agronomy

MDPI

Article

Ammonium Excess Leads to Ca Restrictions, Morphological Changes, and Nutritional Imbalances in Tomato Plants, Which Can Be Monitored by the N/Ca Ratio

Claudia Bonomelli [1,*], Sergio Tonetto de Freitas [2], Camila Aguilera [1], Carola Palma [1], Rebeca Garay [1], Maximiliano Dides [1,3], Natalia Brossard [1] and José Antonio O'Brien [1,3,*]

[1] Departamento de Fruticultura y Enología, Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Catolica de Chile, Vicuña Mackenna 4860, Santiago 7820436, Chile; cpaguilera@uc.cl (C.A.); cppalma@uc.cl (C.P.); rpgaray@uc.cl (R.G.); mdides1@uc.cl (M.D.); ndbrossa@uc.cl (N.B.)

[2] Brazilian Agricultural Research Corporation, Embrapa, Petrolina 56302-970, Brazil; sergio.freitas@embrapa.br

[3] Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Avenida Libertador Bernardo O'Higgins 340, Santiago 8331150, Chile

* Correspondence: cbonomel@uc.cl (C.B.); jobrien@bio.puc.cl (J.A.O.)

Abstract: Both nitrogen and calcium fertilization management are vital for crops, where an imbalance of these elements can cause both physiological and yield problems. It has been proposed that nitrogen absorption, particularly ammonium, is in part dependent on calcium supply. Moreover, the balance between these two nutrients could be a key indicator of plant growth in some species. Tomato, one of the most cultivated crops worldwide, can also be widely affected by nutritional imbalance. Using large amounts of N fertilizers could lead to an imbalance with other nutrients and, thus, detrimental effects in terms of plant development and yield. Here we show that ammonium excess has a negative impact on plant development and results in calcium deficiency. Moreover, a deficit in calcium nutrition not only affects calcium concentration but also leads to a restriction in N uptake and reduced N concentration in the plant. These effects were evident at the seedling stage and also during flowering/fruit set. Using PCA analysis, we integrated both phenotypic and nutritional imbalances in seedlings and grown plants. Interestingly, the Ca/N ratio appears to be a key indicator to monitor appropriate N and calcium nutrition and more importantly the balance between both. Maintaining this balance could be an essential element for tomato crop production.

Keywords: tomato; ammonium; calcium; plant development; nutrition

Citation: Bonomelli, C.; de Freitas, S.T.; Aguilera, C.; Palma, C.; Garay, R.; Dides, M.; Brossard, N.; O'Brien, J.A. Ammonium Excess Leads to Ca Restrictions, Morphological Changes, and Nutritional Imbalances in Tomato Plants, Which Can Be Monitored by the N/Ca Ratio. *Agronomy* **2021**, *11*, 1437. https://doi.org/10.3390/agronomy11071437

Academic Editor: Antonios Chrysargyris

Received: 1 June 2021
Accepted: 12 July 2021
Published: 20 July 2021

1. Introduction

Solanaceae species are globally distributed due to their importance as food crops, and for that reason one of the main focuses in their production is to reach the highest possible yields [1]. Besides genetics and water supply, fertilization management is one of the main factors to obtain higher crop yield and quality. Moreover, these factors are highly influenced by macronutrients such as nitrogen (N) and calcium (Ca).

Nitrogen plays a key role in the synthesis of most macromolecules such as proteins, nucleic acids, hormones, and vitamins [2] and in making part of all the plant structures, especially vegetative tissues. In addition, it is a very mobile element that circulates among the atmosphere, soil, and living organisms [3], including plants. Plants take N from the soil through active transport using specialized transporters located in the plasma membrane of the root epidermal and cortical cells. There are at least 10 transporters that mediate the entry of N, 6 for nitric forms and 4 for ammoniacal forms, as has been observed in *Arabidopsis thaliana* [4,5]. Its mobility favors the assimilation of N because it travels to different plant organs and tissues through the xylem and phloem vessels [6].

Due to its mobility through the tissues, excess N applications are rapidly observed in plants. One of the first signs is the change in the normal coloration of plant tissues, where the green color is intensified by the increase in chlorophyll concentration [7,8]. In addition, there is an increase in vegetative development in terms of the number and length of shoots. Finally, higher concentrations can lead to toxicity, where opposite effects are observed [3]. This toxicity will depend on the N form and the species [9]. Particularly, plants are very sensitive to ammonium, which in tomato can be observed over 2–4 mM NH_4^+ with ammonium as the exclusive N form [10]. This toxicity could be due to a limited assimilation capacity; therefore, after reaching the limit of assimilable N, leaves will begin to necrotize [11]. These are factors that can cause a nutritional imbalance in other organs of interest, such as fruits and roots. This is due to an increase in transpiratory rate; consequently, part of the resources necessary for their development is re-directed to the shoots [12,13].

Calcium is a divalent cation that plays very important roles in plants as a cell wall reinforcing nutrient, secondary messenger in the cytoplasm, and as a counter ion inside storage organelles [14]. This nutrient is also required for proper membrane structure and stability [15]. In addition, higher Ca concentrations have been shown to improve fruit and vegetable storability and reduce losses, which is mainly associated with higher tissue mechanical strength [16,17].

Plant Ca uptake takes place through the apoplast mainly at the root tip where there are no suberized endodermal cells, as well as through the symplast at the suberized endodermal cells where Casparian bands are present [18]. It is believed that apoplastic Ca uptake at the root tip is the most important for plant nutrition due to the fact that Ca does not have to move through the symplast to reach the xylem vessels [19], where it can be distributed through the transpiration stream. Ca deficiency in plants is usually the result of poor Ca uptake, distribution in relation to demand, as well as antagonistic effects with other nutrients. This deficiency can be a consequence of water supply disturbances, salinity, or factors that inhibit transpiration [14]. Ca deficiency induces several physiological disorders such as bitter pit in apple (*Malus domestica*), black heart in celery (*Apium graveolens* L.), tip burn in leafy vegetables, cracking in tomato (*Solanum lycopersicum*) and apple and cherry (*Prunus* spp.) [20–22], as well as blossom end rot (BER) in watermelon, pepper, eggplant, and tomato [14].

It has been reported in some crops that Ca stimulates ammonium absorption [23]. Moreover, in the case of ammonium toxicity, supplementary Ca ameliorates the detrimental effect [24]. In addition, during fruit development, it has been proposed that the fruit competes with other sink tissues within the plant such as leaves, which have the highest transpiration rate and therefore a greater xylem sap and Ca uptake. In this case, N excess is known to increase plant vegetative growth [25], which can generate a greater competition between leaves and fruit for plant Ca uptake.

In this study, we showed that there is a crosstalk between N excess and Ca deficient nutrition in tomato plants at different developmental stages. This leads to changes in physiological and morphological responses in tomato roots and shoots. Furthermore, we found a correlation between ammonium and Ca that is reflected in an altered N/Ca ratio.

2. Material and Methods

This study was carried out at the Fruit Nutrition laboratory, school of Agricultural and Forest Sciences and Plant Development and Biotechnology laboratory, School of Biological Sciences, at Pontificia Universidad Católica de Chile (Santiago, 33°29′ S, 70°36′ W).

2.1. Plant Material and Growth Conditions

Tomato (*Solanum lycopersicum*) cv. Rio Grande seeds were sterilized in Eppendorf tubes using a 2.5% sodium hypochlorite and 0.1% Triton X-100 solution under constant mixing for 10 min. Subsequently, the seeds were washed with sterile water at least three times and sown on Petri dishes containing 0.5× Murashige and Skoog (MS) nutrient

medium with 1% agar. Plates were placed in a growth chamber at 24 °C with 16/8 h day/night photoperiod. At 7 days after germination (DAG), the seedlings were transferred to 200 mL flasks containing 25 mL of media, according to each treatment. For the in vitro experiments, four different nutritional media were evaluated: control, calcium deficiency (10% of control), nitrate excess (+30%), and ammonium excess (+30%). In order to maintain the ion balance, the media used for the treatments were made in the laboratory as described in Table S1 and all four media contained all essential macro- and micro-nutrients. The only difference among the media was the amount of Ca or N forms, as shown in Table S1. All the media were adjusted to pH 5.9 with KOH. The seedlings were kept in the growth chamber for 10 days before evaluation. This experiment was performed in at least 10 biological replicates of 3–4 plants each.

For the pot experiments, twenty-four seeds were sown in Petri dishes with absorbent paper and distilled water. Plates were placed in a growth chamber at 24 °C to germinate for 12 days. The germinated seedlings were then transplanted into 180 cm^3 pots containing peat, perlite, and vermiculite (2:1:1) as the substrate. The pots were placed in a growth chamber at 24 °C until the seedlings reached approximately 10 cm. Subsequently, the plants were transplanted into 0.3 L pots and placed in a greenhouse for 9 days. Finally, the plants were transplanted into 2.8 L pots with the same substrate. For the potted plants, four nutritional treatments were evaluated: control treatment, nitrogen excess (5× urea), calcium deficit, and calcium deficit together with nitrogen excess. In the potted plants, urea was used as the ammonium excess as it is one of the main sources of nitrogen used in crop production. Moreover, unlike ammonium phosphate or ammonium sulfate, it has a lesser impact on soil acidification. The control treatment consisted of plants fertilized once a week with 300 mL of the recommended nutritional solution of macro- and micro-nutrients (Murashige and Skoog Basal Medium with Vitamins). The ammonium excess treatment consisted of fertilization with the same solution supplemented with 1.8 g/L of urea. The Ca deficiency treatment consisted of fertilization with a complete nutritious solution until flowering and thereafter with a nutrient solution of macro- and micro-nutrients without calcium chloride. The interaction treatment consisted of the treatment of ammonium excess until flowering and thereafter with a nutrient solution of macro- and micro-nutrients without calcium chloride and supplemented with 1.8 g/L of urea. The macro- and micro-nutrients of each media are described in Table S2. Although there were some differences in the micro-nutrients, we did not expect an impact on their availability considering that the substrate contained peat and the media were not the only nutrient source. The experiment followed a randomized complete block design (RCBD). Each treatment was composed of three blocks and five biological replicates.

2.2. Phenotypic Analysis

For the in vitro plants, measurements were carried out in the seedlings 10 days after subjecting the plants to the treatments. Three tissues were measured—main root length, stem length, and lateral root length—and the number of lateral roots was counted. Regarding the potted plants, 132 days after germination, when the plant had already started fruit development, a tissue dissection was performed, separating each organ, such as shoots, fruits, and roots, after weighing the entire plant. Once dissected, the root system was measured.

2.3. Microscopy Analysis

In vitro roots of the different treatments were observed through a light microscope. This was achieved with the preparation of paraffin blocks that contained 1 cm long pieces of the middle section of the main root of the plant. Then, cross-sections of 20 µm thick were made to deposit them on slides that were subjected to techniques of staining with safranine and "fast green" to dye dead and living tissues, respectively. Subsequently, the diameter of the metaxylem vessels of each treatment was measured with the Image J program through photographs taken with the Leica ICC50 W microscope camera (Leica,

Heerbrugg, Switzerland) of the prepared samples. In each vessel, two diameters were measured and the average of both was taken because the shape was not perfectly circular.

2.4. Mineral Analysis

For the in vitro plants, two replicates were collected, approximately 1 g of dry matter from whole seedlings, and sent for analysis. At the end of the trial, each plant was separated into stem, leaves, fruit, and roots. Each plant part was weighed separately and sent for analysis. For the determination of the total N concentration, the LEGO CNS-2000 Macro Elemental Analyzer (Leco, Michigan, MI, USA) was used. For the determination of K, Ca, Mg, P, Cu, Zn, and Mn, the plant samples were subjected to 500 °C until conversion to ashes, which were dissolved with HCl (2 M). Later, the samples were used to analyze the nutrient concentrations with the equipment Axial ICP-Optical Emission Spectroscopy Agilent 720 ES—Varian (Victoria, Australia).

2.5. RNA Extraction and cDNA Synthesis

Leaves and roots of the seedlings, 10 days after treatment, were collected and frozen in liquid nitrogen. RNA extraction was performed using Trizol™ Reagent (Invitrogen™, Waltham, MA, USA) following the manufacturer's protocol Later, the RNA samples were treated with dsDNase (EN0771) according to the manufacturer's protocol (Thermo Scientific, Waltham, Massachusetts, MA, USA). The concentration and purity of the RNA samples were determined using the Nanodrop 2000 spectrophotometer. cDNA synthesis was performed by incubating 1000 ng of each RNA sample with 2 µL of Random primer mix (60 uM) and M-MuLV Reverse Transcriptase (M0253S, New England Biolabs, Ipswich, MA, USA)) following the manufacturer's protocol.

2.6. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The qRT-PCR was accomplished with the Brilliant III Ultra-Fast SYBR Green qPCR Kit (Agilent, Santa Clara, California, CA, USA). The cDNA of each gene was amplified using the primers described in Table S3. Gene expression analyses were normalized with the housekeeping gene *GAPDH* [26]. The reaction was performed in an Applied Biosystems StepOnePlus™ Real-Time PCR System in 0.1 mL MicroAmp® Fast 8-Tube Strip (Thermo Fisher Scientific, Foster City, California, CA, USA). The program used for the amplification was a holding stage at 95 °C for 12 min, cycling stage at 95 °C for 15 s, 60 °C for 20 s, and a final stage at 72 °C for 20 s. Gene amplification was accomplished for 40 cycles. Additionally, a melting curve was performed from 60 °C to 95 °C rising 0.3 °C at each phase to confirm the amplification of only one transcript on each gene. For the analysis of data and amplification, StepOne Software v2.3 was used. Finally, data were analyzed using the LinReg program [27].

2.7. Statistical Analysis

The results obtained were analyzed with one-way and factorial ANOVA or Tukey tests, as stated in each figure description. Significant changes were considered when $p \leq 0.05$.

3. Results and Discussion

Different reports have shown that ammonium present in the soil can severely affect plant growth, development, and nutrition [28–30]. As a cation, ammonium not only contributes to nitrogen nutrition in the plant but also, when in excess, can affect other nutrients such as Ca. However, it is not completely clear how the NH_4^+/Ca^{2+} imbalances and ratios affect plant growth and development.

3.1. Calcium Deficiency and Nitrogen Excess Affects Plant Development In Vitro

In order to evaluate N/Ca relations and how these nutrients affect each other, we set up an in vitro experiment with Ca deficit, as well as ammonium and nitrate excesses. Tomato seedlings were grown for 4–5 days in Petri dishes containing solid 1/2 MS media.

At 4–5 days after germination (DAG), the seedlings were transferred to flasks containing 1/2 MS media (control) or the same media with reduced calcium (-Ca) or nitrogen excess (NH_4^+ or NO_3^-). The seedlings were then kept in a growth chamber and were evaluated after 10 days (Figure 1A). Both the aerial part and the roots were measured and weighed, and the different tissues were dissected for analysis.

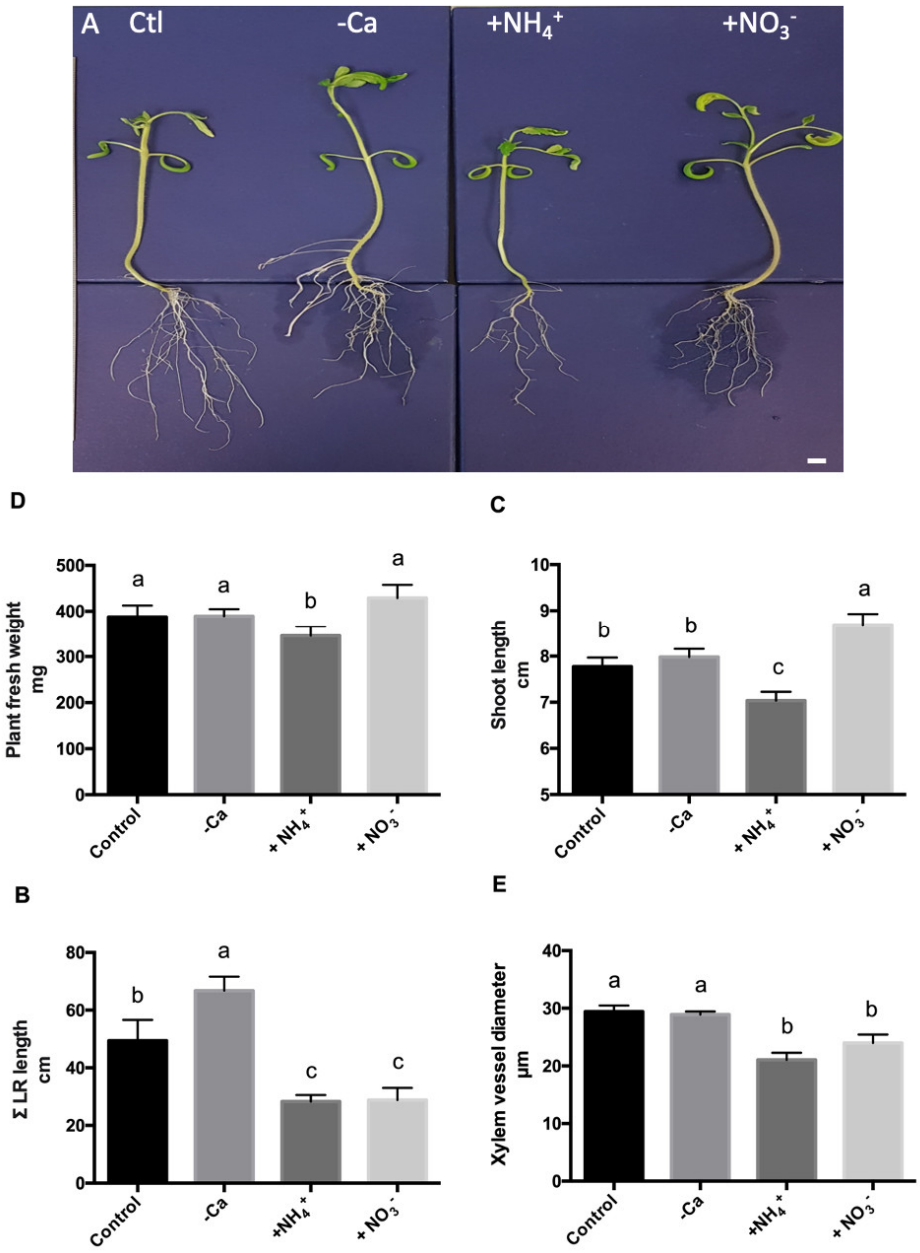

Figure 1. Tomato seedling response to calcium deficit or ammonium and nitrate excesses in in vitro experiment. Tomato seedlings were grown with control media, with a calcium deficit, or with ammonium or nitrate excesses for 10 days in a growth chamber as described in the Materials and Methods section. Tomato images were taken 10 days after sowing the seedlings: (**A**) representative image of seedlings; (**B**) sum of lateral root length; (**C**) shoot length; and (**D**) plant fresh weight. Xylem vessel diameters (**E**) were measured by light microscopy (40×). a, b, c represents statistically significant differences with $p < 0.05$.

Despite the short timeframe of the experimental setup, it was possible to observe different phenotypes in response to each treatment. While no major change was observed in terms of root length, number of lateral roots (LRs), and LR density in any condition

(Figure S1), the length of the lateral roots was clearly altered (Figure 1B). In response to Ca deficit, LR length was significantly increased. On the contrary, N excess had a negative impact on LR growth. In the shoot, the only treatment that showed a positive impact on shoot length was the nitrate excess, opposite to ammonium excess which resulted in reduced shoot length (Figure 1C). This negative impact in shoot growth was also correlated with a slight but significant reduction in plant fresh weight under ammonium excess (Figure 1D). This negative impact in fresh weight and development as a consequence of ammonium could be due to a problem in vasculature development [31,32]. In order to further evaluate this possibility, xylem vessel diameter was also evaluated in plants from the control and ammonium treatments (Figure 1E)). Interestingly, not only ammonium but also nitrate excess showed a reduction in xylem vessel diameter.

3.2. Calcium Deficit and Ammonium Excess Triggers Changes in Nitrate Transporters Expression and Nitrogen Assimilation

Alterations in N availability can affect its transport and metabolism in plants. Thus, we evaluate the expression levels of the dual-affinity transporter *NRT1.1*, the high-affinity nitrate transporters *NRT2.1* and *NRT2.2*, and the gene *Nitrate Reductase* (*NR*) that codes for one of the most important enzymes involved in nitrogen assimilation in plants (Figure 2). The genes *LeNRT1.1* and *LeNRT2.1* showed no expression responses to Ca restrictions and N excess (Figure 2A,B). On the other hand, the relative expression of *LeNRT2.2* was down-regulated in both nitrogen excess conditions, showing a significantly down-regulated *LeNRT2.2* expression in response to NH_4^+ excess (Figure 2C). Interestingly, Ca deficiency also reduced *LeNRT2.2* expression, which could be altering the N uptake. *LeNR* was also analyzed in terms of its relative expression. As expected, ammonium excess resulted in a severe down-regulation of this gene expression.

3.3. Calcium Deficit and Ammonium Excess Results in Nutrient Imbalance and an Altered N/Ca Ratio

The impact of Ca deficit in nitrate transporters expression could be indicating a correlation between Ca deficit and nitrogen uptake. Moreover, it was also expected that ammonium excess could be altering Ca uptake. Therefore, a nutritional analysis was carried out under the same experimental setup. As shown in Figure 3, Ca deficit shows a significant reduction in N content, while N excess results in a significant increase in N content. Ca deficit resulted in lower Ca accumulation in the plant. Each N form applied resulted in opposite effects in plant Ca uptake; while ammonium excess strongly inhibits Ca uptake, nitrate excess seems to stimulate Ca uptake and accumulation in the shoot (Figure 3B). However, taking into consideration that the nitrate excess media has a higher Ca concentration, this could also be independent of nitrate.

Due to the similarities observed in Ca deficit and ammonium excess, we calculated the ratio between N and Ca content. Interestingly, both treatments showed an altered N/Ca ratio compared to the control or nitrate excess treatments (Figure 3C).

3.4. Physiological Changes in Response to Calcium Deficit and Ammonium Excess in Potted Plants Correlate with Treatments In Vitro

Taking into consideration the impact of Ca deficit, ammonium excess, and N/Ca ratio on plant Ca uptake, we set up an experiment with potted plants in a growth chamber in order to further analyze these observations in plants during the latter stages of development.

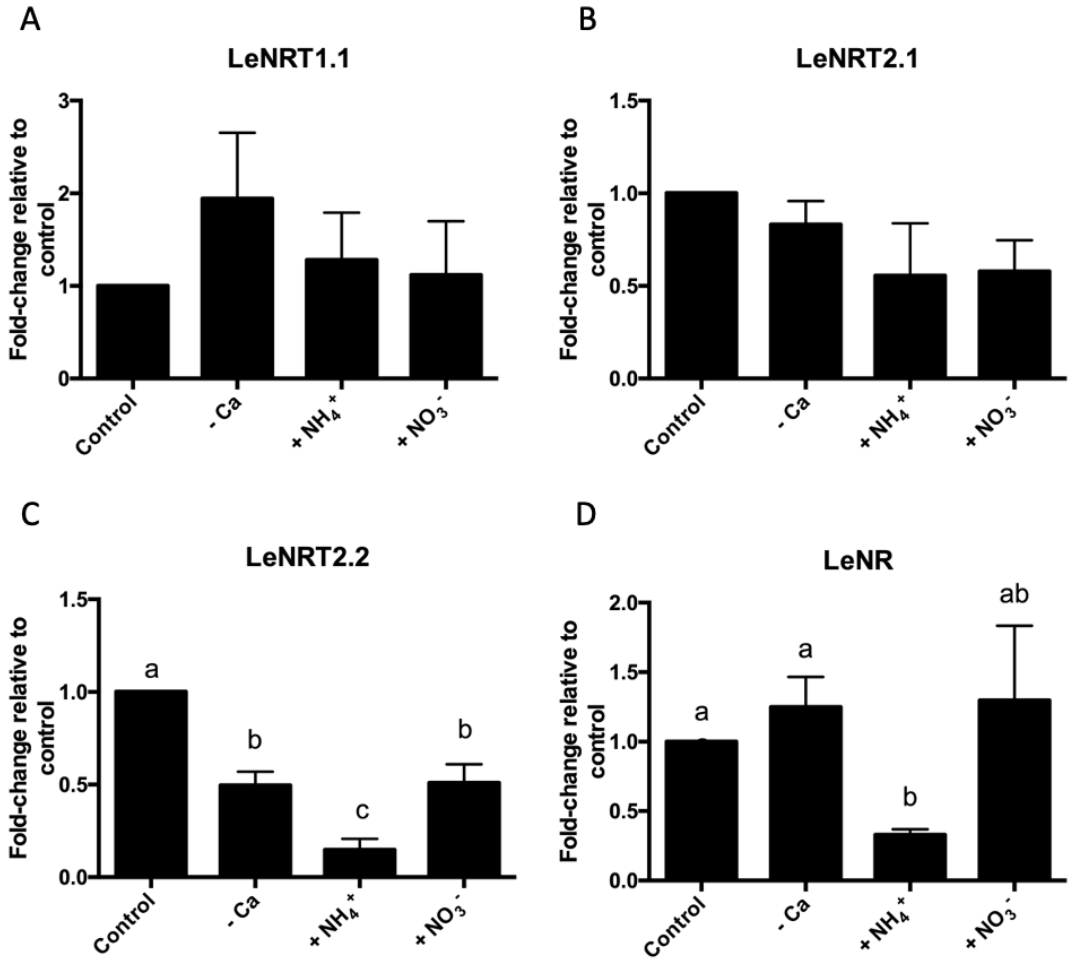

Figure 2. Nitrate transporters and nitrate assimilation gene expression levels were affected by calcium deficit or ammonium and nitrate excesses in in vitro experiment. Tomato seedlings were grown with control media, with a calcium deficit, or with ammonium or nitrate excesses for 10 days in a growth chamber as described in the Materials and Methods section. Tomato seedlings were dissected and root tissue was collected 10 days after sowing. Transcript levels were analyzed by qRT-PCR using *GAPDH* as the housekeeping gene: (**A**) Col-0 WT: fold-change was set for T0; (**B**–**D**) transcript levels from nitrate transporters *LeNRT1.1* (**A**), *LeNRT2.1* (**B**), *LeNRT2.2* (**C**), and nitrate reductase *LeNR* (**D**). Fold-change was set for the control condition. Two-way ANOVA test was performed. Lowercase letters represent statistical differences with a $p < 0.05$. Error bars represent the SEM. Each experiment was performed at least in triplicate with at least 5–10 seedlings in each replicate. a, b, c represents statistically significant differences with $p < 0.05$.

While no changes in plant weight were observed after 11 weeks (Figure 4A), ammonium excess resulted in a significantly smaller root (Figure 4B), which correlated with the results obtained for lateral root length for the same treatment in vitro (Figure 1). Interestingly, the correlation between Ca deficit and ammonium excess observed in the in vitro experiments was also observed in the potted plants. Thus, Ca treatments resulted in a negative impact on N content and also Ca content (Figure 4C,E). Moreover, ammonium excess also resulted in a reduced Ca content compared to the control treatment (Figure 4E).

As observed for the in vitro experiments, the ammonium excess in potted plants also resulted in an altered N/Ca ratio both in shoots and roots (Figure 5). However, the Ca deficit treatment did not result in a significant change in this ratio: a trend was only observed in shoots (Figure 5A). Interestingly, the total N and Ca content in fruits did not show the same results: only the Ca deficit treatment showed a reduced accumulation of both Ca and N (Figure S2).

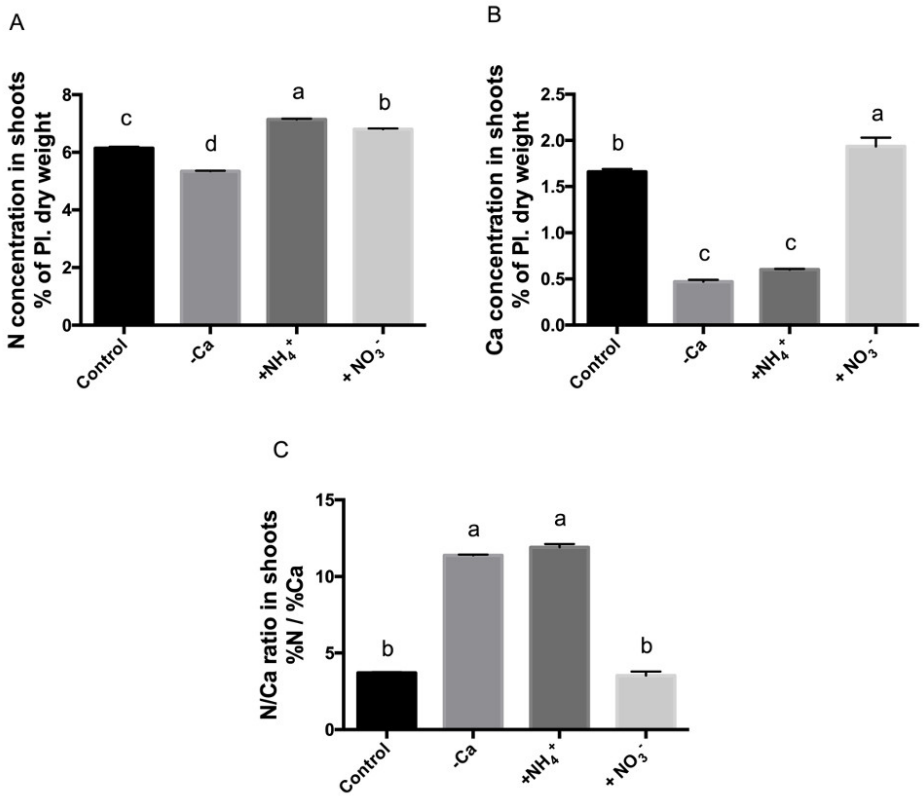

Figure 3. Nitrogen, calcium, and N/Ca ratio were altered by calcium deficit or ammonium and nitrate excesses in in vitro experiment. Tomato seedlings were grown with control media, with a calcium deficit, or with ammonium or nitrate excesses for 10 days in a growth chamber as described in the Materials and Methods section. Tomato seedlings were collected and dried at 45 °C in a dehydrator. Total nitrogen (**A**) and total calcium (**B**) were quantified as a percentage of plant dry weight. (**C**) Nitrogen/calcium ratio was calculated from the nitrogen and calcium percentage for each plant. a, b, c, d represents statistically significant differences with $p < 0.05$.

3.5. Ammonium Excess Correlates with N/Ca Ratio and N Content

To further evaluate the nutritional impact of both in vitro and in-pot treatments, further nutritional analysis was performed, such as P, K, Mn, Mg, Zn, and Cu concentrations (Tables S4 and S5). These data were integrated with the plant dry weight and the N/Ca ratios using principal component analysis (Figure 6). To compare the data between experiments, the nutrient concentrations and phenotyping results were analyzed and normalized. Thus, the nutritional content and dry weight under each treatment were re-calculated as a percentage relative to each control condition (of the corresponding experiment, in vitro or in pots), and the nutritional content was expressed as a percentage of plant dry weight, as indicated in Table S6. As observed in Figure 6, the control conditions for in vitro and in-pot treatments correlate with each other and also with nitrate excess; this was expected as nitrate should not have a negative impact on plant nutrition when compared to Ca deficit or ammonium treatment [33]. In the case of ammonium treatment, it correlated with N content and was negatively correlated with potassium content, which is an indication of the competition with the excess of NH_4^+ [34]. While ammonium treatment in pots correlates with ammonium/calcium treatment, ammonium treatment in vitro only shows a correlation in Figure 2 with the experiments in pots. This could be, at least in part, due to the differences in the developmental stages and the experimental setting. Moreover, there is a direct correlation of the ammonium treatment and the N/Ca ratio, which could be a strong indicator of a nutritional problem.

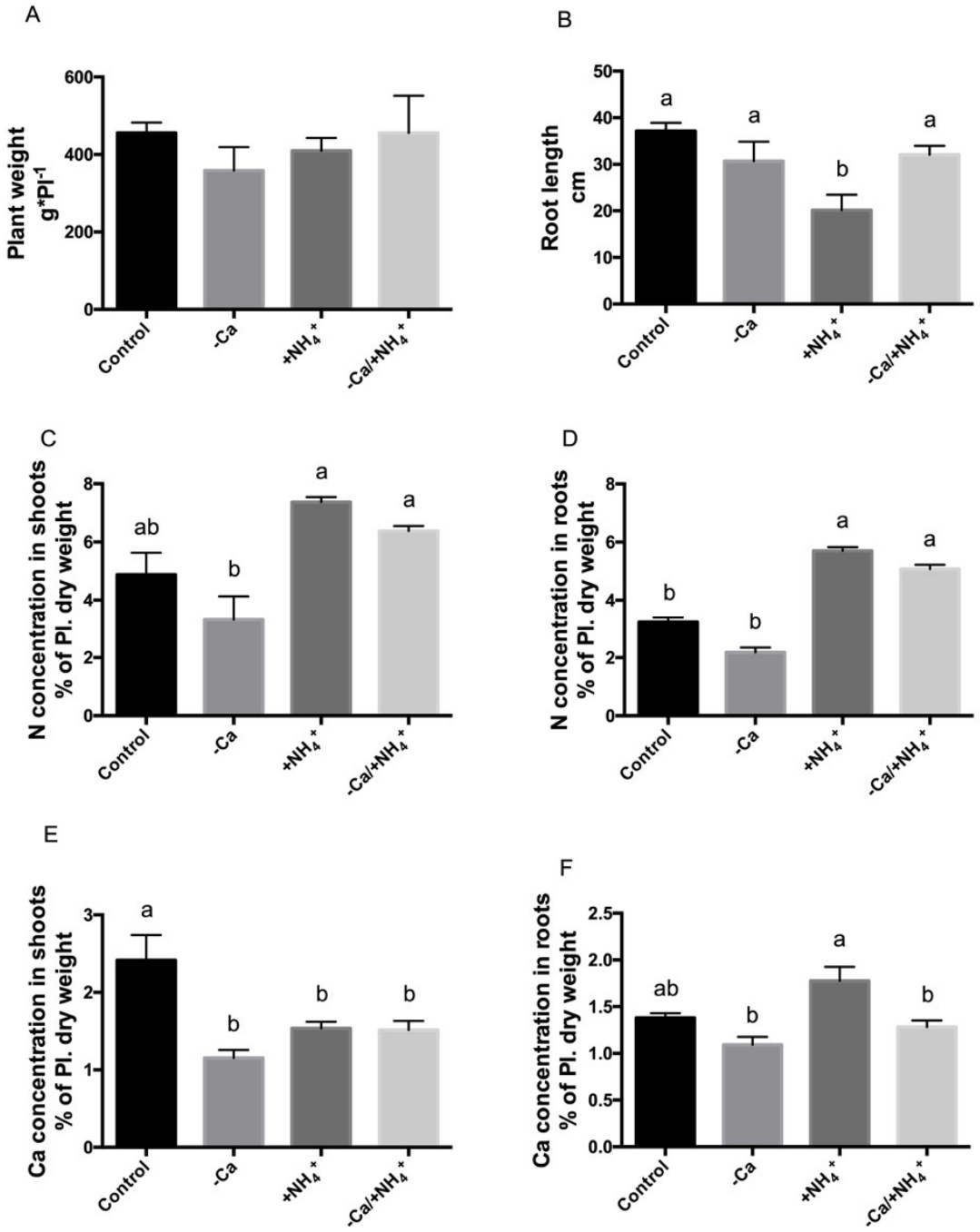

Figure 4. Tomato plants were affected by reduced calcium and/or ammonium excess in in-pot experiments. Tomato plants were watered with a fertilization solution containing all essential nutrients (control) or a fertilization solution with calcium deficit (-Ca), with ammonium excess (+NH_4), or both combined (−Ca/+NH_4). At 132 days from seed, the plants were dissected, and the total plant weight (**A**) and root length (**B**) were analyzed. Total nitrogen (**C**,**D**) and total calcium (**E**,**F**) were determined as a percentage of plant dry weight, for shoot (**C**,**E**) and root (**D**,**F**) tissue. a, b represents statistically significant differences with $p < 0.05$.

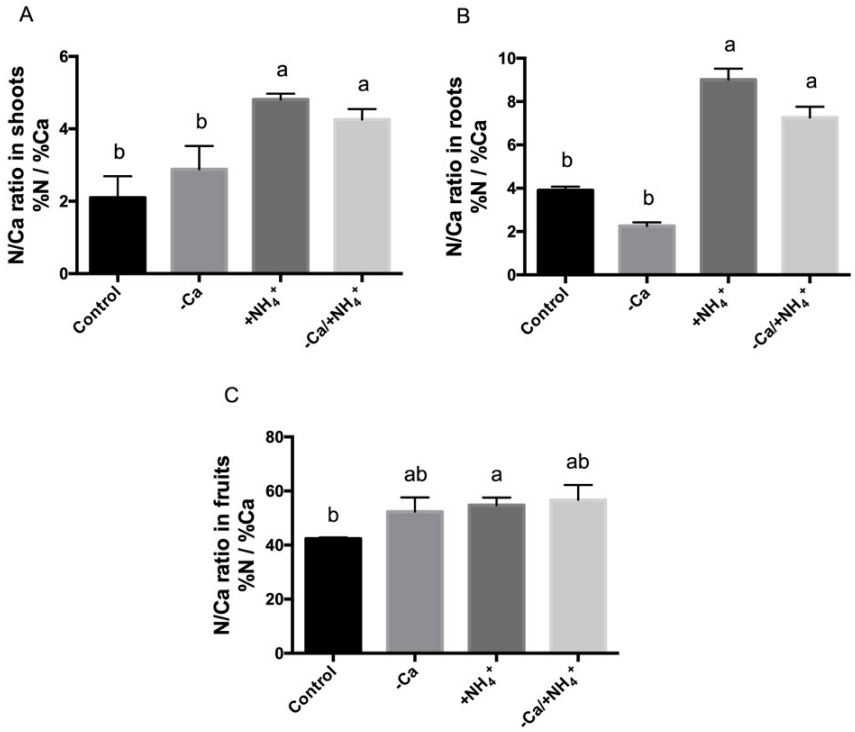

Figure 5. N/Ca ratio was altered in tomato plants with ammonium excess in in-pot experiments. At 132 days after germination, the plants were dissected, and total nitrogen and total calcium were quantified as a percentage of plant dry weight. Nitrogen/calcium ratios were calculated for shoot (**A**), root (**B**), and fruit (**C**) tissue. a, b represents statistically significant differences with $p < 0.05$.

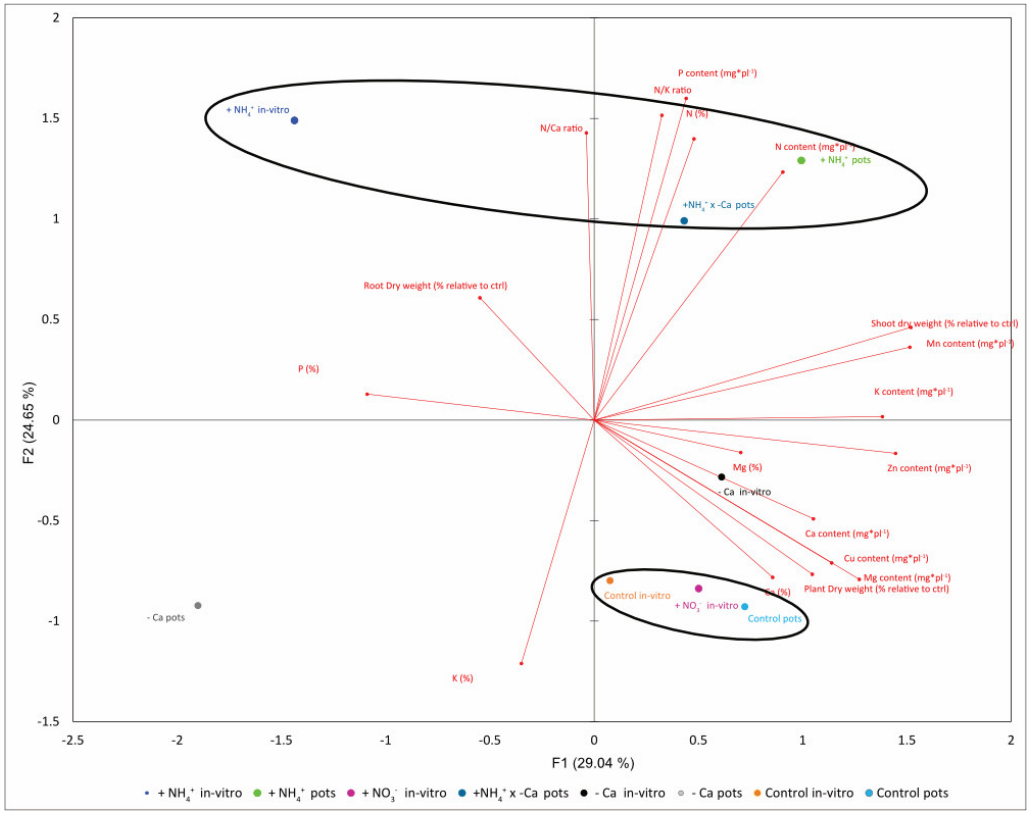

Figure 6. Principal component analysis of physiological and nutritional parameters reveals a relationship of ammonium excess and N/Ca ratio.

4. Discussion

Plants are constantly adapting to ever-changing environmental conditions that could be not favorable and have a negative impact on plant development and yield. For instance, when plants are subjected to nutritional imbalances [35]. Thus, the imbalance of any nutrient could also affect other nutrients, exacerbating the impact in the plant. Nitrogen is a crucial nutrient, but in excess can have a negative impact in the plant [9,10]. However, this depends on the N form. While nitrate is the preferred N form, ammonium is not well tolerated as the main or exclusive N source [9,10]. As expected, a 30% excess of NH_4^+ had a negative impact in plant development, where an excess of ammonium caused a decrease in roots and stem growth, in contrast to nitrate, which, despite the negative effects of lateral root development, allowed a greater growth of aerial tissue (Figure 1). This is in accordance with previously described studies in tomato and *Arabidopsis* [9,10,36]. Glass et al. (2002) showed that in studies of N absorption in tomato, in non-toxic quantities, 50% of the absorbed N was in the form of NH_4^+, even when this nitrogenous form was only 10% of the N available, with the remaining 90% being NO_3-. This is due to the fact that when NH_4^+ is delivered in high quantities, its absorption exceeds the assimilating capacity of the plant and changes the ionic balance, causing changes in the development of tissues, later ending in toxicity [11,37]. On the contrary, the NO_3^- can be stored in the vacuoles of the cells and then be put back into circulation when necessary [38], which explains its lower deterioration capacity and that, in the short term, it complies with its function as a promoter of vegetative growth.

This detrimental impact of ammonium excess could be a consequence of reduced vascular area as these treatments had a higher production rate of cells of smaller size, which is the process that mainly determines vessel area, followed by cell expansion [39]. In situations of nutritional stress related to N, both processes are altered, showing in this case a greater tendency to produce smaller cells than to promote elongation. Indeed, studies have suggested that higher levels of N can inhibit xylem vessel development by changing hormone ratios in the plant and/or by increasing tissue growth rates that could limit xylem expansion in the plant [40,41].

It has been described that Ca stimulates ammonium absorption [23]. Interestingly, we observed this same dependence between calcium and ammonium. Both in vitro and in potted plants, calcium deficit resulted in a mild reduction of nitrogen content. In addition, ammonium excess resulted in a decrease in calcium content. This reduction of N content under calcium deficit could be indicating that calcium deficit has a negative impact on nitrogen uptake. This possibility is further supported by the reduced expression of *LeNRT2.2* (Figure 2). This reduced expression in *LeNRT2.2* was also observed when nitrogen excess was applied, independent of the N source. This was expected as this gene belongs to the *NRT2* family of high-affinity nitrate transporters, which are up-regulated under low nitrogen conditions [42]. This same trend was observed for *LeNRT2.1*; however, the changes observed were not significant. As previously described, the use of NH_4^+ as a main or exclusive N source is detrimental for plant development when used in excess [9,10]. This is not the case for nitrate as a N source, which was evident from the phenotypic analyses. A critical difference between these N sources is in their assimilation, where one of the first steps of nitrate assimilation is mediated by NITRATE REDUCTASE (NR). As expected, ammonium excess results in a strong down-regulation of *LeNR* (Figure 7).

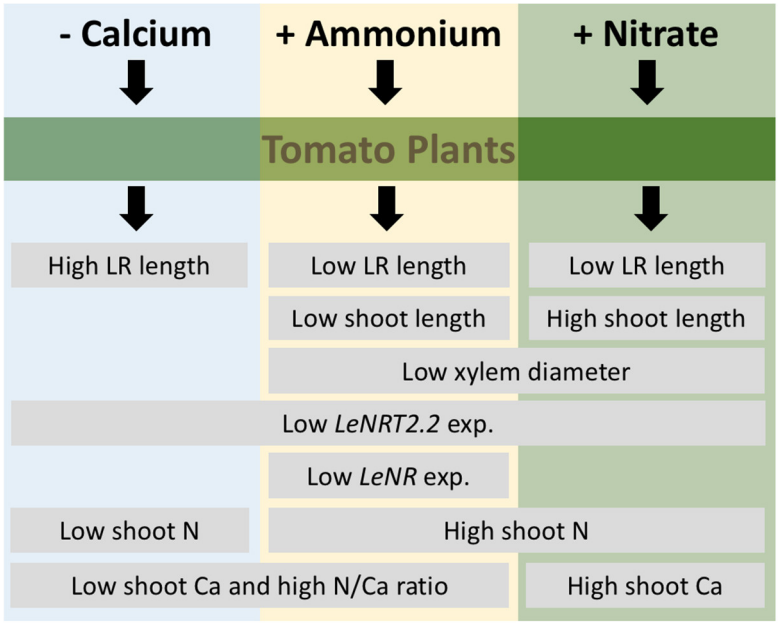

Figure 7. Proposed roles of Ca restrictions (−), as well as ammonium (+) and nitrate (+) excesses on tomato plant physiology and morphology.

In adult potted plants, the main significant difference was observed in root length, where ammonium excess resulted in reduced root length, while no differences were observed under calcium deficit. This is in accordance with the experiments in vitro. It has been previously described that plants with insufficient Ca in the soil show higher levels of *p* in roots, a nutrient that promotes root development, allowing a greater contact surface with the soil [33,43] and thus extracting as much Ca as possible.

The balance between nutrients is key for proper plant development. It has been proposed that interactions between nutrients occur when the supply of one nutrient affects the uptake, distribution, or function of another nutrient and could modify plant growth or yield [44]. Thus, ammonium nutrition has been found to limit the absorption of ions with similar properties, generally divalent cations as Ca, due to competition or antagonism by the active sites of enzymes and membrane transport molecules [44,45]. Some of these transporters are capable of transporting various nutrients, which indicates that their selectivity is based, in part, on the physicochemical similarities between ions [46]. This is in agreement with what was observed in tomato, where the shoots of plants with excess NH_4^+ had the same concentration of Ca as the plants that had low fertilization of this nutrient. On the other hand, also reflected in the results is the bottleneck that is generated at the moment of the absorption [3,47], where the roots of plants with an excess of N present the highest concentration of Ca, which remains in this organ and is not transported to the shoots. The ionic balance can play an essential role in plant nutrition, where increasing anion uptake can stimulate cation uptake and vice versa. This ionic balance effect on plant nutrient uptake was observed in tomato plants exposed to NO_3^- excess, which enhanced Ca uptake and accumulation in the shoot (Figure 7).

This nutrient crosstalk was clearly evident when the N/Ca ratio was analyzed, where the ratio was significantly higher under ammonium excess and calcium deficit. This nutrient ratio has also been studied in fruits, where a correlation was observed with bitter pit apple disorder [48,49].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11071437/s1, Figure S1: Root length and lateral root number are not altered in tomato seedlings under calcium deficit or ammonium and nitrate excesses. Tomato seedlings were grown with control media, with calcium deficit, or with ammonium or nitrate excesses for 10 days in a growth chamber as described in the Materials and Methods section. Tomato images were taken

10 days after sowing the seedlings: (**A**) main root length; (**B**) number of lateral roots; (**C**) lateral root density. Figure S2: Nitrogen and calcium are altered by calcium deficit and not by ammonium and nitrate excesses in fruits. Tomato seedlings were grown with control media, with calcium deficit, or with ammonium or nitrate excesses for 10 days in a growth chamber as described in the Materials and Methods section. Tomato fruits were collected and dried at 45 °C in a dehydrator. Total nitrogen (**A**) and total calcium (**B**) were quantified as a percentage of plant dry weight. Table S1: Nutritional content of in vitro media. Table S2: Nutritional content of in-pot media. Table S3: List of primers used. Table S4: Nutritional data from in vitro experiment used for PCA analysis. Table S5: Nutritional data from potted plants used for PCA analysis. Table S6: Normalized nutritional and phenotypic data used for PCA analysis.

Author Contributions: Conceptualization, methodology, formal analysis, supervision, resources, and funding acquisition, J.A.O. and C.B.; writing—review and editing J.A.O., C.B., S.T.d.F. and C.A.; data analysis–interpretation, J.A.O., C.B., S.T.d.F., N.B. and C.A.; investigation, C.A., C.P., R.G. and M.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

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

References

1. Bénard, C.; Gautier, H.; Bourgaud, F.; Grasselly, D.; Navez, B.; Caris-Veyrat, C.; Weiss, M.; Génard, M. Effects of Low Nitrogen Supply on Tomato (*Solanum Lycopersicum*) Fruit Yield and Quality with Special Emphasis on Sugars, Acids, Ascorbate, Carotenoids, and Phenolic Compounds. *J. Agric. Food Chem.* **2009**, *57*, 4112–4123. [CrossRef]
2. Kusano, M.; Fukushima, A.; Redestig, H.; Saito, K. Metabolomic Approaches toward Understanding Nitrogen Metabolism in Plants. *J. Exp. Bot.* **2011**, *62*, 1439–1453. [CrossRef]
3. Mengel, K.; Kirkby, E.A. *Principles of Plant Nutrition*, 3rd ed.; International Potash Institute: Bern, Switzerland, 1978.
4. Krapp, A. Plant Nitrogen Assimilation and Its Regulation: A Complex Puzzle with Missing Pieces. *Curr. Opin. Plant Biol.* **2015**, *25*, 115–122. [CrossRef]
5. Nacry, P.; Bouguyon, E.; Gojon, A. Nitrogen Acquisition by Roots: Physiological and Developmental Mechanisms Ensuring Plant Adaptation to a Fluctuating Resource. *Plant Soil* **2013**, *370*, 1–29. [CrossRef]
6. Pal'ove-Balang, P. Role of Nitrogen Metabolites on the Regulation of Nitrate Uptake Inmaize Seedlings. *Acta Biol. Szeged.* **2002**, *46*, 177–178.
7. Issawi, M.; Guillaumot, D.; Sol, V.; Riou, C. Responses of an Adventitious Fast-Growing Plant to Photodynamic Stress: Comparative Study of Anionic and Cationic Porphyrin Effect on Arabidopsis Thaliana. *Physiol. Plant.* **2018**, *162*, 379–390. [CrossRef] [PubMed]
8. Jampeetong, A.; Brix, H.; Kantawanichkul, S. Effects of Inorganic Nitrogen Form on Growth, Morphology, N Uptake, and Nutrient Allocation in Hybrid Napier Grass (Pennisetum Purpureum × Pennisetum Americanum Cv. Pakchong1). *Ecol. Eng.* **2014**, *73*, 653–658. [CrossRef]
9. Martínez-Andújar, C.; Ghanem, M.E.; Albacete, A.; Pérez-Alfocea, F. Response to Nitrate/Ammonium Nutrition of Tomato (*Solanum Lycopersicum* L.) Plants Overexpressing a Prokaryotic NH4+-Dependent Asparagine Synthetase. *J. Plant Physiol.* **2013**, *170*, 676–687. [CrossRef] [PubMed]
10. Ben-Oliel, G.; Kant, S.; Naim, M.; Rabinowitch, H.D.; Takeoka, G.R.; Buttery, R.G.; Kafkafi, U. Effects of Ammonium to Nitrate Ratio and Salinity on Yield and Fruit Quality of Large and Small Tomato Fruit Hybrids. *J. Plant Nutr.* **2005**, *27*, 1795–1812. [CrossRef]
11. Gerendás, J.; Zhu, Z.; Bendixen, R.; Ratcliffe, R.G.; Sattelmacher, B. Physiological and Biochemical Processes Related to Ammonium Toxicity in Higher Plants. *J. Plant Nutr. Soil Sci.* **1997**, *160*, 239–251. [CrossRef]
12. Fallahi, E.; Fallahi, B.; Peryea, F.J.; Neilsen, G.H.; Neilsen, D. Effects of Mineral Nutrition on Fruit Quality and Nutritional Disorders in Apples. *Acta Hortic.* **2010**, *868*, 49–59. [CrossRef]
13. Paradiković, N.; Teklić, T.; Horvat, D.; Vinković, T.; Gumze, A. Influence of Different Nitrogen Form Application on the Incidence of BER and Elementary Composition of Bell Peppers. In *Cereal Research Communications*; Springer: Berlin/Heidelberg, Germany, 2007; Volume 35, pp. 897–900. [CrossRef]
14. Taylor, M.D.; Locascio, S.J. Blossom-End Rot: A Calcium Deficiency. *J. Plant Nutr.* **2004**, *27*, 123–139. [CrossRef]
15. Masaharu, K.; Araki, T.; Yoshida, S.; Eguchi, T. Dependence of Calcium Uptake on Water and Respiration in Roots of Tomato Plants (Lycopersicon Esculentum Mill). *Biotronics* **1999**, *28*, 121–130.
16. Hirschi, K.D. The Calcium Conundrum. Both Versatile Nutrient and Specific Signal. Plant Physiology. *Am. Soc. Plant Biol.* **2004**, 2348–2442. [CrossRef]
17. Montanaro, G.; Dichio, B.; Lang, A.; Mininni, A.N.; Nuzzo, V.; Clearwater, M.J.; Xiloyannis, C. Internal versus External Control of Calcium Nutrition in Kiwifruit. *J. Plant Nutr. Soil Sci.* **2014**, *177*, 819–830. [CrossRef]

18. Karley, A.J.; White, P.J. Moving Cationic Minerals to Edible Tissues: Potassium, Magnesium, Calcium. In *Current Opinion in Plant Biology*; Elsevier: Amsterdam, The Netherlands, 2009; pp. 291–298. [CrossRef]
19. White, P.J. The Pathways of Calcium Movement to the Xylem. *J. Exp. Bot.* **2001**, *52*, 891–899. [CrossRef]
20. Kirkby, E.A.; Pilbeam, D.J. Calcium as a Plant Nutrient. In *Plant, Cell & Environment*; John Wiley & Sons, Ltd.: Hoboken, NJ, USA, 1984; pp. 397–405. [CrossRef]
21. White, P.J.; Broadley, M.R. Calcium in Plants. In *Annals of Botany*; Oxford Academic: Oxford, UK, 2003; pp. 487–511. [CrossRef]
22. Kanayama, Y.; Kochetov, A. *Abiotic Stress Biology in Horticultural Plants*; Springer: Japan, 2015; Available online: https://www.springer.com/gp/book/9784431552505 (accessed on 19 March 2021). [CrossRef]
23. Fenn, L.B.; Taylor, R.M. *Calcium Stimulation of Ammonium Absorption in Plants*; Elsevier: Amsterdam, The Netherlands, 1991; Volume 24, pp. 39–47. [CrossRef]
24. Hernández-Gómez, E.; Valdez-Aguilar, L.A.; Cartmill, D.L.; Cartmill, A.D.; Alia-Tajacal, I. Supplementary Calcium Ameliorates Ammonium Toxicity by Improving Water Status in Agriculturally Important Species. *AoB Plants* **2015**, *7*, plv105. [CrossRef] [PubMed]
25. He, Z.L.; Alva, A.K.; Calvert, D.V.; Li, Y.C.; Banks, D.J. Effects of Nitrogen Fertilization of Grapefruit Trees on Soil Acidification and Nutrient Availability in a Riviera Fine Sand. *Plant Soil* **1999**, *206*, 11–19. [CrossRef]
26. Wieczorek, P.; Wrzesińska, B.; Obrepalska-Steplowska, A. Assessment of Reference Gene Stability Influenced by Extremely Divergent Disease Symptoms in *Solanum Lycopersicum* L. *J. Virol. Methods* **2013**, *194*, 161–168. [CrossRef]
27. Ruijter, J.M.; Ramakers, C.; Hoogaars, W.M.H.; Karlen, Y.; Bakker, O.; van den Hoff, M.J.B.; Moorman, A.F.M. Amplification Efficiency: Linking Baseline and Bias in the Analysis of Quantitative PCR Data. *Nucleic Acids Res.* **2009**, *37*. [CrossRef]
28. Swietlik, D. The Interaction between Calcium Chloride and Ammonium-Nitrogen on Growth, Nitrogen Uptake and Translocation in Apple and Sour Orange. *Acta Hortic.* **2006**, *721*, 159–164. [CrossRef]
29. Mokhele, B.; Zhan, X.; Yang, G.; Zhang, X. Review: Nitrogen Assimilation in Crop Plants and Its Affecting Factors. *Can. J. Plant Sci.* **2012**, 399–405. [CrossRef]
30. Sun, H.; Li, J.; Song, W.; Tao, J.; Huang, S.; Chen, S.; Hou, M.; Xu, G.; Zhang, Y. Nitric Oxide Generated by Nitrate Reductase Increases Nitrogen Uptake Capacity by Inducing Lateral Root Formation and Inorganic Nitrogen Uptake under Partial Nitrate Nutrition in Rice. *J. Exp. Bot.* **2015**, *66*, 2449–2459. [CrossRef] [PubMed]
31. Rančić, D.; Quarrie, S.P.; Pećinar, I. Anatomy of Tomato Fruit and Fruit Pedicel during Fruit Development. *Microsc. Sci. Technol. Appl. Educ.* **2010**, *2*, 851–861.
32. Andrews, M.; Raven, J.A.; Lea, P.J. Do Plants Need Nitrate? The Mechanisms by Which Nitrogen Form Affects Plants. *Ann. Appl. Biol.* **2013**, *163*, 174–199. [CrossRef]
33. Saure, M.C. Why Calcium Deficiency Is Not the Cause of Blossom-End Rot in Tomato and Pepper Fruit—A Reappraisal. *Sci. Hortic.* **2014**, *174*, 151–154. [CrossRef]
34. Nunes Júnior, E.S.; De Medeiros, J.F.; De Oliveira, F.D.A.; Lima, L.A.; Bezerra, F.M.S.; De, R.; Alves, C. Nitrogen and Potassium Fertigation in Bell Pepper Cultivated in Greenhouse Using Fertigation Managements 1. *Rev. Bras. Eng. Agrícola Ambient.* **2017**, *21*, 186–190. [CrossRef]
35. Hagassou, D.; Francia, E.; Ronga, D.; Buti, M. Blossom End-Rot in Tomato (*Solanum Lycopersicum* L.): A Multi-Disciplinary Overview of Inducing Factors and Control Strategies. *Sci. Hortic.* **2019**, *249*, 49–58. [CrossRef]
36. Liu, Y.; Lai, N.; Gao, K.; Chen, F.; Yuan, L.; Mi, G. Ammonium Inhibits Primary Root Growth by Reducing the Length of Meristem and Elongation Zone and Decreasing Elemental Expansion Rate in the Root Apex in Arabidopsis Thaliana. *PLoS ONE* **2013**, *8*, 61031. [CrossRef] [PubMed]
37. Flores, P.; Carvajal, M.; Cerdá, A.; Martínez, V. Salinity and Ammonium/Nitrate Interactions on Tomato Plant Development, Nutrition, and Metabolites. *J. Plant Nutr.* **2001**, *24*, 1561–1573. [CrossRef]
38. Rossato, L.; Lainé, P.; Ourry, A. Nitrogen Storage and Remobilization in Brassica Napus L. during the Growth Cycle: Nitrogen Fluxes within the Plant and Changes in Soluble Protein Patterns. *J. Exp. Bot.* **2001**, *52*, 1655–1663. [CrossRef]
39. Trapani, N.; Hall, A.J.; Weber, M. Effects of Constant and Variable Nitrogen Supply on Sunflower (Helianthus Annuus L.) Leaf Cell Number and Size. *Ann. Bot.* **1999**, *84*, 599–606. [CrossRef]
40. Aloni, R. Role of Hormones in Controlling Vascular Differentiation and the Mechanism of Lateral Root Initiation. *Planta* **2013**, *238*, 819–830. [CrossRef]
41. Dražeta, L.; Lang, A.; Cappellini, C.; Hall, A.J.; Volz, R.K.; Jameson, P.E. Vessel Differentiation in the Pedicel of Apple and the Effects of Auxin Transport Inhibition. *Physiol. Plant.* **2004**, *120*, 162–170. [CrossRef]
42. O'Brien, J.A.; Vega, A.; Bouguyon, E.; Krouk, G.; Gojon, A.; Coruzzi, G.; Gutiérrez, R.A. Nitrate Transport, Sensing, and Responses in Plants. *Mol. Plant* **2016**, *9*, 837–856. [CrossRef] [PubMed]
43. Li-Li, G.; Sheng-Feng, W.; Zi-Fei, L.; Jin-Sheng, H.; Rong-Le, L.; Hong, W. Two Cultivars of Peanut (*Arachis Hypogaea*) Seedlings Show Different Tolerance to Calcium Deficiency. *J. Plant Nutr.* **2015**, *4167*. [CrossRef]
44. Rietra, R.P.J.J.; Heinen, M.; Dimkpa, C.O.; Bindraban, P.S. Effects of Nutrient Antagonism and Synergism on Yield and Fertilizer Use Efficiency. *Commun. Soil Sci. Plant Anal.* **2017**, *48*, 1895–1920. [CrossRef]
45. Kowalska, I.; Sady, W.; Leja, M. Effect of Nitrogen Form and Type of Polyethylene Film Covering Tunnel on Nutrient Content of Hydroponically Grown Sweet Pepper. *Veg. Crop. Res. Bull.* **2009**, *71*, 69–78. [CrossRef]

46. White, P.J. *Ion Uptake Mechanisms of Individual Cells and Roots: Short-Distance Transport*; Elsevier Ltd.: Amsterdam, The Netherlands, 2011. [CrossRef]
47. Wang, H.-Y.; Wang, Y.-H.; Du, C.-W.; Xu, F.-S.; Yang, Y.-H. Effects of Boron and Calcium Supply on Calcium Fractionation in Plants and Suspension Cells of Rape Cultivars with Different Boron Efficiency. *J. Plant Nutr.* **2003**, *26*, 789–806. [CrossRef]
48. Bonomelli, C.; Mogollón, R.; de Freitas, S.T.; Zoffoli, J.P.; Contreras, C. Nutritional Relationships in Bitter Pit-Affected Fruit and the Feasibility of Vis-NIR Models to Determine Calcium Concentration in "Fuji" Apples. *Agronomy* **2020**, *10*, 1476. [CrossRef]
49. Do Amarante, C.V.T.; Miqueloto, A.; de Freitas, S.T.; Steffens, C.A.; Silveira, J.P.G.; Corrêa, T.R. Fruit Sampling Methods to Quantify Calcium and Magnesium Contents to Predict Bitter Pit Development in "Fuji" Apple: A Multivariate Approach. *Sci. Hortic.* **2013**, *157*, 19–23. [CrossRef]