Ammonium enhances resistance to salinity stress in citrus plants

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ABSTRACT

In this work, we demonstrate that NH_4^+ nutrition in *citrange Carrizo* plants acts as an inducer of resistance against salinity conditions. We investigated its mode of action and provide evidence that NH_4^+ confers resistance by priming abscisic acid and polyamines, just as enhancing H_2O_2 and proline basal content. Moreover it observed a diminished Cl^- uptake as well as an enhanced *PHGPx* expression after salt stress. Control and N-NH_4^+ plants have shown optimal growth, however it was observed that N-NH_4^+ plants have displayed greater dry weight and total lateral roots than control plants, but that differences are not seen for primary roots length. Our results reveal that N-NH_4^+ treatment induces a similar phenotypical response to the recent stress-induced morphogenetic response (SIMRs). The hypothesis is that N-NH_4^+ treatment triggers mild chronic stress in *citrange Carrizo* plants, which might explain the SIMR observed. Moreover, we observed modulators of stress signaling, such as H_2O_2 in N-NH_4^+ plants, which could acts as an intermediary between stress and the development of the SIMR phenotype. This observation suggests that NH_4^+ treatments induce a mild stress condition that primes the *citrange Carrizo* defense response by stress imprinting and confers protection against a subsequent salt stress.

KEY WORDS

Ammonium nutrition; citrus; salinity and SIMR.

ABBREVIATIONS

AOS, allene oxide synthase; DAB, diaminobenzidine; DW, dry weight; FW, fresh weight; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; HFBA, heptafluorobutyric acid; *PHGPx,* phospholipid hydroperoxide glutathione peroxidase; *PR5*, osmotin-like; PAs, polyamines; PUT, putrescine; *RD22,* responsive to dessication 22; ROS, reactive oxygen species; SIMRs, stress induced morphogenetic response; SPD, spermidine; SPM, spermine.

INTRODUCTION

Nitrate (NO_3^-) and ammonium (NH_4^+) are the main inorganic nitrogen (N) resources absorbed by the roots of higher plants. Nitrogen is used to form glutamine, a precursor of many amino acids, like nucleic acids, alkaloids and polysaccharides, as well as secondary metabolites like PAs (Bagh et al.,, 2004). It is generally accepted that many plants display optimal growth and development if nitrogen is available in the form of NO_3^- (Coruzzi and Bush, 2001). Although NO_3^- uptake consumes more energy than NH_4^+, only a few plant species display optimal growth when N is available in only the NH_4^+ form (Marschner, 1995). Camañes et al., (2009) demonstrated that citrus plants prefer to absorb NH_4^+ more than NO_3^- when both N forms are present in the nutrient solution, which is probably due to less energy in the assimilation ion process. NH_4^+ is a paradoxical nutrient ion because, despite being a major N source and an important intermediate in many metabolic reactions, there are reports that high concentrations of this ion in either soil or the nutrient solution may lead to an "ammonium syndrome". This may include leaf chlorosis, lower plant yield production and root/shoot ratio, lower cation content, acidification of the rizosphere and changes in several metabolites levels, such as amino acids or organic acids (Britto and Kronzucker, 2002). In spite of the information available about the appearance of toxic symptoms due to NH_4^+ nutrition, different studies have produced contradictory results. This could be explained by each plant's specific and varietal characteristics, and by experimental conditions. Thus, there is a wide range of plant responses to NH_4^+ nutrition; there are some species that are tolerant to high NH_4^+ doses, such as rice (Wang et al., 1993), and some very sensitive species which practically cannot survive under NH_4^+ nutrition, such as tomato or barley (Britto et al., 2001).

Salinity is amongst the most significant environmental factors responsible for substantial losses in agricultural production worldwide and it is one of the serious problems confronting sustainable agriculture in irrigated production systems in arid and semiarid regions

(Marschner 1995; Ravindran et al., 2007). Nearly 20% of the world's cultivated area and about half of the world's irrigated lands are affected by this stress (Munns and Tester, 2008). This is a critical problem especially in citrus since they are one of the most globally important horticultural crops considered salt sensitive (Al-Yassin 2005). Salinity causes several injuries in citrus such as tissue burning, loss of yield, leaf abscission and finally plant death (Romero-Aranda et al., 1998). Identifying successful strategies that enhance salinity resistance to this plant species is of both agronomic and economic interest. This complex environmental stress presents three different components: an ionic component linked to the accumulation of ions, which become toxic at high salt concentrations (mainly Na^+ and Cl^-) in the cytoplasm, leading to ionic imbalance; an osmotic component due to the compartmentalization of this toxic ion in the vacuole. When this compartmentalization occurs in cells, the cytosol water potential must be lowered to balance the low-external water potential, thus ensuring water intake in the plant cell and avoiding macromolecule damage (Ellouzi et al., 2011). Apart from the toxic and osmotic effects of salinity, a high cellular NaCl concentration causes enhanced formation of reactive oxygen species ROS (Hernandez and Almansa, 2002). ROS are highly reactive and, in the absence of any scavenging mechanism, they can provoke major alterations in normal metabolism through oxidative damage to lipids, proteins and nucleic acids (Foyer and Noctor, 2005). However, transient ROS formation apart from causing oxidative damage when present at high concentrations can play a signaling protective role in the short term (Dat et al., 2000). The role of some ROS, such as signal molecules in biotic or abiotic stress, is of biological significance because the production of these molecules could benefit the plants brought into a state of acclimation (Foyer et al., 1997, Jubany-Marí et al., 2009). Similarly, plant hormones play an important role in response to unfavorable environmental conditions. They are involved in signaling response to drought and salinity by the activation of acclimation processes such as stomatal closure, regulation of hydraulic conductivity and regulation of

developmental processes that affect stress tolerance, such as senescence abscission (Sakamoto et al., 2008). Plants are sessile organisms that have developed an extensive array of defensive responses. An important aspect related to response to a range of biotic and abiotic stress is the phenomenon of priming (Van der Ent et al., 2009). Preliminary stress exposure or stress imprinting is indeed necessary to induce priming, which makes the plants more resistant to future biotic or abiotic stress (Conrath et al., 2006; Bruce et al., 2007; Galis et al., 2009). Priming state can be induced by different biological or chemical stimuli. Some quemical inducers are 2,6-dichloro isonicotinic acid (INA), benzo-(1,2,3)-thiadiazole-7-carbotionic acid S-methyl ester (BTH), β-aminobutyric acid (BABA) (Oostendorp et al., 2001; Conrath et al., 2002) and hexanoic acid, which by root treatment protects tomato plants and Arabidopsis against *Botrytis cinerea* (Vicedo et al., 2009; Kravchuk et al., 2011). Regarding abitotic stress, BABA has been shown to confer plant protection against salinity and drought (Jakab et al., 2005; Macarisin et al., 2009). Moreover, stress can also boost plant stress tolerance through induction of acclimation responses. Tolerance can be linked to an array of morphological, physiological and biochemical responses which lower the stress exposure limit and damage, or facilitate the repair of damaged systems (Mittler 2002). After exposure to stress, various changes take place, leading to different phenotypes depending on the type of stress, its duration or experimental conditions. However, a common response in all these responses occurs, which is known as "stress-induced morphogenic responses" (SIMRs). Exposure of plants to mild chronic stress could cause induction of these specific SIMRs. These responses are characterized by blockage of cell division in the main meristematic tissues, inhibition of elongation and redirected outgrowth of lateral organs. Furthermore, it is believed that this process brings about a rise in ROS species and alters different hormones (Potters et al., 2007). The induced resistance against abiotic stress in citrus and other woody species has not been explorer, and understanding the molecular mechanisms beneath this

process will provide the necessary insights to exploit this phenomenon in sustainable agriculture.

In this work, we demonstrate that NH_4^+ nutrition in *citrange Carrizo* plants enhanced resistance to salinity conditions. We investigated its mode of action, and provide evidence that NH_4^+ primes *citrange Carrizo*'s defenses by enhancing abscisic acid (ABA), PAs and potentiating H_2O_2 and proline basal content as well as diminished Cl^- uptake.

MATERIAL AND METHODS

Plant material, growth conditions and nutrition treatments

citrange Carrizo seeds (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L.) (Beniplant, Valencia, Spain) were allowed to germinate in vermiculite in a growth chamber under the following environmental conditions: light/dark cycle of 16/8 h, temperature of 20/24°C, light intensity of 200 μmol m^{-2} s^{-1} and relative humidity of 70%. Seeds were irrigated twice a week with distilled water. After 6 weeks, seedlings were irrigated for two months with Hoagland solution lacking nitrogen (Hoagland and Arnon, 1950) complemented with 1 mM NH_4NO_3 (control treatment) and 5 mM of N-NH_4^+ [$(NH_4)_2SO_4$)] (NH_4^+ treatment). Then 1.5 mM K_2SO_4 and 3 mM $CaSO_4$ were added to compensate for the absence of K^+ and Ca^{2+}. The pH of the nutrient solution was adjusted to 6.0 with 1 mM KOH.

Prior to the experiments, 3-month-old plants with a single shoot were selected for uniformity of size, and transferred to an aerated complemented Hoagland solution for 7 days in hydroponic culture devices.

To salt stress, 90 mM NaCl were added to the hydroponic solution and renewed twice weekly. Samples were taken for individual analysis at 2 h and 14 d after addition of salt to the hydroponic solution. At the end of the experiment, the phenotype was determined by the percentage of leaves with symptoms of necrosis or burns.

Growth and damage of salt values

The DW and length primary roots were measured at each sampling. The lengths of the individual primary roots of individual seedling were measured directly. Dry weight refers to the total roots (primary, secondary and tertiary) of the individual seedlings.

To measure the damage provoked by high salinity in the medium soil, we also established three damage levels: healthy leaves, chlorotic leaves (level 1), leaves with necrosis (level 2) and burnt leaves (level 3).

Quantitative RT-qPCR analysis

Gene expression by quantitative real-time PCR (RT-qPCR) was performed using RNA samples extracted from leaf tissue using the Total Quick RNA cells and tissues kit (Talent; http://www.spin.it/talent). Citrus leaves tissue samples for RNA isolation were collected at 2 h and 14 d after NaCl treatment. Leaf tissue was collected from treated and untreated plants. The RT-qPCR conditions were those described by Flors et al. (2007). A list of the primers used in the RT-qPCR is shown in Table 1, using *GAPDH* gene expression of citrus how an internal standard.

Chromatographic analysis.

Hormone extraction and quantification were performed as described in Flors et al. (2008). Briefly, fresh material was frozen in liquid nitrogen. Before extraction, a mixture of internal standards containing 100 ng [^{2}H6]-ABA and 100 ng [^{2}H4]-SA was added. Dry tissue (0.05 g) was immediately homogenized in 2.5 mL of ultrapure water. After centrifugation (5000 g, 40 min), the supernatant was recovered and adjusted to pH 2.8 with 6% acetic acid, and subsequently partitioned twice against an equal volume of diethyl ether. The aqueous phase was discarded, the organic fraction evaporated in a Speed Vaccuum Concentrator (Eppendorf;

www.eppendorf.com) at room temperature, and the solid residue re-suspended in 1 mL of a water/methanol (90:10) solution and filtered through a 0.22 μm cellulose acetate filter. A 20 μl aliquot of this solution was then directly injected into the HPLC system.

For PAs analysis, fresh material was frozen in liquid nitrogen. Before extraction, according to the method of (Sánchez-López et al., 2009) a mixture of internal standards containing $^{13}C_4$-putrescina and 1,7-diaminoheptane, was added. Dry tissue (0.02 g) was homogenized in 2 mL of 2% perchloric acid. After centrifugation (5000 g, 40 min), the supernatant was separated 2 mL of 2% perchloric acid were add to the pellet and centrifugation was repeated. Then both supernatants were collected and a mixture of 10% MeOH and HFBA 25 mM was added until 6 mL. Next 1 mL of the mixture was taken and filtered through a 0.45 μm cellulose acetate filter. A 20 μl aliquot of this solution was directly injected into the HPLC system. Analyses of hormone and PAs sample were carried out using a Waters Alliance 2690 HPLC system (Milford, MA, USA) with a nucleosil ODS reversed-phase column (100 x 2 mm i.d.; 5 mL; Scharlab, Barcelona, Spain; http:// www.scharlab.es). The chromatographic system was interfaced to a Quatro LC (quadrupole-hexapole-quadrupole) mass spectrometer (Micromass; http://www.micromass.co.uk). The MASSLYNX NT software version 4.1 (Micromass) was used to process the quantitative data from calibration standards and plant samples.

Chloride analyses

Chloride measurements were taken by automatic titration using a chloridemeter (Model 926, Sherwood Scientific Ltd., Cambridge, UK), as described in López-Climent et al. (2008). Briefly, ground plant material was incubated overnight in a mixture of 0.1 N HNO_3 (Panreac, Barcelona, Spain) and 10% glacial acetic acid (Panreac) under continuous shaking. The

supernatant was filtered through Whatmann #1 filter paper and 0.5 mL used for determinations.

Proline analyses

Leaf proline content was determined by a spectrophotometric assay, as described in Bates et al. (1973). Briefly, 50 mg of frozen plant material was extracted in 5mL of 3% sulphosalicylic acid (Panreac). After centrifugation at 4000×g and 4 ºC, 1 mL of supernatant was combined with 1 mL of glacial acetic acid (Panreac) and 1 mL of ninhydrin (Panreac) solution. The combined solution was incubated at 80 ºC in a water bath for 1 h and the resulting mixture was partitioned against 2 mL of toluene after a cooling period. Absorbance at 520 nm was read in the organic layer against a blank. Determinations were performed using commercial proline as a standard (Sigma-Aldrich, Madrid, Spain).

DAB staining and H_2O_2 quantification.

$N\text{-}NH_4^+$ and control plants were exposed to 90 mM NaCl for 2 h, and the salt-stressed leaves were stained in 1 mg of DAB per milliliter at pH < 3 for 24 h in the dark and were subsequently destained in 95% ethanol. Later, samples were rehydrated with distilled water. DAB staining intensities were quantified from digital photographs by the number of dark-brown DAB pixels in relation to the total pixels corresponding to plant material, using GIMP2 program.

Statistical analysis

Statistical analysis was carried out using the Statgraphics software support. Data are expressed as means and standard error. Mean values were compared by an LSD (least significant difference) test. All experiments were repeated at least three times.

RESULTS

Development of the *citrange Carrizo* plants grown under the NH_4^+ condition

Three-month-old *citrange Carrizo* plants were grown for 2 months with Hoagland solution lacking nitrogen, but complemented with 1 mM NH_4NO_3 (control plants) and 5 mM $(NH_4)_2SO_4$ (N-NH_4^+plants). Although other studies have shown that some species develop toxic symptoms when only NH_4^+ nutrition is applied (Gerendás et al., 1997; Lasa et al., 2001), the *citrange Carrizo* N-NH_4^+ plants displayed optimal growth, estimated on the basis of biomass production (Fig. 1A). Moreover, we observed that N-NH_4^+ plants developed a darker green color, and their chlorophyll content was 13.12% higher than in the control plants (data not shown). Likewise, the N-NH_4^+ plants showed vigorous root growth, estimated on the basis of the DW of total roots, which was higher in the N-NH_4^+ plants if compared with the control plants (Fig. 1C). However, primary root length did not differ between the control and the NH_4^+ treated plants (Fig. 1B). The secondary and tertiary lateral roots of the N-NH_4^+ plants developed more than control plants. NH_4^+ treatment increased the number of lateral roots per DW of the total roots of the individual seedlings, as well as the number of total roots per primary root length (Figs. 1D and E).

NH_4^+ treatment enhances *citrange Carrizo* resistance to salt stress.

The increased levels of NaCl in the watering solution led to different levels of damage in plants. To achieve salt stress, 90 mM NaCl were added to the hydroponic solution to the control and the N-NH_4^+ plants over a 14-day period. In order to check how NH_4^+ nutrition could affect the response of *citrange Carrizo* plants to salt stress, necrosis and burns on leaves were estimated. The result was expressed as percentage of damaged leaf in relation to the total leaves per plant by establishing the following damage levels: healthy leaves, chlorotic leaves (level 1), leaves with necrosis (level 2) and burnt leaves (level 3) (Fig. 2). In this case,

significant differences between the control and the N-NH_4^+ salt-treated plants were noted as the N-NH_4^+ plants displayed 30% more healthy leaves than the control plants. Both the control and the N-NH_4^+ plants had a similar percentage of chlorotic and necrotic leaves but, interestingly, the level of burnt leaves was higher in the control plants.

NH_4^+ treatment reduced *citrange Carrizo* toxic and osmotic stress

Salinity tolerance in *Citrus* is strongly related to leaf chloride accumulation. It is well-known that Cl^- toxicity, rather than Na^+ toxicity, is the primary factor involved in the molecular responses of citrus plant leaves to salinity (Brumós et al., 2009). We observed that the Cl^- concentration in leaves increased in both treatments, in the control and the N-NH_4^+ plants upon salt stress (Fig. 3A). The highest leaf Cl^- concentration occurred in the control plants at 14d, at which time the Cl^- concentration noted for the leaves of the N-NH_4^+ plants had reduced by 24% when compared with the control plants leaves.

It is well-known that soil with high salt concentrations is virtually dry because the available water is trapped by ions. Proline has been considered to play an important role in plant response to salinity (Gaspar et al., 2002) since it acts as a compatible solute that adjusts the osmotic potential in the cytoplasm (Bartels and Sunkar, 2005). In order to assess the effectiveness of N-NH_4^+ treatment against osmotic stress induced by NaCl, we tested proline content in leaves. The basal proline content differed between the control and the N-NH_4^+ plants (Fig. 3B). The N-NH_4^+ plants had higher proline content at 2 h and 14 d in the absence of salt if compared with the control plants. Interestingly the proline content of both treatments significantly increased after salinity, with no statistically significant differences between the control and the N-NH_4^+-treated plants. Proline content increased by 19.53% in the control plants upon salinity treatment, while it increased by only 9.30% in the N-NH_4^+ plants.

NH_4^+ treatments enhance H_2O_2 accumulation

In this work, we used DAB staining to establish how NH_4^+ treatment affects cellular oxidative stress. The H_2O_2 staining based on the *in vivo* reaction of H_2O_2 with DAB allows a rapid estimation of H_2O_2 accumulation in leaves (Thordal-Christensen et al., 1997). Our results indicate that the N-NH_4^+ plants show higher initial levels of H_2O_2 accumulation than the control plants in the absence of salt stress. Increased H_2O_2 accumulation was noted 2 h after treatment with 90 mM NaCl. Although higher H_2O_2 accumulation was seen in the control plants in response to salt stress, the highest levels of H_2O_2 accumulation were observed for the N-NH_4^+ plants treated with NaCl (Fig. 4)

NH_4^+ treatment induces the main hormone signaling pathways

In order to establish whether enhanced resistance of NH_4^+ is mediated by the induction of the ABA-, salicylic acid- (SA) and jasmonic acid - (JA) signaling pathways, the *RD22*, *PR5* and *AOS* marker genes expressions were analyzed by RT-qPCR (Fig. 5). These genes have been previously reported to be salt stress inducible in different species (Zhu et al., 1995; Nylander et al., 2001; Pendranzani et al., 2003). We observed that NH_4^+ treatments induced *RD22* mRNA accumulation in the absence of salt stress and that *RD22* mRNA accumulation in response to salt stress lightly increased after salinity in both treatments (Fig. 5A). NH_4^+ treatment induced *PR5* mRNA accumulation in the absence of salt stress, however, upon salinity stress both treatments induced the *PR5* expression at 14 d, but greater inductions were observed in the control plants than in the N-NH_4^+ plants (Fig. 5B). We also checked JA-dependent signaling pathway transduction after NH_4^+ treatment in *citrange Carrizo* plants. NaCl treatment induced *AOS* mRNA accumulation in both treatments, but the N-NH_4^+ plants exhibited greater accumulation upon salinity when compared with the control plants (Fig. 5C). The expression patterns of the markers genes for the ABA, SA and JA pathways indicate

that all the pathways were more induced in the NH_4^+ treated plants than in the control ones. In order to further confirm the possible role of the different signaling pathways in NH_4^+ resistance, we analyzed the hormonal levels in both the control and the N-NH_4^+ treated plants at 14 d after salinity stress. The basal ABA levels differed between control and the N-NH_4^+ plants in the absence of salt, but were higher in the N-NH_4^+ plants (Fig. 6A). The control plants revealed increased ABA accumulation 14 d after salt stress, as expected, but no such increase was observed in the N-NH_4^+ plants upon salt stress. These initial ABA accumulations suggest that the resistance induced by NH_4^+ treatment could be mediated by this hormone, which plays a role in defense signaling in osmotic and salt stresses (Jakab et al., 2005). The basal SA levels did not differ between the control and the NH_4^+-treated plants before salinity, but ranged between 25 ng g^{-1} and 38 ng g^{-1} FW (Fig. 6B). Interestingly, the control plants displayed a significant increase in SA accumulation at 14 h after salinity which was not observed in the N-NH_4^+-treated plants upon salinity. No differences in JA levels were observed in either the control or the NH_4^+-treated plants in the absence or the presence of salt stress (data not shown).

NH_4^+ treatments reduced the oxidative damage caused by salt stress. Polyamines content and *PHGPx* expression

PAs play a key role in plant responses to salinity. These compounds have been tested not only as antioxidants, but also as osmoprotectors under salinity conditions (Groppa et al., 2001; Chattopadhayay et al., 2002; Kakkar and Sawhney, 2003). In order to determine whether NH_4^+ treatment affects PAs content, leaf samples were analyzed at 14 d in the control and the N-NH_4^+ plants, and also in these plants after salt stress. In the N-NH_4^+ plants, the concentrations of Put, Spd and Spm were higher than in the control plants in the absence of salt. It is interesting to note that the Put titer increase was especially important. The Put

concentration in the control plants was 6.16 ng mg $^{-1}$ FW, while it was 146.98 ng mg $^{-1}$ FW in the N-NH_4^+ plants. However, the Put, Spd and Spm contents remained unaffected in the control and the N-NH_4^+ plants after salt stress (Table 2). Put accumulations in N-NH_4^+ plants suggest that the resistance induced by NH_4^+ treatment could be mediated by this polyamine, which reduces salt-inducible oxidative damage (Groppa and Benavides, 2008).

In order to establish whether the enhanced resistance of N-NH_4^+ treatment to salt stress is mediated by the induction of antioxidant activity pathways, the *PHGPx* gene expression was analyzed by RT-qPCR. It has been previously reported that *PHGPx* is a unique intracellular antioxidant enzyme that directly reduces phospholipid hydroperoxides produced in cell membranes under salt conditions, and has been considered the main line of enzymatic defense against oxidative biomembrane damage in mammalian cells (Chun-Juan et al., 2009). Although the *PHGPx* expression was unaffected in the absence of salt, N-NH_4^+ treatment enhanced this expression at 14 d after salt stress.

DISCUSSION

In this study, we have analyzed influences of NH_4^+ nutrition on *citrange Carrizo* plants undergoing 90 mM NaCl. *citrange Carrizo* plants were grown with 1 mM NH_4NO_3 (control plants) and 5 mM de N-NH_4^+ (N-NH_4^+ plants), they showed optimal growth in both treatments. However, we observed that the N-NH_4^+ plants had greater DW and total lateral roots than the control plants; yet these differences were not noted for primary roots length. It is commonly accepted that the root system is critical for nutrient and water uptake from soil, and that it displays considerable plasticity in response to development and environment signals (Li et al., 2010). Primary root growth is often diminished in stressful soil environments, such as those deficient in phosphate (Svistoonoff et al., 2007) or with excess aluminum (Jones and Kochian, 1995). Previous results have shown that stunted root systems

are a significant symptom of NH_4^+ toxicity and confirmed that NH_4^+ in soil inhibits primary root growth, being cell elongation but not cell division, the principal target in NH_4^+ inhibition (Li et al., 2010). We have demonstrated that NH_4^+ treatment induces lateral root development in *citrange Carrizo* plants. This is supported by the findings of Yang et al. (2011), who observed that NH_4^+ stimulated root hair branches in Arabidospsis which may be directly due to a response to NH_4^+ toxicity or NH_4^+-induced stress signals. Moreover, it has been suggested that ROS may be an NH_4^+-induced stress signal leading to the formation of hair branching. Moreover, it demonstrates that *citrange Carrizo* plants grown under N-NH_4^+ conditions are more resistant to salinity stress. Salinity stress was induced by the addition of 90 mM NaCl for 14 d to the control and the N-NH_4^+ plants. After checking for any damage induced by salinity, we found a differential response to salt stress, and the N-NH_4^+ plants presented less damage than the control plants. These data suggest that N-NH_4^+ treatment produces some response mechanism which benefits *citrange Carrizo* plants to better tolerate exposure to 90 mM NaCl. Salinity is a complex environmental stress that presets three different components: an ionic component linked to the accumulation of ions, mainly Cl^- in citrus plants (Brumós et al., 2009); an osmotic component due to compartmentalization of this toxic ion in the vacuole, which triggers accumulation of low molecular-weight osmolytes (Zhu et al., 1998); and increased ROS formation, which is considered the primary event under a variety of stress conditions (Hernandez and Almansa, 2002). N-NH_4^+-treated plants are capable of reduced Cl^- leaves accumulation after 14 d of salt exposure. The high shoot Cl^- level in the salt-treated control plants indicates this ion's poor capacity to prevent translocation to shoots. However, N-NH_4^+ treatment helps avoid leaves from accumulating Cl^-, probably by the inhibition of chlorid channel-like (CLC) proteins, as observed in barley (Lopes and Araus, 2008). Moreover, soil with high salt concentrations is practically dry because ions trap any available water. To overcome this problem, citrus responds by

overproducing compatible osmolites such as proline (Bañuls and Primo-Millo, 1992). For a long time, proline has been considered an inert compatible osmolyte which protects subcellular structures and macromolecules upon osmotic stress (Kishor et al., 2005). Several studies have shown that proline is a potent ROS scavenger associated with the prevention of apoptotic-like PCD (Chen and Dickman, 2005). Proline content in the N-NH_4^+ plants at 2 h and 14 d in the absence of salt is higher than in the control plants. Yet under salt conditions, both treatments showed an increased proline accumulation. The highest basal proline content in the N-NH_4^+ plants could confer initial protection to salt stress since proline accumulation in stressed plants has been associated with enhanced tolerance to abiotic stress conditions (Szabados and Savouré, 2009). Furthermore, it is well-known that salinity increases cellular ROS accumulation (Hernandez and Almansa, 2002). Although ROS can induce severe cellular damage, these molecules are important in signaling, since control, among others, the expression of stress tolerance (Foyer and Noctor, 2005). Control and N-NH_4^+ *citrange Carrizo* plants display considerably increased H_2O_2 accumulation 2 h after salinity stress. It is noteworthy that the initial H_2O_2 levels were higher in the N-NH_4^+ plants than in the control ones. This result supports the idea that H_2O_2 could act as a stress signal in the N-NH_4^+-treated plants.

Several studies have suggested that NH_4^+ nutrition induces a stress response in several species (Lasa et al., 2001). Here, we confirmed that N-NH_4^+ treatment enhance resistance to salt stress. Moreover, it also found that N-NH_4^+ treatment induced a similar phenotypical response to the recently stress-induced morphogenetic response (SIMRs) (Potters et al., 2007, 2009). We hypothesize that N-NH_4^+ treatment triggers mild chronic stress in *citrange Carrizo* plants which may account for the SIMRs noted. SIMRs is part of a general acclimation strategy characterized by blockage of cell division in main meristematic tissues, inhibition of elongation, redirected outgrowth of lateral organs (Potters et al., 2009), increase in

antioxidants that prevent damage caused by ROS, and accumulation of foliar molecules which act as modulators of stress signals (Gould and Lister, 2006). This work demonstrates that the N-NH_4^+-treated plants clearly provoke an increase in lateral organs by augmenting the weight and number of lateral roots. N-NH_4^+ plants do not increase primary root length, however these plants showed an increase of modulators of stress signaling, such as H_2O_2, which could be intermediated between stress and the development of the SIMRs phenotype (Potters et al., 2007). This observation suggests that NH_4^+ treatment results in an enhanced resistance to salinity, possibly due to plants being previously exposed to mild stress which could be the prime *citrange Carriz*o defenses by stress imprinting, thus conferring plants resistance (Bruce et al., 2007).

On the one hand, we also investigated the effect of NH_4^+ nutrition on the expression of the *RD22, PR5*, and *AOS* marker genes involved in the stress response. We noted that N-NH_4^+ treatment increase the accumulation of the three marker genes. These results may indicate that the N-NH_4^+ plants have a more active defense pathway than the control plants. Moreover, salt treatment mainly increased *PR5* accumulation in the control plants, but the expression of the other marker genes in the control and the N-NH_4^+ plants was practically unaffected, which may be directly due to acclimated stage that NH_4^+ nutrition confers to citrus plants.

The analysis of hormones and metabolites in relation to plant responses to salinity reveal that ABA plays a role in the response against salt stress in the N-NH_4^+ plants. In this work, we found that the N-NH_4^+ plants have higher ABA levels than the control plants in the absence of salt. This fact is supported by the findings of Lopes and Araus (2008), who studied the gene expression profiles of barley seedlings fertilized with NH_4^+, NH_4^+ and NO_3^-, or with NO_3^- they observed that an epoxycarotenoid dioxygenase gene (involved to ABA synthesis) was upregulated in NH_4^+, probably due to NH_4^+ treatments which may invoke stress responses. Previous results have shown that stomatal closure occurs when barley plants are exposed to

NH_4^+ for long periods. The fact that NH_4^+ nutrition increases ABA accumulation in leaves may induce ABA-signaling. ABA signaling plays an important role in adaptation to abiotic stress and in the regulation of several genes, thought to be involved in dehydratation or salt tolerance as well as in stomatal closure (Zhu 2002). However, signaling in response to salinity seems to not depend solely on ABA. SA has long since been known to be a signal molecule in inducing defense mechanisms in plants (Shah 2003, Halim et al., 2007). Under our experimental conditions, the control plants exhibit SA accumulation at 14 d after salt stress; this accumulation correlates well with the SA-marker gene, *PR5*, since it was overexpressed in the control plants at the same time. On the other hand, several studies support that SA binds directly to the catalase enzymes inhibiting its activity in several plants species (Sanchez-Casas and Klessig, 1994; Horvath et al., 2002). This inhibition of catalase activity has been proposed to explain an increased H_2O_2 level upon SA accumulation (Chen et al., 1993), and H_2O_2 is responsible for ROS accumulation and induction of cell death (Overmyer et al., 2003). This fact can explain our results since the control plants, with greater SA accumulation, were more affected by salinity. Jackab et al. (2005) observed that BABA-induced salt stress tolerance mediated by ABA-dependent signaling in Arabidopsis and this response is independent of functional SA signaling. In this work, we also show antagonism between ABA and SA since the N-NH_4^+ plants treated with NaCl showed a faster, stronger ABA accumulation which could inhibit SA accumulation. Hence, salinity resistance in the N-NH_4^+ plants might be mediated by ABA accumulation, which is a regulator of salt tolerance. N-NH_4^+ treatment could increase ABA accumulation in leaves, thus conferring *citrange Carrizo* plants resistance to later salinity conditions.

The analysis of PAs in our study has determined that N-NH_4^+ treatment induces a faster, stronger Put accumulation at 2 h (data not shown) and at 14 d in the absence of salt. It is commonly accepted that some species develop toxic symptoms when NH_4^+ nutrition is

applied (Gerendás et al., 1997; Lasa et al., 2001), while a negative effect on plant growth has been observed with this kind of nutrition (Claussen and Lenz, 1999; Walch-Liu et al., 2000). Ammonium nutrition decreases essential cations content (Britto and Kronzucker, 2002), probably due to competition with NH_4^+ in the uptake process. Although the uptake of cations other than NH_4^+ is sometimes reduced, NH_4^+ uptake usually increases under NH_4^+ nutrition. Finally, plants may have excessive total cation content in comparison with anion content (Clark 1982). Gerendás et al. (1997) suggest that plants could accumulate PAs to compensate for the lack of some cations other than NH_4^+, hence they could contribute to cellular ionic balance maintenance. Furthermore PAs mainly Put, has an important role in abiotic stress since it reduces salt-induced oxidative damage by increasing the activities of antioxidant enzymes and by lowering lipid peroxidation (Tang and Newton, 2005). In this work, we also reveal that NH_4^+ treatment leads to a greater induction of gene *PHGPx*. *PHGPx* is a unique intracellular antioxidant enzyme that directly reduces the phospholipid hydroperoxides produced in cell membranes, and has been considered the main line of enzymatic defense against oxidative biomembrane damage in mammalian cells (Chun-Juan et al., 2009). Furthermore, *PHGPx* gene expression levels have been recorded to increase in plant tissues in response to pathogen infections (Criqui et al., 1992), high salinity (Li et al., 2001), heavy metals (Li et al., 2001), and extreme temperatures (Chen et al., 2004), suggesting the important roles that play in the defense responses of plants to biotic and abiotic stresses. Transient expression of *LePHGPx* protects tobacco leaves from salt and heat stress, and suppresses the apoptotic pathway induced by Bax (Chen et al., 2004). Our results suggest that the resistance to salinity that we found in the N-NH_4^+-treated plants could be mediated by a stronger accumulation of Put and the *PHGPx* transcript, which might induce resistance to the oxidative damage induced by salinity.

In conclusion, collectively these results indicate that NH_4^+ treatment enhances *citrange Carrizo* defense against salinity stress. This suggests that NH_4^+ treatment produces mild chronic stress and therefore induces the SIMRs in *citrange Carrizo*. Activation of the response related to SIMRs due to NH_4^+ toxicity led to the "acclimation stage", which leads to better adaptation to subsequent salt stress. This response initially brings about increased H_2O_2 accumulation which could act as a modulator of stress signal. Besides this, NH_4^+ treatment lowers Cl^- accumulation in leaves reducing its toxic effect and produces a higher basal proline content which might confer initial protection against salt stress. Moreover, the N-NH_4^+ treated plants have more active defense pathways than the control plants, and have activated ABA accumulation, which could prime ABA-signaling and PAs, mainly Put, which, in turn, could contribute to cellular ionic balance maintenance and reduce salt-induced oxidative damage. Furthermore, the N-NH_4^+ citrus seedlings display enhanced antioxidant machine activity, thus increasing *PHGPx* transcription. Together, this observation suggests that NH_4^+ treatments induce a mild stress condition that primes the *citrange Carrizo* defense response by stress imprinting and confers protection against a subsequent salt stress. The use of nutritional compounds like NH_4^+ could be an interesting alternative to the use of chemical compounds to induce plant resistance. In addition, this fact may help to alleviate the toxicity caused by salinity, one of the major problems currently on citrus crop.

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Table 1. Primers sequences

Primer name	Forward primer	Reverse primer
GAPDH	5′- ggaaggtcaagatcggaatcaa - 3′	5′ cgtccctctgcaagatgactct -3′
AOS	5′- cgaatttcaatccccaagaa-3′	5′- ttggtgggttgttcatcaga-3′
PHGPx	5′- catcacagtgtggcttgacc -3′	5′- tgctggatttcagatgcttg -3′
PR5	5′- tgggggactactccaatgtc -3′	5′- atcctcctggaaccctcaat-3′
RD22	5′- ttggaaaaggacttgcatcc-3′	5′- atgccagcgtcttcacactc-3′

Table 2. Polyamine levels expressed in μg g^{-1} FW in control and N-NH_4^+ plants upon salinity. Leaves were collected at 14 days after addition of NaCl (90 mM). Levels were determined in freeze-dried material by HPLC-MS. Data show the average of three independent experiments of a pool of 30 plants per experiment ± SE. Letters indicates statistically significant differences ($p < 0.05$).

	Put		**Spd**		**Spm**	
Control	6.16 ± 1.9	a	29.51 ± 1.41	b/c	11.76 ± 2.57	a
NH_4^+	146.98 ± 27.87	b	36.06 ± 1.93	c	19.83 ± 2.36	b
Control + NaCl	3.04 ± 1.12	a	24.35 ± 2.99	a/b	12.89 ± 2.61	a
NH_4^+ + NaCl	160 ± 14.68	b	30.65 ± 3.43	b/c	18.77 ± 4.02	a

FIGURE LEGENDS

Fig. 1. Effect of NH_4^+ nutrition on the growth of *citrange Carrizo* plants. (A) Biomass production, (B) root length, (C) root DW, lateral roots development expressed as: (D) number

of lateral roots/root DW and (E) number of lateral roots/root length. Data are from a representative experiment that was repeated three times with similar results. Values are the mean of 50 seedlings. Asterisk indicates statistically significant differences ($p< 0.05$).

Fig. 2. Effect of NH_4^+ treatment on the *citrange Carrizo* plants treated with NaCl (90 mM) for 14 d. The result is expressed as % of damage at different levels: healthy leaves, chlorotic leaves (level 1), intermediate leaf necrosis (level 2) and burnt leaves (level 3). Data are from a representative experiment that was repeated three times with similar results. Values are the mean of 50 seedlings.

Fig. 3. Effect of NH_4^+ treatment on the Cl^- and proline content in the *citrange Carrizo* plants treated with NaCl (90 mM) for 2 h and 14 d. (A) Cl^- content expressed in mg Cl^- g^{-1} DW and (B) Proline accumulation expressed in μmol proline g^{-1} FW. Data show the average of three independent experiments of a pool of 30 plants ± SE. Letters indicate statistically significant differences ($p < 0.05$).

Fig. 4. H_2O_2 staining, estimated by using DAB staining in the leaves of the control and the N-NH_4^+ *citrange Carrizo* treated with NaCl (90 mM) for 2 h. (A) Quantitative H_2O_2 measurement on the basis of brown pixels from digital photographs and (B) Brownish areas are indicative of H_2O_2 accumulation. Data are from a representative experiment that was repeated three times with similar results. Values are the mean of 10 seedlings. Letters indicates statistically significant differences ($p < 0.05$).

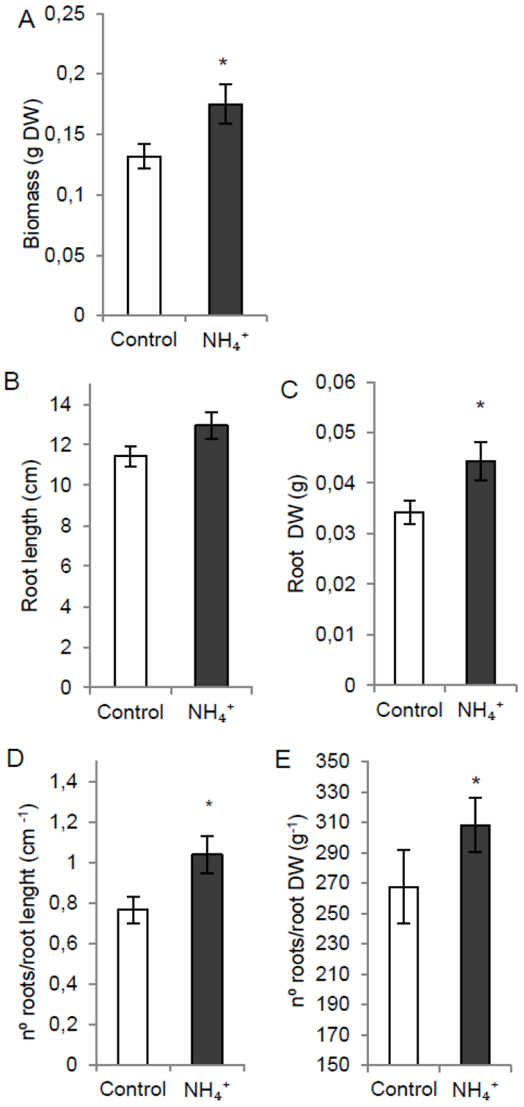
Fig. 5. Hormone levels in the control and N-NH_4^+ *citrange Carrizo* plants upon salinity. Leaves were collected at 14 d after addition of NaCl (90 mM). (**A)** ABA and (B) SA levels were determined in freeze-dried material by HPLC-MS. Data show the average of three

independent experiments of a pool of 30 plants per experiment ± SE. Asterisk indicates statistically significant differences ($p < 0.05$).

Fig. 6. Effect of NH_4^+ treatment on the gene expression in *citrange Carrizo* plants upon salt stress. Total RNA was isolated from leaves at 14 d after addition of NaCl (90 mM) converted into cDNA, and was subjected to a RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression measured in the same samples. The relative level of (A) *RD22*, (B) *PR5* and (C) *AOS* were analyzed in the control and the N-NH_4^+ citrus plants. The data show the average of three independent experiments obtained with a pool of 10 plants per point ±SE. The experiment was repeated three times with similar results.

Fig. 7. Effect of NH_4^+ treatment on the *PHGPx* expression in the *citrange Carrizo* plants upon salt stress. Total RNA was isolated from leaves at 14 d after addition of NaCl (90 mM) converted into cDNA, and was subjected to a RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression measured in the same samples. The data show the average of three independent experiments obtained with a pool of 10 plants per point ±SE. The experiment was repeated three times with similar results.

FIGURES



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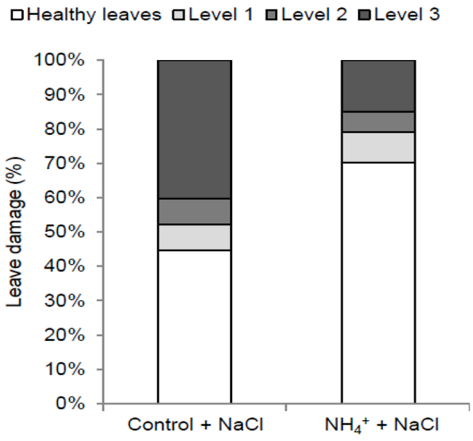

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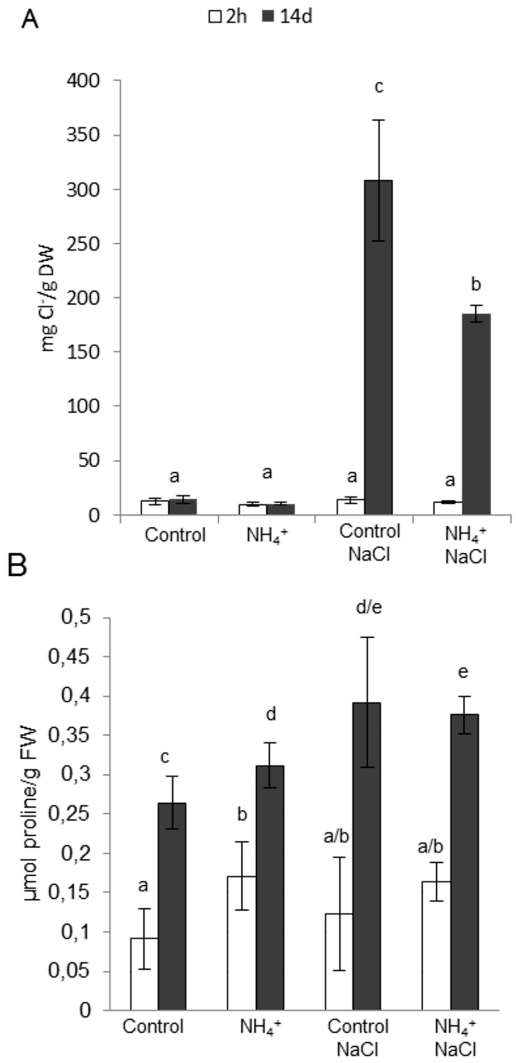

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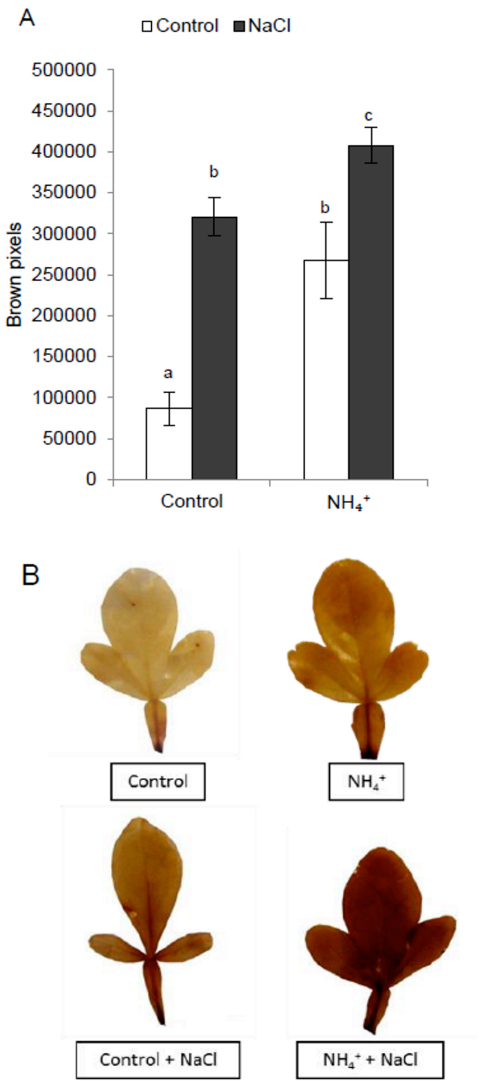

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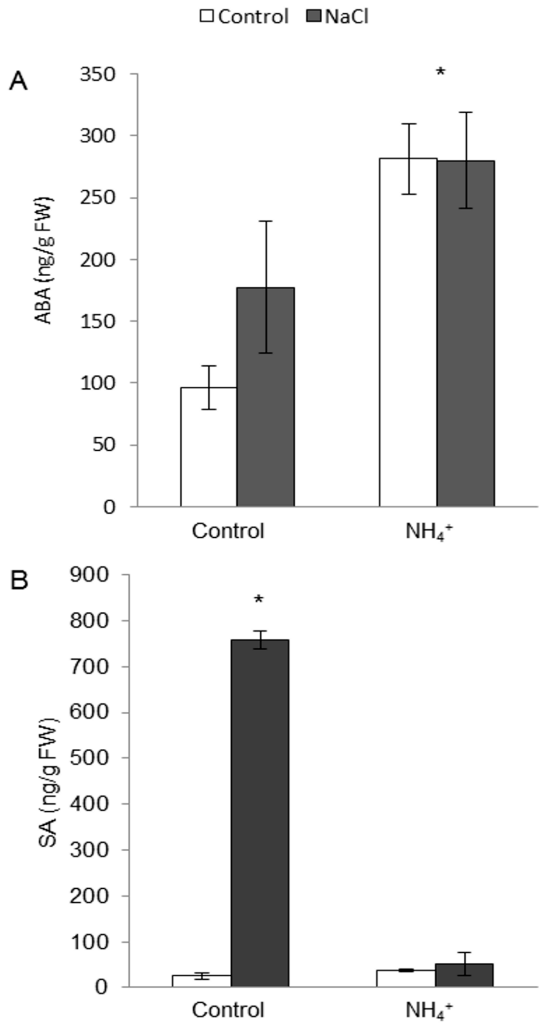

Fig. 5. Hormone levels in the control and N-NH_4^+ *citrange Carrizo* plants upon salinity. Leaves were collected at 14 d after addition of NaCl (90 mM). (**A)** ABA and (B) SA levels were determined in freeze-dried material by HPLC-MS. Data show the average of three independent experiments of a pool of 30 plants per experiment ± SE. Asterisk indicates

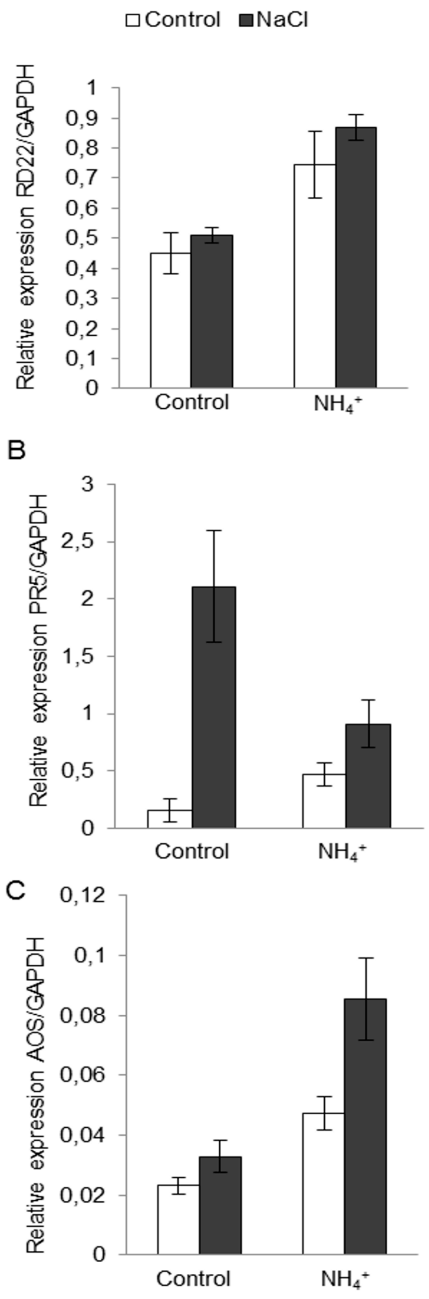

Fig. 6. Effect of NH_4^+ treatment on the gene expression in *citrange Carrizo* plants upon salt stress. Total RNA was isolated from leaves at 14 d after addition of NaCl (90 mM) converted into cDNA, and was subjected to a RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression measured in the same samples. The relative level of (A) *RD22*, (B) *PR5* and (C) *AOS* were analyzed in the control and the N-NH_4^+ citrus plants. The data show

the average of three independent experiments obtained with a pool of 10 plants per point ±SE. The experiment was repeated three times with similar results.

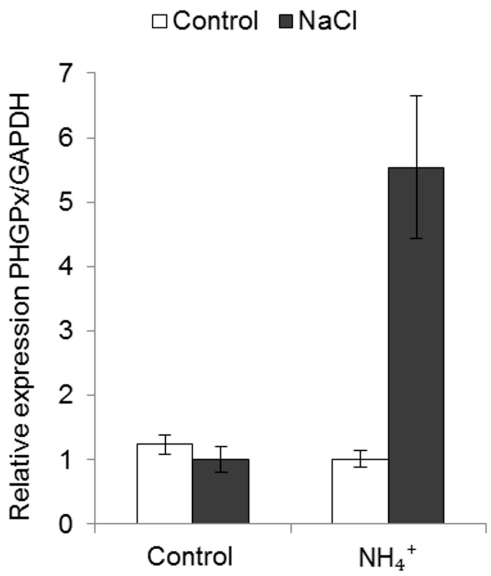

Fig. 7. Effect of NH_4^+ treatment on the *PHGPx* expression in the *citrange Carrizo* plants upon salt stress. Total RNA was isolated from leaves at 14 d after addition of NaCl (90 mM) converted into cDNA, and was subjected to a RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression measured in the same samples. The data show the average of three independent experiments obtained with a pool of 10 plants per point ±SE. The experiment was repeated three times with similar results.