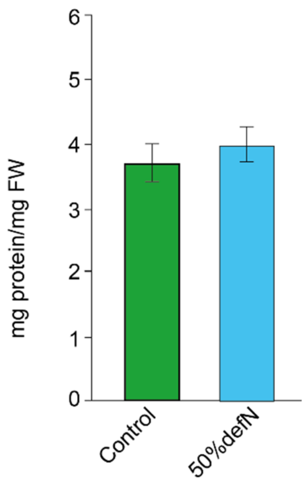

Figure 1. Total protein content in leaves of citrus plants grown with control solution and half-nitrogen containing solution. Data was obtained from 5 different plants of each condition.

3.3. Enzymatic Activities

Given that total protein content did not change between control plants and plants treated with 50% nitrogen-containing solution, it suggested that in both conditions, the uptake of nitrogen by roots was not altered. To explore that hypothesis, the enzymatic activities of nitrate reductase and nitrite reductase, the main enzymes implied in nitrogen assimilation, were studied. In the same way as regards the total protein content, there were no significant differences between control plants and plants exposed to 50% nitrogen-containing solution (Figure 2).

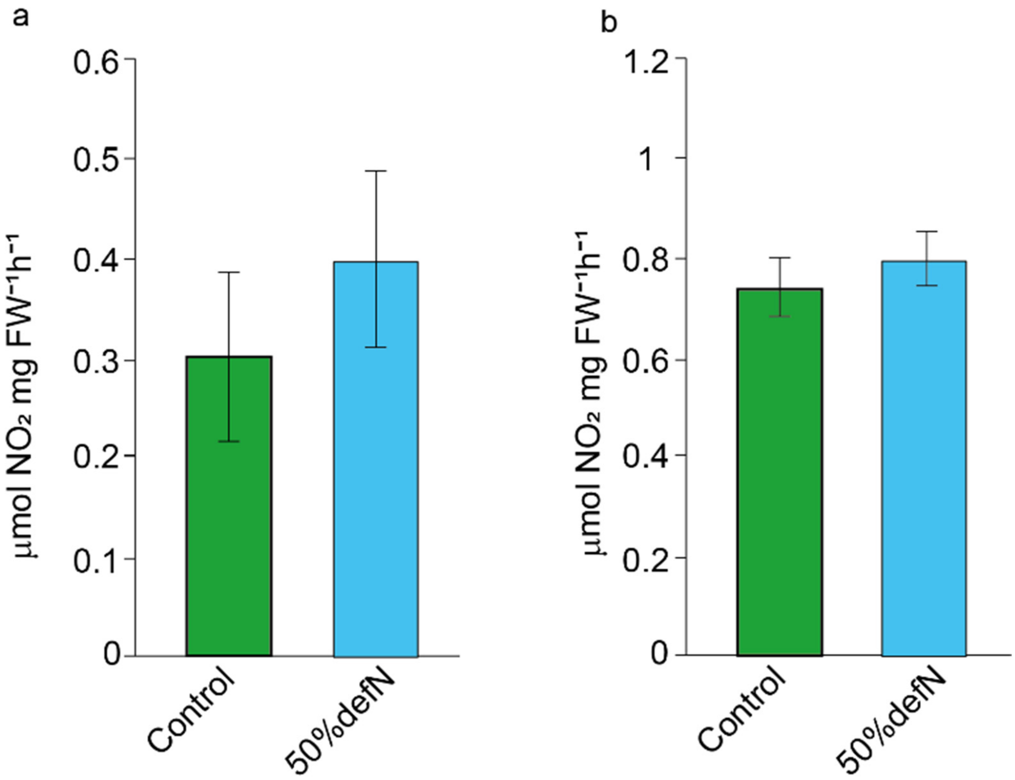

Figure 2. Nitraterreductase activity (**a**) and nitriterreductase activity (**b**) in leaves grown with control solution and half-nitrogen containing solution. Data was obtained from 5 different plants of each condition.

4. Discussion

All the data presented in this work suggest that citrus plants, both the ones watered with full nitrogen-containing solution (considered as control) and the ones watered with half-nitrogen containing solution, responded in a similar way. That might suggest that nutrition with half-nitrogen containing solution is sufficient to permit citrus plants to grow and develop normally. The fact that several authors [6–8] have used Hoagland solution, or variants, to water their plants did not imply that studies for optimizing a precise nutrient solution for citrus plants were made. According to Chen et al., 2020 [11], citrus plants were able to obtain nitrogen through both nitrate and ammonia salts, and, whereas some authors have taken this fact into account [12,13], others have not [6,14,15]. Therefore, it would be necessary to obtain a consensus about the compounds and concentrations used in order to work with citrus plants, at least in controlled conditions.

As for the data shown in this work, there were no significant differences in any of the analysed parameters between control plants and plants treated with half-containing nitrogen solution. That allows us to conclude that it is possible to reduce the nitrogen nutrition of citrus plants without any symptom of plant stress. In this way, more studies should be done to determine the best form and level of nitrogen in each developmental stage.

Author Contributions: Conceptualization, P.G.-G. and M.C.; methodology, P.G.-G.; software, P.G.-G.; validation, P.G.-G. and M.C.; formal analysis, P.G.-G.; investigation, P.G.-G.; resources, P.G.-G.; data curation, M.C.; writing—original draft preparation, P.G.-G.; writing—review and editing, P.G.-G. and M.C.; visualization, M.C.; supervision, M.C.; project administration, M.C.; funding acquisition, M.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Comunidad Autónoma de la Región de Murcia, by the project RIS3MUR-CERONO3 MAR MENOR (Ref. 2I20SAE00081).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not supplementary data is attached to this paper.

Conflicts of Interest: The authors declare no conflict of interest.

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