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Proceeding Paper

Determining the Response of Citrus Plants to Reduced Nitrogen Fertilization †

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Abstract: It is well known that there is a greater demand for food due to a larger global population. To cope with this situation, the conventional agriculture uses various strategies, being the most important the use of nitrogen-based fertilizers. However, the 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 in various crops searching the optimization of 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 the rootstock citrus crops (*Citrus macrophylla*) grown in controlled temperature and light conditions. The 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 parameters (total proteins and enzymatic activities of nitrogen-fixing enzymes such as nitrate reductase and nitrite reductase). Data obtained showed that there were no significant differences between plants grown under conditions of 100% of nitrogen content and plants grown with 50% of nitrogen content in all the analysed parameters, except that plants grown with 50% of nitrogen content showed less leaf area than plants grown with 100% of 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

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1. Introduction

The population of the planet is increasing developing an overpopulation during the next years. Therefore, it is critical to augment the quantity of food to avoid famines without damaging the environment. Several strategies have been established in agriculture to cope with that 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) is the main limiting nutrient for plant growth and (b) 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, the overuse of N-based fertilizers is linked to important environmental problems and even can provoke a reduction of the nutritional quality of crops due to and 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 analyse the nitrogen use efficiency (NUE) in citrus, a meta-analysis performed by Qin et al., 2016 [3] showed that 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 an N rate ranging from 354 to 534 kg ha^{-1} [4]. In accordance with these results, the N uptake of citrus trees was estimated in 30% N fertilizer [5]. These results were obtained in citrus plant grown on soil, but there is little information about the optimal nitrogen fertilization in citrus plants grown in controlled conditions. Although some authors have used them as object of study [6–8] the nitrogen needs has not been studied. Most of the previous papers previously mentioned used the Hoagland solution or variants 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 parameters (total proteins and enzymatic activities of nitrogen-fixing enzymes).

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, during 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 as control solution. Then, plants were divided in 2 groups: control and 50% nitrogen deficiency, and 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 during 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, 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 during 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 were added in this order 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 was used methyl viologen. 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 it is represented in Table 1, plants watered with 50% of nitrogen showed no significant differences with plants watered with 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 was sufficient to permit plant growth even in case of half-nitrogen exposure. To explore that hypothesis, total protein content both in control plants and plants exposed to 50% nitrogen was analysed. As it was expected, the total protein content was similar with both treatments (Figure 1).

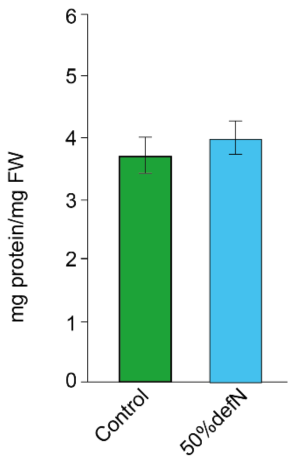

Figure 1. Total protein content in leaves of citrus plants grown with control solution and half-nitrogen containing solution. Data was obtained from 5 different plants of each condition.

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 than to the total protein content, there were no significant differences between control plants and plants exposed to 50% nitrogen-containing solution (Figure 2)

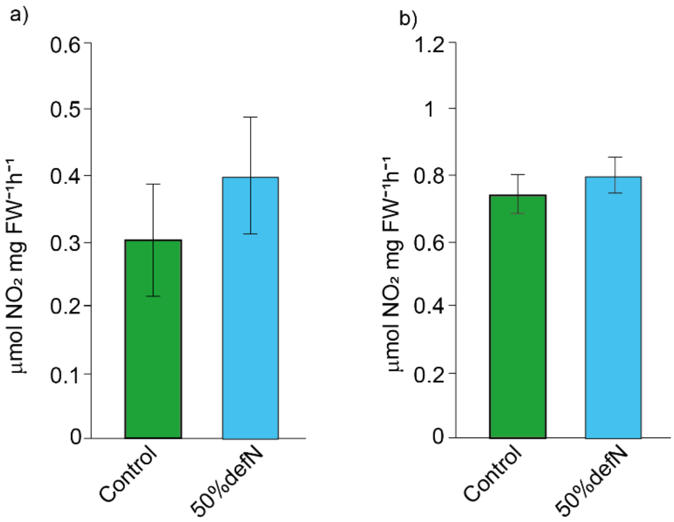

Figure 2. nitraterreductase activity (**a**) and nitriterreductase activity (**b**) in leaves grown with control solution and half-nitrogen containing solution. Data was obtained from 5 different plants of each condition.

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 optimising 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 into account this fact [12,13], others not [6,14,15]. Therefore, it would be necessary a consensus about the compounds and concentrations used in order to work with citrus plants, at least in controlled conditions.

As for the data showed 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 for determining best form and level of nitrogen in each developmental stage.

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