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Effects of Salinity on 'Fino 95' Lemon Trees Inoculated with Arbuscular Mycorrhizal Fungi

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Abstract: An experiment was conducted with two-year-old 'Fino 95' lemon plants (*Citrus limon* Burm. f. cv. Fino) grafted on *C. macrophylla* (*Citrus macrophylla* Wester) to study the effects of salinity on plants inoculated with arbuscular mycorrhizal fungi (AMF). Half of the inoculated (+AM) and non-inoculated (−AM) plants were irrigated with half-strength Hoagland solution, and the remainder were inoculated with half-strength Hoagland solution + 30 mM NaCl. Ninety-eight days later, results showed that AMF had alleviated the negative effect of salinity on growth. Inoculation with AMF provided some protection against the damage that salinity caused on cellular membranes and improved the plant water status and turgor under saline conditions (Ψ_x and Π increased by 16% and 48%, respectively). The responses of mineral nutrition to salinity and AMF treatments were complex. P concentrations in the leaves and roots of +AM plants were lower than in those of −AM plants, but inoculation improved Ca^{2+} (by 20%), Mg^{2+} (24%), Fe^{2+} (21%), and Zn^{2+} (7%) nutrition in roots and also the Mg^{2+}/Na^{+} ratio in leaves (33%), reducing the antagonistic effect of Na^{+} on Mg^{2+} nutrition in salt-treated plants. AMF could protect plants against salt stress through the maintenance of the gas exchange capacity and due to a better antioxidant response. All these positive effects of AMF contributed to mitigating the harmful effects of salinity stress on the plant growth performance of lemon trees grafted on *C. macrophylla* rootstock under salinity conditions.

Keywords: *Citrus macrophylla*; mineral nutrition; physiological responses; *Rhizophagus irregularis*; *Funneliformis mosseae*

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Citation: Navarro, J.M.; Pérez-Tornero, O.; Morte, A. Effects of Salinity on 'Fino 95' Lemon Trees Inoculated with Arbuscular Mycorrhizal Fungi. *Horticulturae* **2024**, *10*, 612. https://doi.org/10.3390/horticulturae10060612

Academic Editors: Stefania Toscano, Daniela Romano and Qiang-Sheng Wu

Received: 26 March 2024
Revised: 5 June 2024
Accepted: 6 June 2024
Published: 9 June 2024

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1. Introduction

Citrus species stand out as the most productive and widely consumed fruit globally, constituting a vital component of the economy as an evergreen tree cultivated in 140 countries that span across various regions [1]. These regions can be predominantly found in warm temperate zones [2], which offer favorable conditions for citrus cultivation, albeit they are often characterized by arid, hot summers. A prime example of this is the south-eastern region of Spain, renowned for its significant citrus output, attributed to a warm Mediterranean climate and a fertile soil, which collectively foster an optimal growing environment [3,4]. However, the low precipitation levels of these areas create the necessity of implementing supplementary irrigation systems. Many of these arid and semi-arid regions grapple with groundwater salinization issues stemming from aquifer overexploitation and seawater intrusion. As a consequence, the available irrigation water presents with subpar quality due to the high concentrations of soluble salts, posing challenges for citrus cultivation, as citrus trees are particularly sensitive to salinity [5,6]. Moreover, in the Mediterranean regions, the temperature rise induced by climate change is projected to increase evapotranspiration, decrease rainfall, and, consequently, elevate irrigation demands

for this crop, which already exhibits high irrigation requirements. With climate change becoming increasingly widespread, the diminishing freshwater resources pose a threat to the sustainability of irrigated agriculture and, therefore, to food security [7,8]. Thus, in citrus-producing regions, climate change is anticipated to exacerbate the already significant issues of drought and salinity stress in citrus.

On the other hand, in the northern hemisphere, fresh lemon (*Citrus limon* [L.] Osbeck) is mainly produced in the Mediterranean area. Specifically, in Spain, it is mainly grown in the southeastern region of the country, which is responsible for almost 90% of its production [9]. Among commercial citrus rootstocks, *Citrus macrophylla* is one of the most commonly used in lemon orchards in southeastern Spain, since growers used it to replace sour orange, which is strongly affected by the tristeza disease [10]. Due to its low vigor compared with other rootstocks, it presents a higher drought-stress tolerance, and its use is recommended for regions such as southeastern Spain, where the availability of water is not assured [11].

Salinity poses a significant environmental constraint that limits the productivity of citrus, classified as a salt-sensitive crop. The response of citrus to soil salinity varies depending on the rootstock, the scion, the soil composition, the irrigation method, and the climatic conditions. Moderate levels of salinity can disrupt physiological processes, diminish growth, and reduce fruit yield in citrus trees [5,12–14]. Salinity can have an impact on citrus plants through various mechanisms, including the inhibition of CO_2 assimilation due to elevated concentrations of Cl^- and Na^+ in leaves [15]. Additionally, it can lead to nutrient imbalances by impeding the uptake of essential elements such as Ca^{2+}, Mg^{2+}, and K^+ [16], while also triggering an increase in intracellular inorganic and organic solutes as the plant seeks osmotic adjustment to maintain leaf turgor [17]. Saline stress prompts plants to restrict stomatal opening, partly through abscisic acid (ABA) signaling, resulting in a decreased photosynthetic activity [18,19]. This alteration in photosynthesis disrupts the electron transport chain and heightens the production of reactive oxygen species. Variations in the response to salinity among citrus varieties and rootstocks have been linked to differences in their capacity to regulate transpiration and photosynthesis [20].

Mycorrhizal fungi, which develop a symbiotic relationship with plant roots, are believed to alleviate salt stress in host plants. Arbuscular mycorrhizal fungi (AMF) have been shown to enhance citrus plant growth and vigor, mitigating the damage caused by soil salinization [21–27]. The increased plant growth under salinity following mycorrhizal colonization with *Rhizophagus irregularis*, *Funneliformis mosseae,* or *Paraglomus occultum* described in these studies has been attributed to a more efficient nutrient uptake, particularly in the case of phosphorus (P), and physiological processes such as increased carbon dioxide exchange rates, transpiration, stomatal conductance, and water use efficiency, as well as an improved osmotic adjustment through organic and inorganic osmolytes, scavenging reactive oxygen species (ROS) through antioxidant enzymes and antioxidants, soil structural improvements, etc.

The research hypothesis of this study is that the inoculation of AMF can mitigate the adverse effects of salinity on citrus plants, particularly when considering both the rootstock and the scion components in commercial orchards. This hypothesis is based on previous findings indicating that AMF colonization has alleviated NaCl stress in commonly used citrus rootstocks in Spain [22,23]. However, in commercial orchards, citrus trees comprise both rootstock and scion. The novelty of this study is the use of AMF to alleviate the negative effects of salinity in typical commercial citrus plants. For this purpose, we used grafted citrus plants and evaluated the growth, mineral nutrition, and physiological responses of the plant in two-year-old lemon trees grafted on *C. macrophylla*, one of the rootstocks that had been previously studied, which is considered as a Cl^- and Na^+ accumulator under saline conditions [28,29]. This study aims to provide a comprehensive understanding of how AMF inoculation can alleviate the effects of salinity on commercial citrus plants that are commonly used in citrus orchards. The findings could offer valuable insights for developing sustainable agricultural practices, providing citrus growers with tools to

enhance crop productivity and quality under saline conditions, and thereby addressing a significant challenge in the Mediterranean agriculture.

2. Materials and Methods

2.1. Plant Culture and Experimental Design

Uniformly sized, two-year-old 'Fino 95' lemon trees (*Citrus limon* [L.] Burm. fil.), grafted on *C. macrophylla* rootstock and obtained from a commercial nursery, were transplanted to plastic pots (3 L) on 15 March 2021. Soil was collected from the Estación Experimental de Torreblanca (Murcia, Spain), sieved (5 mm), diluted with quartz sand (1:3, soil/sand, *v*/*v*) and then sterilized in an autoclave for 1 h at 100 °C three times on alternating days. The sterilization bags with the substrate were closed and, after each sterilization step, they were stored in a clean and cool place. Original soil was analyzed by preparing a saturated paste with the following characteristics: organic carbon 1.38%; organic matter 2.37%; organic N 0.12%; C–N ratio 11.2; Olsen P 72.1 mg L^{-1}. The nutrient concentrations (g L^{-1}) were as follows: K 0.23; Ca 0.59; Mg 0.17; NO_3^- 0.62; SO_4^{2-} 1.19; HCO_3^- 0.18; Cl^- 1.08. Plants were placed in a greenhouse providing average day/night temperatures of 30–32/13–15 °C. Relative humidity was maintained at approximately 75%.

Twenty-five grams of *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler and *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler in equal proportions (a mix of spores, mycorrhized roots, and substrate) as the mycorrhizal inoculum, propagated with the hybrid of *Sorghum bicolor* (L.) Moench and *Sorghum sudanense* (Piper) Hitch (*Sorghum bicolor* × *sudanense*) as the trap plant, were used to inoculate plants. In half of the pots (+AM), the inoculum was placed just around the root system and the pot was filled with the substrate. The inoculum was supplied by the Mycology-Mycorrhizas Laboratory, Department of Plant Biology, of the University of Murcia (Spain). Non-inoculated plants (−AM) were watered every 2 days with 500 mL of half-strength modified Hoagland's solution [30] to maintain the moisture content of the substrate. Inoculated plants (+AM) were also watered regularly with the same solution, but without P. Seedlings were acclimated for 3 months and then were subjected to salinity treatments. For both the +AM and −AM seedlings, half of them were selected randomly for the non-salt treatment (0 mM NaCl), and the other half received the salt stress treatment (30 mM NaCl). The salt stress treatment was applied in steps of 10 mM NaCl per day to avoid an osmotic shock.

Plants were placed randomly in the greenhouse, and the experiment was laid out in a completely randomized block design. Experimental treatments consisted of factorial combinations of two components: mycorrhization (−AM and +AM) and salinity (0 and 30 mM NaCl). Each of the four treatments had 10 replicates, giving a total of 40 pots. Trees were harvested on 20 September 2021, 98 days after starting the NaCl treatments.

2.2. Determination of Physiological Parameters

Gas interchange parameters (net CO_2 assimilation rate [*A*], transpiration [*E*], and stomatal conductance [g_s]) were measured one day before harvest using an infrared gas analyzer Li-6400 (Li-Cor, Lincoln, NE, USA) equipped with a broad leaf chamber (6.0 cm^2). All of the measurements were taken at a molar air flow of 300 µmol mol^{-1}, a reference CO_2 concentration of 400 µmol mol^{-1}, and a photosynthetic photon flux density of 1200 µmol m^{-2} s^{-1}. The intrinsic gas exchange efficiency was calculated as A/g_s.

Stem water potential (Ψ_x) was measured at noon (12:00–14:00) with a Schölander-type pressure chamber (3000; Soil Moisture Equipment Corp., Santa Barbara, CA, USA; [31]) using mature leaves that had been enclosed in aluminum-foil-covered plastic envelopes at least 2 h before measurement. After Ψ_x measurement, leaves were tightly enclosed in aluminum foil and frozen at −18 °C. After thawing, leaf osmotic potential (Π) was measured in expressed cell sap collected from a syringe at 25 ± 1 °C and placed in an osmometer (Digital Osmometer, Wescor, Logan, UT, USA). Similar fresh leaves were hydrated by placing their petioles in distilled water at 4 °C for 24 h; after they were

frozen at −18 °C, osmotic potential at full turgor (Π^{100}) was determined by the same procedure that has been previously described.

2.3. Plant Growth

The relative growth rate (RGR) of the trunk section of the scion variety was calculated as in Tattini et al. [32], measuring the trunk when salinity treatments started and at the end of the experiment: $RGR = (ln\ M_2 - ln\ M_1)/(t_2 - t_1)$, where M is the trunk section of the measured variety and $t_2 - t_1$ is the time elapsed between measurements.

At harvest, plant roots were carefully separated from the substrate and washed with distilled water. The fresh root systems were divided into two parts: one of them was used for the determination of mycorrhization, and the other was oven-dried to calculate the dry weight (DW) and analyze the nutrients. The leaves and stems were separated and weighed fresh. The total leaf area was measured using an Area Meter LI-3100C (LI-COR, Lincoln, NE, USA). The relative water content (RWC) of the leaves was measured. After the roots and stems were oven-dried at 60 °C for 48 h (until constant weight) and the leaves were freeze-dried, their DWs were determined.

2.4. Plant Analysis

The dried plant tissues were ground and stored for chemical analysis. They were ashed at 550 °C, ashes were dissolved in 0.7 N HNO_3, and then P, Na, K, Mg, Ca, Fe, Cu, Zn, and Mn were analyzed by an inductively coupled plasma optical emission spectrometer Varian MPX Vista (Varian, Palo Alto, CA, USA). Chloride was extracted with bi-distilled water, using the method of Guilliam [33], and determined by ionic chromatography with a Dionex ICS-3000 (Dionex, Sunnyvale, CA, USA) ion chromatograph equipped with a conductivity detector and an AS11-HC anion exchange column.

Quaternary ammonium compounds (QAC) were measured by the Grieve and Grattan [34] method. Samples were extracted with deionized H_2O and, after dilution with 2 N H_2SO_4, periodide crystals were formed with cold KI-I_2 reagent. The absorbance at 365 nm was measured after periodide crystals were dissolved in 1,2-dichloroethane.

Free proline was analyzed by a modification of the acid ninhydrin method [35]. Fifty mg of freeze-dried leaves were extracted with 10 mL of 5-sulfosalicylic acid, shaken for 30 min with an orbital shaker, and centrifuged at 5000 rpm for 15 min. After the addition of glacial acetic acid and acid ninhydrin, color was developed at 100 °C, the chromophore was extracted with toluene, and the absorbance was measured at 520 nm.

Lipid peroxidation was determined by measuring malondialdehyde (MDA) using the Heath and Packer [36] method. Dry tissue was homogenized with a thiobarbituric acid–trichloroacetic acid mixture. After 45 min at 95 °C and centrifugation at 4000× g for 35 min, the absorbance was read at 532 and 600 nm to correct the unspecific turbidity.

2.5. Determination of Mycorrhizal Colonization

Root samples from every tree were taken from the middle part of the root system to make an estimation of mycorrhizal colonization. They were cleaned and stained with trypan blue according to the method of Phillips and Haymann [37] but using lactic acid instead of lactophenol. One hundred root segments per plant were mounted on slides, squashed by pressing on the coverslips, and quantified for AM colonization according to McGonigle et al. [38].

2.6. Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) procedures with the SPSS software package (SPSS 7.5.1 for Windows, standard version, 1996). A two-way ANOVA procedure was used to discriminate the effects of the inoculation with AMF, the salinity level, and their interaction.

3. Results

3.1. Mycorrhizal Colonization and Plant Growth Analysis

Trees inoculated with AMF showed a high percentage of root colonization, with no significant effect of the salt level on the percentages of colonization (Table 1, Figure 1). There was no mycorrhizal colonization in the non-inoculated plants.

Table 1. Root colonization, vesicular colonization, and arbuscular colonization of roots of 'Fino 95' lemon plants grafted on *C. macrophylla* and grown at two levels of salinity under greenhouse conditions.

NaCl (mM)	Root Colonization (%)	Vesicular Colonization (%)	Arbuscular Colonization (%)
0	89	54	65
30	85	55	53
ANOVA	ns	ns	ns

Non-significant differences at the 0.05 level of probability are indicated as ns.

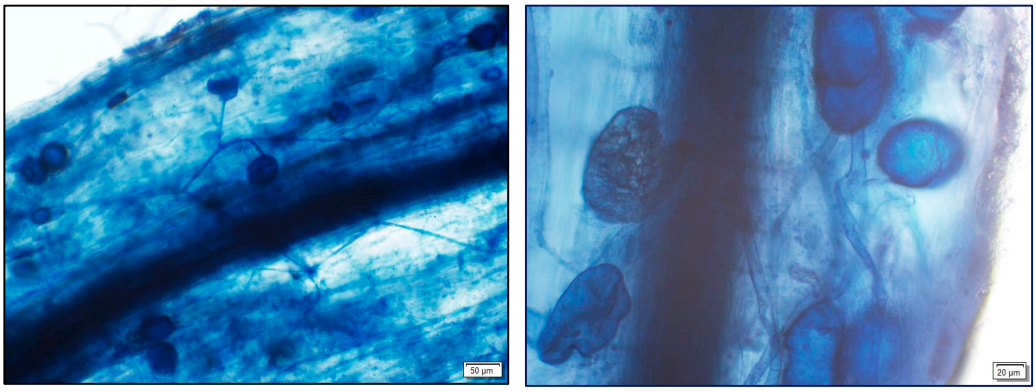


Figure 1. Arbuscular mycorrhizal colonization of the mycorrhizal roots, details of hyphae and vesicles.

Salinity decreased the plant growth of the lemon trees grafted on *C. macrophylla* rootstock in a significant way, but this effect was different in −AM and +AM plants (Figure 2). Whereas salinity decreased the growth of −AM plants (23% of plant dry weight and 35% of RGR trunk section; Figure 2), no growth reduction was observed in +AM plants.

3.2. Physiological Changes

Our study revealed the change in leaf water potential in response to NaCl treatment and mycorrhizal inoculation (Table 2). Salinity significantly decreased the Ψ_x in both −AM and +AM plants. The reduction in Ψ_x due to salinity was greater in −AM plants (67%) than in +AM plants (19%), so AMF inoculation improved plant water status under saline conditions, increasing Ψ_x with regard to −AM plants (Table 2). In this experiment, only +AM plants under 30 mM NaCl slightly decreased their Π in order to maintain turgor potential (Table 2). In fact, salinity treatment reduced turgor in −AM plants, and only +AM plants maintained turgor values that were similar to those of the control (Table 2). The significant decrease in the osmotic potential at full turgor (Π^{100}) by salinity observed in +AM plants highlighted the active solute accumulation in these plants (Table 2).

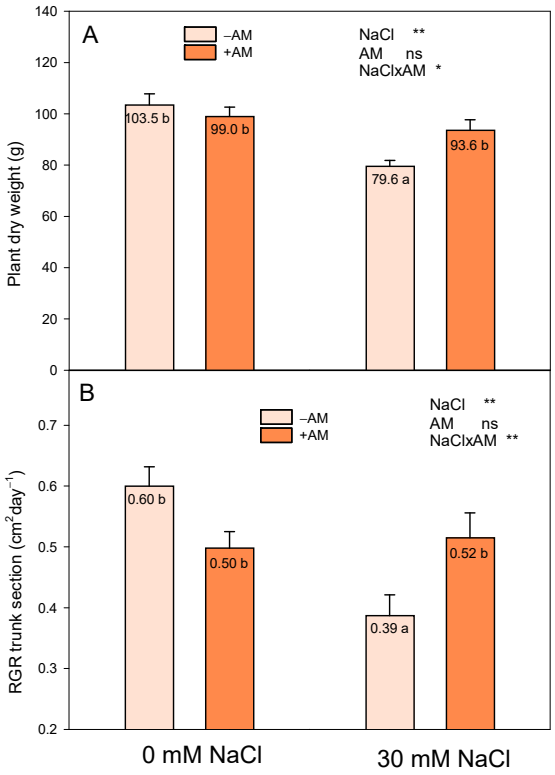


Figure 2. Effect of inoculation with AMF (−AM non-inoculated plants, +AM inoculated plants) on total plant biomass (**A**) and RGR trunk section (**B**) of 'Fino 95' lemon plants grown at two levels of salinity (0 and 30 mM NaCl) under greenhouse conditions. * and ** indicate significant differences at the 0.05 and 0.01 levels of probability, respectively. Non-significant differences at the 0.05 level of probability are indicated as ns. Different letters indicate significant differences between treatments according to Duncan's multiple range test at the 95% confidence level.

Table 2. Effect of the inoculation with AMF (−AM non-inoculated plants, +AM inoculated plants) on water potential (Ψ_x, MPa), osmotic potential (Π, MPa), turgor potential (P, MPa), osmotic potential at full turgor (Π^{100}, MPa), and malondialdehyde concentration (MDA, µmol kg^{-1} DW) of 'Fino 95' lemon plants grown at two levels of salinity (0 and 30 mM NaCl) under greenhouse conditions.

NaCl (mM)		Ψ_x	Π	P	Π^{100}	MDA
0		−0.75	−1.72	0.94	−1.66	116.1
30		−1.06	−1.78	0.74	−1.74	133.7
AMF						
−AM		−0.92	−1.78	0.81	−1.68	125.5
+AM		−0.89	−1.72	0.87	−1.72	124.3
NaCl × AMF						
0	−AM	−0.69 d	−1.81 a	1.02 b	−1.64	111.8 a
	+AM	−0.81 c	−1.63 b	0.86 b	−1.67	120.4 b
30	−AM	−1.15 a	−1.75 ab	0.60 a	−1.71	139.1 c
	+AM	−0.97 b	−1.81 a	0.89 b	−1.77	128.3 b
ANOVA						
NaCl		***	ns	**	*	***
AMF		ns	ns	ns	ns	ns
NaCl × AMF		***	*	***	ns	**

*, **, and *** indicate significant differences at the 0.05, 0.01, and 0.001 levels of probability, respectively. Non-significant differences at the 0.05 level of probability are indicated as ns. Different letters indicate significant differences between treatments (lines) according to Duncan's multiple range test at the 95% confidence level.

In our experiment, 'Fino 95' lemon plants did not accumulate proline as part of their osmotic response to the salinity treatment, and the increase in Π in +AM plants treated with 30 mM NaCl was partly due to a higher accumulation of QAC than in non-salinized plants (Figure 3).

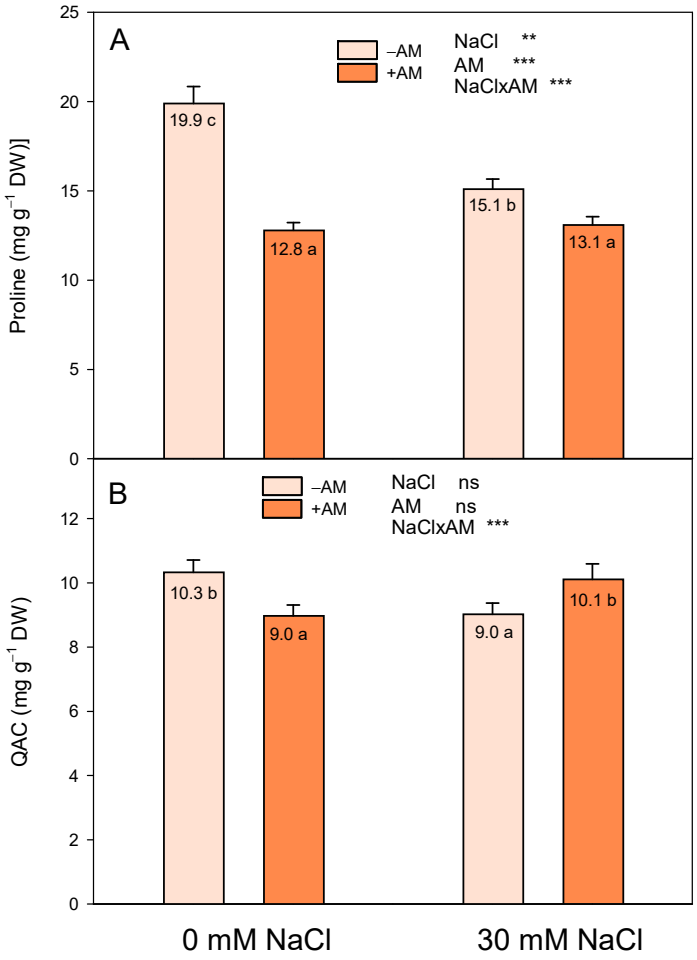


Figure 3. Effect of inoculation with AMF (−AM non-inoculated plants, +AM inoculated plants) on the leaf concentrations of proline (**A**) and quaternary ammonium compounds (QAC) (**B**) of 'Fino 95' lemon plants grown at two levels of salinity (0 and 30 mM NaCl) under greenhouse conditions. ** and *** indicate significant differences at the 0.01 and 0.001 levels of probability, respectively. Non-significant differences at the 0.05 level of probability are indicated as ns. Different letters indicate significant differences between treatments according to Duncan's multiple range test at the 95% confidence level.

In our experiment, MDA data showed that salinity produced a significantly higher lipid peroxidation in −AM plants, whereas oxidative damage due to salinity was reduced in +AM plants (Table 2).

3.3. Nutritional Changes

Under saline conditions, citrus plants transported Cl^- to leaves, as shown in the present research by the increase in foliar Cl^- in these conditions (Table 3). However, no effect of the AMF on the foliar Cl^- concentrations under control or saline conditions was found in our experiment (Table 3). Salinity increased the root Na^+ concentrations and did not reduce K^+ in roots nor in leaves. AMF did not modify Na^+ concentrations in roots or leaves but increased the foliar K^+ concentration under non-saline conditions (Tables 3 and 4). However, this surge was not enough to increase the K^+/Na^+ ratio in the leaves of control plants.

Similarly, the decrease in foliar K^+ due to AMF under saline conditions was not enough to reduce the K^+/Na^+ ratio of these plants. However, the K^+/Na^+ ratio was increased by AMF under non-saline conditions but was not modified with 30 mM NaCl (Table 4). As AMF did not increase the abundance of K^+ in roots or leaves nor did they decrease that of Na^+ under salinity, K^+/Na^+ ratios were not higher in +AM plants under saline conditions.

Table 3. Effect of inoculation with AMF (−AM non-inoculated plants, +AM inoculated plants) on the leaf nutrient concentrations and leaf ionic balance of 'Fino 95' lemon plants grown at two levels of salinity (0 and 30 mM NaCl) under greenhouse conditions. Cl^-, Na^+, P, and K expressed as mmol kg^{-1} DW; Cu and Mn expressed as mg kg^{-1} DW.

NaCl (mM)	Cl^-	Na^+	P	K^+	K^+/Na^+	Ca^{2+}/Na^+	Mg^{2+}/Na^+	Cu	Mn
0	99.8	198.5	39.7	570	2.95	2.70	0.68	27.0	22.3
30	143.5	190.2	36.3	604	3.24	3.07	0.59	37.8	24.7
AMF									
−AM	120.2	201.5	50.3	580	3.06	3.00	0.57	34.4	25.6
+AM	123.1	187.2	25.7	594	3.13	2.76	0.70	30.5	21.3
NaCl × AMF									
0 −AM	99.4	200.4	52.2	526 a	2.79	2.78	0.64	28.5	24.8
0 +AM	100.2	196.5	27.2	613 bc	3.10	2.62	0.72	25.6	19.8
30 −AM	141.0	202.5	48.5	634 c	3.32	3.23	0.51	40.2	26.5
30 +AM	146.0	169.5	24.2	574 ab	3.16	2.91	0.68	35.3	22.8
Anova									
NaCl	**	ns	ns	ns	ns	ns	ns	*	ns
AMF	ns	ns	***	ns	ns	ns	*	ns	***
NaCl × AMF	ns	ns	ns	***	ns	ns	ns	ns	ns

*, **, and *** indicate significant differences at the 0.05, 0.01, and 0.001 levels of probability, respectively. Non-significant differences at the 0.05 level of probability are indicated as ns. Different letters indicate significant differences between treatments according to Duncan's multiple range test at the 95% confidence level.

Table 4. Effect of inoculation with AMF (−AM non-inoculated plants, +AM inoculated plants) on the root nutrient concentrations and root ionic balance of 'Fino 95' lemon plants grown at two levels of salinity (0 and 30 mM NaCl) under greenhouse conditions. Cl^-, Na^+, P, and K expressed as mmol kg^{-1} DW; Cu and Mn expressed as mg kg^{-1} DW.

NaCl (mM)	Cl^-	Na^+	P	K^+	K^+/Na^+	Ca^{2+}/Na^+	Mg^{2+}/Na^+	Cu	Mn
0	116.5	121.9	74.4	465	3.78	7.98	1.52	30.8	74.5
30	126.5	317.7	74.5	444	1.43	2.88	0.44	28.3	93.4
AMF									
−AM	129.9	216.3	109.6	450	2.49	4.79	0.85	28.3	91.8
+AM	113.2	223.3	39.4	459	2.72	6.07	1.11	30.8	76.1
NaCl × AMF									
0 −AM	132.8	129.7	109.4	441	3.41 b	6.84 b	1.28 b	31.0	73.4 a
0 +AM	100.2	114.2	39.4	488	4.15 c	9.12 c	1.76 c	30.5	75.5 a
30 −AM	126.9	303.0	109.8	458	1.56 a	2.74 a	0.42 a	25.6	110.2 b
30 +AM	126.1	332.4	39.3	430	1.30 a	3.03a	0.46a	31.1	76.6 a
Anova									
NaCl	ns	***	ns	ns	***	***	***	ns	**
AMF	ns	ns	***	ns	ns	**	***	ns	*
NaCl × AMF	ns	ns	ns	ns	**	*	**	ns	**

*, **, and *** indicate significant differences at the 0.05, 0.01, and 0.001 levels of probability, respectively. Non-significant differences at the 0.05 level of probability are indicated as ns. Different letters indicate significant differences between treatments according to Duncan's multiple range test at the 95% confidence level.

Inoculated plants of this experiment had higher root concentrations of Ca^{2+} and Mg^{2+} than −AM plants for both salinity treatments (Figure 4). Nonetheless, in plants

exposed to salinity, the increase in these nutrients due to AMF was not enough to improve the Ca^{2+}/Na^{+} and Mg^{2+}/Na^{+} ratios of the roots (Table 4). Although the Ca^{2+} and Mg^{2+} concentrations of mycorrhizal lemon roots were increased under saline conditions, the root Na^{+} levels were also heightened (although not significantly) and, as a consequence, the Ca^{2+}/Na^{+} and Mg^{2+}/Na^{+} ratios of the +AM and −AM plants were similar (Table 4). However, the Mg^{2+}/Na^{+} ratio increased in the leaves of mycorrhizal lemon plants exposed to salt stress.

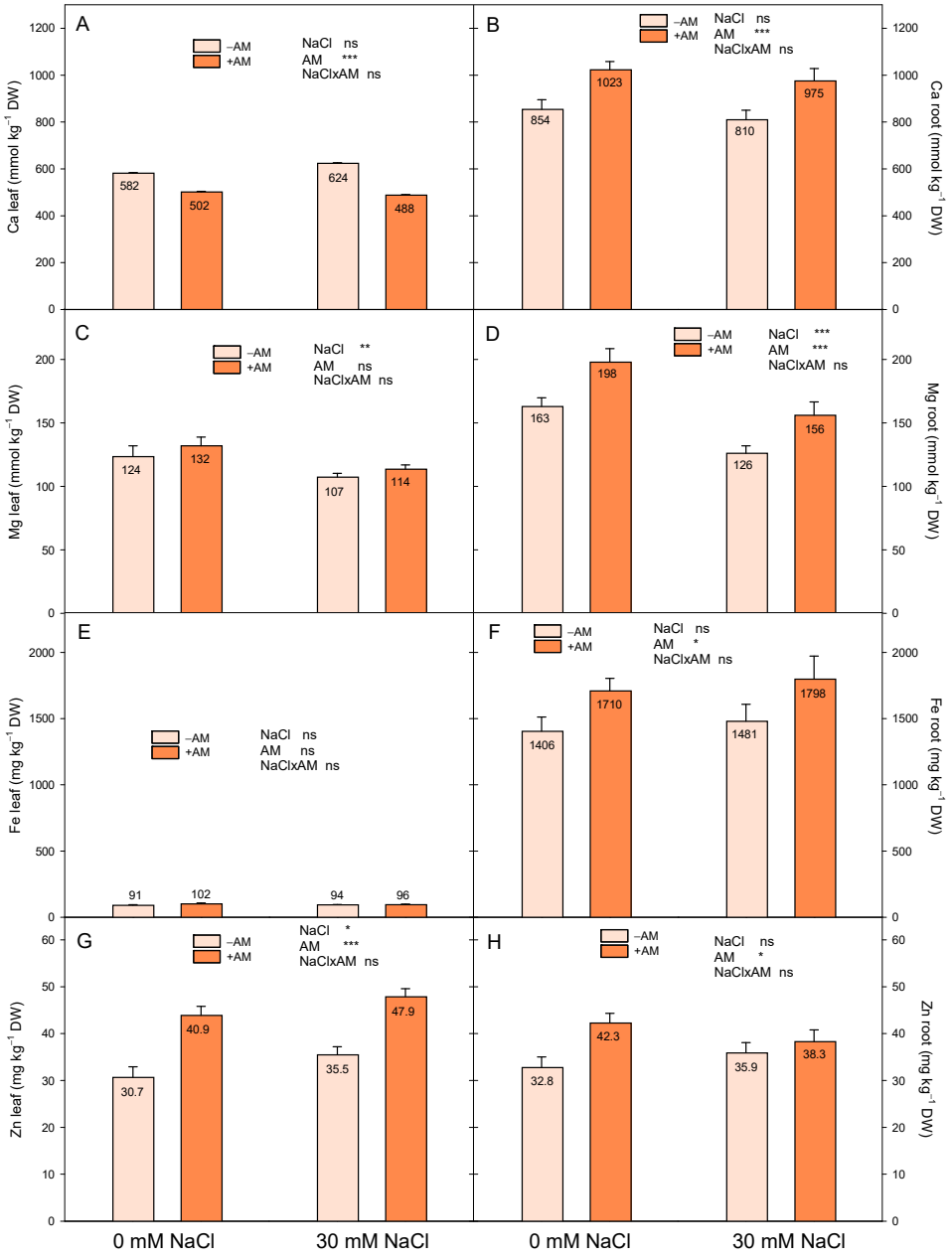


Figure 4. Effect of inoculation with AMF (−AM non-inoculated plants, +AM inoculated plants) on the leaf (**A**,**C**,**E**,**G**) and root (**B**,**D**,**F**,**H**) concentrations of Ca (**A**,**B**), Mg (**C**,**D**), Fe (**E**,**F**), and Zn (**G**,**H**) of 'Fino 95' lemon plants grown at two levels of salinity (0 and 30 mM NaCl) under greenhouse conditions. Ca and Mg expressed as mmol kg^{-1} DW; Fe and Zn expressed as mg kg^{-1} DW. *, ** and *** indicate significant differences at the 0.05, 0.01 and 0.001 levels of probability, respectively. Non-significant differences at the 0.05 level of probability are indicated as ns.

The concentrations of P and Mn in the roots and leaves of +AM plants were lower than those found in −AM plants (Tables 3 and 4). With regard to the Fe concentration, +AM plants had increased root levels of Fe, in both the control and saline treatments, but their leaf Fe concentration was not modified (Figure 4). On the other hand, Zn was significantly increased by AMF in both roots and leaves of control and salinized plants (Figure 4).

3.4. Gas Interchange Parameters

The present study indicated an inhibitive effect of salt stress on the photosynthetic rate and stomatal conductance (Figure 5). However, this effect of salinity on A and g_s depended on the inoculation, since both parameters were significantly decreased by the exposure to 30 mM NaCl in −AM plants, remaining unaltered in +AM plants (Figure 5). We observed similar g_s values in +AM and −AM plants under saline conditions and a lower reduction in g_s in +AM plants than in −AM plants with increasing salinity. On the other hand, inoculation significantly decreased the photosynthetic capacity of the plants under control conditions (Figure 5). No effects were observed on the transpiration rate (E) nor on the intrinsic photosynthetic efficiency (A/g_s).

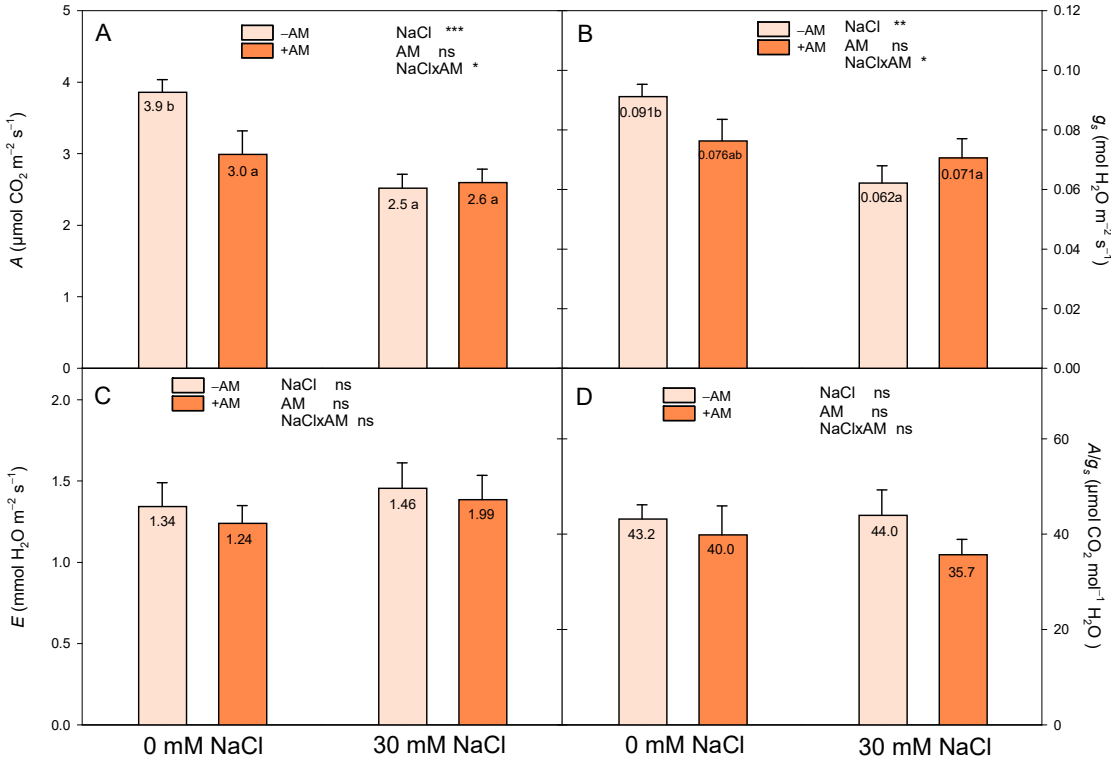


Figure 5. Effect of inoculation with AMF (−AM non-inoculated plants, +AM inoculated plants) on the net CO_2 assimilation rate (A, µmol CO_2 $m^{-2}s^{-1}$) (**A**), stomatal conductance (g_s, mol H_2O $m^{-2}s^{-1}$) (**B**), transpiration (E, mmol H_2O $m^{-2}s^{-1}$) (**C**), and intrinsic gas exchange efficiency (A/g_s, µmol CO_2 mol^{-1} H_2O) (**D**) of 'Fino 95' lemon plants grown at two levels of salinity (0 and 30 mM NaCl) under greenhouse conditions. *, ** and *** indicate significant differences at the 0.05, 0.01 and 0.001 levels of probability, respectively. Non-significant differences at the 0.05 level of probability are indicated as ns. Different letters indicate significant differences between treatments according to Duncan's multiple range test at the 95% confidence level.

4. Discussion

Some previous reports in citrus plants showed a decrease in root mycorrhizal colonization with salinity when compared with no-NaCl treatment [39–41], which was attributed to both a reduced hyphal extension of AMF and a decreased availability of inoculum sporulation due to a poorer development of spores. However, the salinity levels used in

these previous studies were much higher (75, 100, or even 150 mM NaCl) than those used in this experiment (30 mM NaCl), and we only observed a slight and non-significant decrease in the arbuscular colonization due to salinity.

Our results indicated that AMF mitigated the harmful effects of salinity stress the on plant growth performance of lemon trees grafted on *C. macrophylla* rootstock. A similar positive resistance to soil salinity was previously observed in citrus plants by some authors when *R. irregularis* and/or *F. mosseae* were used [23,25,27,41]. In all of these studies, it was concluded that mycorrhizal citrus plants had a better growth than non-mycorrhizal ones under salinity conditions. A meta-analysis of the published studies to determine how AMF influences plant responses under salt-stressed conditions indicated that mycorrhizal inoculation had a significant impact on some physiological and biochemical variables of the plant related to plant growth, photosynthesis, and defense against oxidative damage under salinity stress [42]. In this meta-analysis, it was confirmed that AMF inoculation increases the biomass of host plants and that the effect of AMF on plant growth is more prominent under salinity stress than under normal conditions. The different mechanisms that AMF utilizes to enhance salt tolerance in host plants have been summarized in several reviews [43–48]: enhancing nutrient uptake, inhibiting a high uptake of Na^+ and Cl^- and their transport to plant shoots, improving water uptake, accumulating proline and polyamines or having a better oxidant response. Other physiological mechanisms induced by AMF may include osmotic adjustment, which helps to maintain the leaf turgor pressure, and effects on photosynthesis, transpiration, stomatal conductance, and water use efficiency. In our experiment, some of these mechanisms have been involved in the beneficial effect of AMF on the growth of 'Fino 95' lemon plants under saline conditions.

First of all, a positive effect of AMF on the plant water status was observed. Under salinity stress, this is the first cause of plant growth suppression. The reduction in Ψ_x in both −AM and +AM plants by salinity is associated with the reduction in water potential in the root environment and, therefore, with the water absorption capacity of the plant. The lower reduction in Ψ_x due to salinity in +AM plants indicated that AMF inoculation improved plant water status under saline conditions. Changes in leaf water potential under saline conditions stimulated by AMF have been previously described [41,43,47]. The higher absorption of water of +AM plants could be explained by an improved hydraulic conductivity attributed to AMF-induced altered root morphology, as well as their ability to explore soil well beyond depletion due to the external hyphae, which can help the host plant to absorb more water from soils [49,50]. Furthermore, the better water status in +AM plants may be also explained by the AMF-regulated expression of aquaporin (AQP) genes present in the leaves and roots of salt-stressed plants. AQPs are the crucial pathway to transport water and nutrients, and AMF upregulated their expression in the host under salinity conditions, revealing the important regulation of plant water by AMF [41,51]. Recent studies in trifoliate orange have shown that fourteen AQPs were dramatically induced by AMF inoculation under salt stress [41], revealing that AMF can increase the expression of AQP genes under salt stress, which improves the membrane's water permeability and stimulates the host's water transport.

Additionally, mycorrhizal plants under salt stress showed a better osmoregulation. Under saline conditions, plants can use osmoregulation as a mechanism to tolerate salt stress, lowering their osmotic potential due to the accumulation of osmolytes [5,14]. Under saline conditions, +AM plants reduced their Π, and this reduction was due to an active solute accumulation, not to a concentration effect for a possible dehydration of plants. This lowering of Π allowed them to maintain the turgor potential within similar values to those of control plants (Table 2). Some of the key osmolytes that help plants to maintain cell turgor are proline and QAC [52]. A large number of plant species accumulate proline in response to salinity, since it plays a role in their defense against salinity stress. In our experiment, the protection of +AM plants against salinity was not mediated by an increased accumulation of proline, since lower proline concentrations were found in these plants. Some authors have stated that these lower proline concentrations in +AM plants occur because they

suffer less stress than −AM plants [53,54]. In fact, in our experiment, +AM plants under salt stress had a better water status, as revealed by their higher Ψ_x with regard to −AM plants. In addition, the inoculation of AMF has been reported to modulate the biosynthesis of glycine betaine, probably the most thoroughly studied QAC, thereby protecting and improving photosynthetic efficiency [55,56]. In our experiment, the decrease in Π in +AM plants treated with 30 mM NaCl was partly due to a higher accumulation of QAC than in non-salinized plants (Figure 3). Neither Na^+ nor Cl^- concentrations were involved in the osmotic adjustment observed in +AM plants in the 30 mM NaCl treatment.

With regard to the effect of AMF on the inhibition of a high uptake of Na^+ and Cl^-, no effects were found. The salinity level used in the present experiment was low, so it is possible that Na^+ concentrations in roots and leaves were not high enough for the AMF to regulate Na^+ uptake, and the results show no effect of the fungi on the Na^+ concentrations in roots or leaves under control or saline conditions (Tables 3 and 4). Previous studies have suggested the possibility of a buffering effect of AMF on the uptake of Na^+ when the content of the latter is within acceptable limits [43,57]. Moreover, the lack of an accumulation of Na^+ in shoots in parallel to Cl^- in our experiment could be related to the scion vigor. In grafted plants, the rootstock is not the only element that controls the Cl^- and Na^+ transport to the shoot; the scion may also have an influence on their accumulation in leaves [12,58]. The grafting of lemon scion varieties on sour orange and on *C. macrophylla* produced variable effects on the Na^+ and Cl^- concentrations of the leaf [58], so the mechanism by which some rootstocks reduce the concentration of ions seems to depend on the vigor of the scion and on water requirements [12].

However, AMF can modify the altered nutrient uptake that can occur due to the high concentration of Na^+ and Cl^-. Maintaining a high K^+/Na^+ ratio in inoculated plants is important for proper metabolic functioning [59]. In citrus plants, mycorrhizal symbiosis enhances salinity tolerance through the selective absorption of K^+/Na^+ but not through their selective transport from roots to leaves, which is reduced by mycorrhizal treatment under salt stress [60]. In our experiment, since AMF did not increase the abundance of K^+ in roots or leaves nor did they decrease that of Na^+ under salinity, plant inoculation offered no advantage with regard to maintaining higher K^+/Na^+ ratios in +AM plants under saline conditions. On the other hand, it has been shown that AMF selectively takes up other elements, such as Mg^{2+} and Ca^{2+}, while diminishing the uptake of potentially toxic Na^+ [21,57,61] to keep the internal Mg^{2+}/Na^+ and Ca^{2+}/Na^+ ratios within narrow limits that help to maintain the integrity of the membrane and protect the host plants against salt damage. Although Ca^{2+} and Mg^{2+} concentrations in mycorrhizal lemon roots were increased under saline conditions, Na^+ levels in the root were also heightened and, as a consequence, the rise in these nutrients was not enough to improve the Ca^{2+}/Na^+ and Mg^{2+}/Na^+ ratios of the roots. However, the Mg^{2+}/Na^+ ratio increased in the leaves of mycorrhizal lemon plants exposed to salt stress, which suggests that mycorrhizal colonization alleviated salt stress by reducing the antagonistic effect of Na^+ [62].

The positive effect of AMF under saline conditions can also be related to the concentration of some micronutrients. An excessive amount of Na^+ and Cl^- in the soil contributes to limiting the availability of Fe, Zn, and Cu [63]. However, extraradical hyphae can bridge the zone of nutrient depletion adjacent to the root and thus increase the availability of elements of low mobility, such as Zn, by transporting them from more distant sites across the hyphae to the plant root [64,65]. This can be the reason why Zn concentration in both roots and leaves of control and salinized plants was significantly increased by AMF (Tables 3 and 4). In addition, AMF contributed to better Fe nutrition in both the control and saline treatments. On the contrary, although mycorrhizal hyphae also increased the availability of the immobile Cu in the soil by translocating it from more remote sites through the hyphae to the plant root [64], we found no effect of AMF on the Cu concentration in roots and leaves. The lower Mn concentrations found in +AM plants could be due to a reduction in Mn uptake by hyphae in +AM plants. The solubility of Mn in the soil may decrease due to changes in the oxidation state influenced by the pH of the mycorrhizosphere, or

to a decreased Mn^{4+} reducing potential in the rhizosphere of mycorrhizal plants, likely attributable to a smaller population of Mn-reducing organisms [66,67].

Another important nutrient that can be modified by AMF is P. Mycorrhizal association increases the acquisition of P by the host plant [68], and the improved growth of mycorrhizal plants in saline conditions is primarily related to a mycorrhiza-mediated enhancement of the host plant P nutrition [23,69,70]. In the present experiment, +AM plants were not fertilized with P, and we expected results to be similar to those previously found in other experiments where +AM plants had not been fertilized with P; besides that, they had high P concentrations because of the P input coming from the fungus [22,71]. All of these experiments were conducted in pots containing substrate composed by soil/sand (1:3) where, due to the high levels of $CaCO_3$ (19%, typical of southeastern Spain), a significant portion of the high soil P concentration (72 ppm Olsen P) could be in insoluble form. However, we anticipated that the fungal hyphae secretion of acid and alkaline phosphatases [42] would release and solubilize enough P to provide an adequate P nutrition throughout the experiment, as has been observed in previous experiments [22,71]. Contrary to preceding results, the concentrations of P in the roots and leaves of +AM plants were lower than those found in −AM plants (Tables 3 and 4). However, +AM plants grown without P fertilization were able to take up high amounts of P (25.7 mg kg^{-1} DW, Table 3). Even if these P concentrations were below the optimum range of P for adult citrus trees (37–50 mg kg^{-1} DW, [72]), they were sufficient to sustain the proper growth of citrus lemon plants (Figure 2) and adequate to support the healthy growth of young citrus seedlings without any deficiency symptoms. In a previous experiment performed with *C. macrophylla* plants fed with 0 mM of P, foliar concentrations of P in both +AM and −AM plants subjected to P starvation were even lower than those found in this experiment, and no visual deficiency symptoms were found in plants [73]. The results found in our experiment showed that, even if AMF can counteract to some extent the imposed P starvation, the application of a regular P fertilization to −AM plants certainly affected the P availability and may explain the results obtained with this particular combination of plants and AMF. In light of these results and due to the P concentrations found in +AM plants, we suspect that this P starvation could have produced a new stress situation; although this circumstance did not affect plant growth, it probably modified the balanced AMF/plant relationship.

Another physiological mechanism that AMF may alter under salinity stress is photosynthesis. Salt stress impedes photosynthesis and brings an enormous decline in crop productivity. However, our results showed that AMF could protect plants against salt stress through the maintenance of the gas exchange capacity, since there was no decrease in A due to salinity. According to previous results, we observed similar g_s values in +AM and −AM plants under saline conditions and a lower reduction in g_s in +AM plants than in −AM plants with increasing salinity, which suggests a decreased reduction in CO_2 diffusion through the stomata and, hence, a better water status in +AM plants [74,75]. Consistent with our results, a positive effect of AMF colonization on the gas exchange capacity of salinity-exposed citrus plants was previously observed [22,24,76]. The positive role of AMF symbiosis in the alleviation of the negative effects of salinity on the photosynthetic capacity of the plant has been summarized in several reports [42,47,74]: an improvement in water status that results in maintaining a higher stomatal conductance and, consequently, a better CO_2 assimilation; due to a higher expression of Rubisco, an increase in its activity, which allows the abatement of the intercellular CO_2 concentration and ensures the protection of the photosynthetic capacity; maintenance of the integrity of PSII as a result of a higher concentration of polyamines and glycine betaine, which stabilizes PSII pigment–protein complexes, as well as the protection of the activities of enzymes such as Rubisco; the maintenance of a higher chlorophyll and carotenoid concentration by improving the uptake of Mg^{2+}, enhancing photosynthetic efficiency via a proficient conversion of harvested light into chemical energy; and the minimization of NPQ. In our experiment, salinity did not decrease the photosynthetic rate in +AM plants, probably due to some of these advantages that AMF provides to plants to alleviate the effects of salinity. In this way, +AM

plants under 30 mM NaCl improved their water status with regard to –AM plants (Table 2), and they had higher g_s than −AM plants (Figure 5); additionally, +AM plants under salinity treatment had a higher QAC concentration (Figure 3), which probably helped to maintain the integrity of PSII; and finally, the higher concentration of Mg^{2+} in the plant (Figure 4) could be probably related with a higher photosynthesis rate through a higher chlorophyll concentration.

On the other hand, inoculation significantly decreased the photosynthetic capacity of the plants under control conditions, probably due to the fact that the relationship established between the plants and AMF under non-saline conditions was not a mutualistic relationship as balanced as those we found in previous studies with the same rootstock [22]. For a given plant species, this relationship could differ among growth stages. Some plant species show a much greater benefit from an association with an AM fungus in the seedling stage than being adult plants [75]. In our previous studies, the colonization of *C. macrophylla* plants by *R. irregularis* and *F. mosseae* significantly improved gaseous interchange parameters at the seedling stage [22]. In the present experiment, we used older *C. macrophylla* plants; moreover, these plants were grafted with 'Fino 95' lemon, which could have had an influence on their response to AMF. In addition to all of this, the AMF–host plant relations are context-dependent and can be mediated by environmental conditions [76,77]. As Jin et al. [78] pointed out, the C cost analyses of plants suggest that the effectiveness of AMF varies from mutualistic to parasitic, which depends primarily on the soil nutrient availability. Under our P starvation conditions, AMF did not improve plant growth in non-saline conditions, even when plants received most likely a major part of P via the AMF pathway, as was previously found [79–81]. Under these conditions, the photosynthesis rate was reduced, so the amount of C available for the plant and the fungus also decreased, reducing plant growth. The C-for-nutrient trade is the key factor in predicting the outcome of AMF symbioses, but our results showed a complex "cost/benefit" analysis.

Finally, as per our results, AMF mitigated the harmful effects of salinity stress on plant growth due to a better antioxidant response. Exposure to salt stress can lead to a detrimental impact on membrane integrity due to the generation of reactive oxygen species (ROS), which induce the release of electrolytes from cellular membranes, consequently disrupting the functionality of those membranes and upsetting the metabolic balance [82]. The lipid membrane of cells is damaged by oxidative burst, resulting in lipid peroxidation that can be measured by the content of malondialdehyde (MDA), the final product. In our experiment, no accumulation of MDA was observed in inoculated plants by salinity, so AMF alleviates salt-induced oxidative damage by protecting membrane lipids from ROS oxidation, as previously observed [83,84]. Furthermore, AMF-inoculated plants have higher levels of polyunsaturated fatty acids in membranes, thus enhancing plasma membrane integrity, reducing cellular component leakage, and leading to decreased ROS production [55].

5. Conclusions

The results of this study support the research hypothesis that the inoculation of AMF can mitigate the adverse effects of salinity on citrus plants, particularly in terms of plant growth and physiological responses. Whereas AMF did not consistently improve mineral nutrition or gas exchange parameters, their positive impact on plant growth under saline conditions is noteworthy. However, the complex nature of the relationship between AMF and citrus plants suggests that further research is needed to fully understand their interaction, particularly considering factors such as plant age and the influence of the scion on the rootstock response. Future studies should focus on exploring the chemical signaling between the root and the aerial parts to comprehensively understand the effect of the rootstock on the scion. This will provide deeper insights into the intricate dynamics of AMF–plant interactions and their implications for improving citrus cultivation practices.

This research underscores the significance of utilizing AMFs as viable options as biofertilizers in the cultivation of citrus trees, especially in regions where saline water is commonly used for irrigation. By harnessing the symbiotic relationship between plants

and AMFs, citrus growers can mitigate the detrimental effects of salinity on crop productivity, offering valuable insights for developing sustainable agricultural practices in the Mediterranean regions. Future research endeavors should explore the optimization of the symbiotic relationship between AMFs and citrus plants to maximize their potential benefits and address the remaining challenges in Mediterranean agriculture.

Author Contributions: Conceptualization, J.M.N., O.P.-T., and A.M.; methodology, J.M.N., O.P.-T., and A.M.; formal analysis, J.M.N.; investigation, J.M.N., O.P.-T., and A.M.; data curation, J.M.N. and O.P.-T.; writing—original draft preparation, J.M.N.; writing—review and editing, J.M.N. and A.M.; funding acquisition, J.M.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by 15369/PI/10 Fundación Séneca (Region of Murcia, Spain) and POI07-12 (Instituto Murciano de Investigación y Desarrollo Agrario y Medioambiental), as well as the FEDER Funds.

Data Availability Statement: Data will be made available upon request. The raw data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: We acknowledge Eva Arques for her help with the analysis..

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

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