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# Ascorbate Supplementation: A Blessing in Disguise for Tomato Seedlings Exposed to NiO Nanoparticles

**Sofia Spormann [1,2,†], Filipa Sousa [1,2,†], Fátima Oliveira [2], Vasco Ferreira [2], Bárbara Teixeira [3], Cláudia Pereira [1,2], Cristiano Soares [1,2,*] and Fernanda Fidalgo [1,2]**

[1] GreenUPorto-Sustainable Agrifood Production Research Centre/Inov4Agro, Faculty of Sciences, University of Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal
[2] Biology Department, Faculty of Sciences, University of Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal
[3] CIC-Colégio Internato dos Carvalhos, Rua do Padrão 83, 4415-284 Carvalhos, Portugal
* Correspondence: cfsoares@fc.up.pt
† These authors contributed equally to this work.

**Abstract:** While nanomaterials offer wide-ranging solutions, their intensified use causes environmental contamination, posing ecotoxicological risks to several organisms, including plants. It becomes important to understand the phytotoxicity of NMs and find sustainable strategies to enhance plant tolerance to these emerging contaminants. Thus, this study aimed to evaluate the potential of ascorbic acid (AsA) in increasing the tolerance of in vitro grown tomato seedlings to nickel oxide nanomaterials (nano-NiO). Seeds of *Solanum lycopersicum* cv. Micro-Tom were germinated in culture medium containing 30 mg/L nano-NiO, 150 mg/L AsA, or a combination of both. A control situation was included. Surprisingly, single AsA administration in the medium impaired the growth of tomato seedlings and increased the lipid peroxidation of biomembranes. Nonetheless, plant development was more severely repressed by nano-NiO, with evident macroscopic effects that did not translate into serious redox disorders. Still, proline and AsA levels diminished in response to nano-NiO, while glutathione and phenols increased. Despite the negative effects of AsA on non-stressed plants, nano-NiO-induced stress was counteracted by AsA supply, with enhanced levels of glutathione and phenols. Overall, the supplementation with AsA proved to be a "blessing in disguise" for plants under nano-NiO-induced stress, improving antioxidant capacity and activating other defense mechanisms.

**Keywords:** nanomaterials; ascorbic acid; *Solanum lycopersicum* L.; axidative stress; antioxidant system



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## 1. Introduction

Nanotechnology provides the ability to explore the physical, chemical, electrical, and mechanical properties of nano-scaled materials—nanomaterials (NMs) [1]—typically with dimensions of less than 100 nm [2]. NMs possess a large range of applications in medicine, engineering, science, and pharmaceutical industry, putting nanotechnology in the top areas of interest in recent years [3]. NMs may arise in nature due to chemical reactions, volcanic eruptions, forest fires, and rock erosion events [1,4]; however, their anthropic production and emission into the environment have been increasing, making NMs hazardous, emergent contaminants, namely in agricultural soils and irrigation waters [5]. In fact, due to their small size, high biochemical reactivity, and ability to cross biological membranes, NMs can pose ecotoxicological risks to plants and soil organisms, which should not be overlooked from an agronomic point of view [4,6]. With the recently expanded use of nano-designed fertilizers and pesticides in agriculture, the occurrence of surface runoff events or atmospheric deposition are common causes of NMs accumulation in soils and water [7]. Metal oxide NMs (metal-NMs), which include nickel oxide NMs (nano-NiO), are among the most used nano-formulations [8,9], presenting promising contributions

to the fields of catalysis, gas sensors, lithium-ion batteries, magnetism, and supercapacitors, which have even been associated with low toxicity, cheapness, and environmental friendliness [9]. Despite that, recent reports are more pessimistic regarding the status of metal-NMs as emerging environmental contaminants capable of inducing phytotoxicity (see Soares et al. [10,11], Malandrakis et al. [12], and references therein). Given their sessile nature and direct contact with the soil, plants are particularly vulnerable to metal-NMs, which may accumulate in tissues, putting agricultural productivity at risk and perpetuating their presence throughout the food chains [4,6,12–16]. Besides the inherent aspects of NMs regarding size, structure, and reactivity, their phytotoxicity features are also greatly dependent on the presence of the metal ions released over time, thus increasing the risks of metal-NMs [17].

Although nickel (Ni) is one of the essential micronutrients required for plant growth and development, when exposed to higher levels of this heavy metal (HM), plants suffer from metal-induced toxicity [18–21]. Contaminant levels of Ni in plants cause interference with the uptake of other essential nutrients and induce cytotoxic and genotoxic effects [18–21]. Ni-stress is usually accompanied by redox imbalances and reduced efficiency of the antioxidant (AOX) mechanisms, leading to conditions of oxidative stress and consequent growth inhibition and foliar chlorosis [18,19]. Although the phytotoxicity of Ni has been widely studied in several plant species [18,20,22–24], studies that evaluate the effects of Ni nano-formulations on plants' physiology are still scarce [11,23,25].

In vitro plant tissue culture technology is being widely used as a tool of research [26], offering an alternative to other plant growth systems, by providing strictly controlled conditions of growth, including a proper supply of nutrients, controlled pH of the medium, and assured aseptic conditions, which prevents microbial infestations that would otherwise introduce variability among samples. Besides, the composition of the medium can be easily manipulated, allowing plant tissues to directly contact specific supplements at very well-tuned concentrations [27]. Some commonly used supplements for plants growing under in vitro conditions are adsorbent and AOX agents, such as activated charcoal, ascorbic acid (AsA), citric acid, cysteine, and silver nitrate [27].

AsA is the most abundant AOX metabolite in plants given its ability to directly neutralize the toxic effects of the ROS (such as singlet oxygen—$^1O_2$, superoxide anion—$O_2^{\bullet-}$, and hydroxyl radical—$^{\bullet}OH$), while concomitantly acting as an electron donor in an enzymatic reaction that leads to the elimination of hydrogen peroxide ($H_2O_2$) [28]. In addition, AsA actively participates in various cellular processes, such as mitosis, cell elongation, senescence, and cell death [28]. For these reasons, AsA stands as one of the most interesting compounds in the research for strategies that could increase plants' tolerance to abiotic stresses [29,30]. Concerning the toxicity imposed by metals on plants, Zhang et al. [31] reported that the exogenous application of AsA alleviated cadmium (Cd) toxicity, by reducing oxidative damage by boosting AOX defenses and remarkably decreasing Cd uptake. Similar findings in Cd-stressed wheat plants were also recently observed by Zhou et al. [32]. AsA has also been shown to be an efficient mitigator of stress imposed by lead (Pb) and chromium (Cr), alleviating their negative effects by reducing their uptake, and activating AOX mechanisms, decreasing the accumulation of ROS and consequently attenuating oxidative damage [33–35]. Nonetheless, to the best of our knowledge, the exogenous application of AsA to mitigate Ni toxicity has not been explored yet.

Bearing all of this in mind, with the goal of assessing the potential of AsA in increasing the tolerance of tomato plants to nano-NiO, in vitro grown plantlets of *Solanum lycopersicum* L. cv. Micro-Tom were exposed to 30 mg $L^{-1}$ nano-NiO alone or in combination with 150 mg $L^{-1}$ AsA. After 19 days, several biometric and biochemical parameters were quantified to better understand the impacts of nano-NiO as well as the potential of AsA as a stress ameliorative strategy.

## 2. Material and Methods

### *2.1. Experimental Design*

Seeds from *Solanum lycopersicum* L. cv. Micro-Tom, obtained from FCUP's collection, were surface disinfected (10 min in 70% (v/v) ethanol and 7 min in 20% (v/v) commercial bleach, containing 0.05% (m/v) Tween 20, followed by three series of rinsing with sterile deionized water) and let to germinate on agar-solidified half-strength Murashige and Skoog (MS; [36]) culture medium (Duchefa Biochemie B.V., Haarlem, The Netherlands), supplemented with 1.5% (m/v) sucrose. Three different treatments were considered, besides the control (CTL): AsA, nano-NiO, and nano-NiO + AsA. For that purpose, the culture media was either supplemented with 150 mg $L^{-1}$ AsA, 30 mg $L^{-1}$ nano-NiO, or with both 30 mg $L^{-1}$ nano-NiO and 150 mg $L^{-1}$ AsA. Nano-NiO concentration was selected based on a previous study by Pinto et al. [23], in which tissue explants were cultured in medium containing 15 mg $L^{-1}$ nano-NiO. This concentration was increased to 30 mg $L^{-1}$, given that the present work used whole seedlings, which are normally less sensitive than the tissue explants used in the aforementioned study. The characterization of nano-NiO can be found in Soares et al. [37]. The nano-NiO stock solution (1 g $L^{-1}$) was previously sonicated for 15 min by ultrasound in a water bath, to avoid aggregation of NMs. Regarding AsA, the concentration of 150 mg $L^{-1}$, which corresponds to approximately 0.85 mM, was selected based on the bibliography, namely on the several reports cited in the review by Ortiz-Espín et al. [38]. The pH of the culture media was adjusted to 5.7–5.8 and 0.625% (m/v) agar was added before autoclaving at 121 °C for 25 min.

Flasks containing three tomato seeds each were placed in a growth chamber, under controlled conditions of temperature (25 ± 2 °C), photoperiod (16 h light/8 h dark), and light (photosynthetically active radiation—PAR: 60 µmol $m^{-2}$ $s^{-1}$). For each treatment (CTL; AsA; nano-NiO; nano-NiO + AsA), 8 replicates (flasks) were considered.

### *2.2. Biometry Assessments and Visual Observation of Symptoms*

Twenty-eight days after inoculation, i.e., nineteen days after germination, plantlets were collected and used to assess biometric parameters (maximum length of roots and biomass of roots and shoots, expressed in cm and g of fresh weight (f.w.), respectively). Then, plant material from 4 biological replicates of each treatment was immediately processed to be used in confocal microscopy techniques, as described in Section 2.5, while the remainder was frozen and ground in liquid nitrogen, subdivided into aliquots of approximately 200 mg, and stored at −80 °C for subsequent biochemical analysis.

### *2.3. Extraction and Quantification of Photosynthetic Pigments*

Photosynthetic pigments were extracted from frozen aliquots of shoot tissues (ca. 200 mg) in 80% (v/v) acetone and were quantified according to Lichtenthaler [39]. After a 10 min centrifugation at 1400 g, the absorbance (Abs) of the supernatant was read at 663, 647, and 470 nm, and the content in chlorophylls (Chl *a* and *b*) and carotenoids (Car) was estimated based on the formulas of Lichtenthaler [39]. Results were expressed in mg $g^{-1}$ fresh weight (f.w.).

### *2.4. Quantification of Lipid Peroxidation (LP)*

The membrane damage was assessed in terms of LP, by the quantification of malondialdehyde (MDA). Frozen aliquots of shoots of approximately 200 mg were homogenized in 0.1% (m/v) trichloroacetic acid (TCA), under cold conditions, using a bead miller (Bead Ruptor 12 Homogenizer (Omni International Inc., Georgia, USA)). MDA was spectrophotometrically quantified, according to Heath and Packer [40], and expressed as nmol MDA $g^{-1}$ f.w., using the molar extinction coefficient (ε) of 155 $mM^{-1}$ $cm^{-1}$.

### *2.5. Detection and Quantification of $H_2O_2$*

$H_2O_2$ levels were quantified in vitro according to Alexieva et al. [41], using homogenates obtained from frozen aliquots of shoots (ca. 200 mg f.w.) extracted with

0.1% (m/v) TCA under cold conditions, using a bead miller (Bead Ruptor 12 Homogenizer (Omni International Inc.)). The content of $H_2O_2$ was spectrophotometrically quantified (390 nm) based on the degree of potassium iodide (KI) oxidation. Levels were calculated from a standard curve, and results were expressed as nmol $H_2O_2$ $g^{-1}$ f.w.

Moreover, the presence of $H_2O_2$ was also detected in vivo through confocal laser scanning microscopy (CLSM) in cells from the primary root elongation zone, using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich), by adapting the methods of Sandalio et al. [42]. For this purpose, root sections (ca. 2 cm from the root apex) were selected and incubated at 37 °C for 30 min in a 25 µM DCF-DA solution prepared in 10 mM Tris-HCl buffer (pH 7.5). Plant material was then washed in 10 mM Tris-HCl buffer (pH 7.5) to remove excess DCF-DA. Root sections were finally mounted between a slide and a coverslip, in aqueous medium. Observations and image acquisition were performed using a Laser Point Scanning Confocal System (Leica TCS SP5-Leica Microsystems), detecting the fluorescent probe by monitoring the excitation and emission wavelengths of 480 and 530 nm, respectively. Images were analyzed and processed using the ImageJ-Fiji program [43], by measuring the signal intensity of each pixel on a grayscale, where black corresponds to the absence of signal and white to the maximum amount of signal. Measurements were performed on the whole cells, except for cell walls, to avoid the influence of their autofluorescence.

### *2.6. Extraction and Quantification of Soluble Proteins*

Total soluble proteins were extracted from frozen aliquots of shoots (ca. 200 mg) using the Bead Ruptor 12 Homogenizer (Omni International Inc.) in 100 mM potassium phosphate (PK) buffer (pH 7.3), supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA), 8% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM L-AsA, and 2% (m/v) polyvinylpolypyrrolidone (PVPP). To prevent sample overheating, tubes were incubated on ice for 1 min between each homogenization cycle. Then, all homogenates were centrifuged at 16,000× *g*, for 35 min at 4 °C, the supernatants were collected and transferred to new tubes for soluble protein quantification [44], and the kinetic assessment of the activity of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.8.1.7) was performed as described in Section 2.7.6.

### *2.7. Assessment of AOX Responses*

#### 2.7.1. Total Phenols

The content of total phenols was determined according to the adapted methods of Zafar et al. [45]. Frozen aliquots of shoots (ca. 200 mg) were homogenized on ice in 80% (v/v) methanol, using a mortar and pistil with quartz sand. Then, 180 µL of Folin–Ciocalteu reagent were added to 40 µL of diluted SN (1:2). After incubating for 5 min at room temperature, 180 µL of 7.5% (m/v) sodium carbonate ($Na_2CO_3$) were added, followed by an incubation in the dark for 1 h, at room temperature. Total phenols were spectrophotometrically quantified (725 nm), and results were expressed in µg of gallic acid $g^{-1}$ f.w., using a calibration curve obtained with gallic acid solutions of known concentration.

#### 2.7.2. Glutathione (GSH)

The levels of total GSH were evaluated following procedures optimized by Soares et al. [46], in shoot aliquots (ca. 200 mg f.w.) homogenized on ice in 3% (m/v) sulfosalicylic acid. GSH content was spectrophotometrically determined (412 nm) by its oxidation by the sulfhydryl reagent 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB). The concentrations of GSH were calculated from a calibration curve previously prepared with solutions of known GSH concentration. Results were expressed in µmol GSH $g^{-1}$ f.w.

#### 2.7.3. Proline (Pro)

Pro levels were quantified from shoot aliquots (ca. 200 mg f.w.), according to a protocol described by Bates et al. [47], based on the complexation of Pro with acid ninhydrin (detectable at 520 nm). As before, the extraction procedure was accomplished by a series of homogenization in the bead miller (Bead Ruptor 12 Homogenizer (Omni International Inc.)). The Pro content was estimated through a calibration curve, obtained with different solutions of known concentration of Pro. Results were expressed as μg $g^{-1}$ f.w.

#### 2.7.4. Ascorbate-Reduced (AsA) and Oxidized (DHA) Forms

Quantification of ascorbate was based on the methods described by Gillespie and Ainsworth [48]. Frozen aliquots of shoots (ca. 200 mg) were homogenized, under cold conditions, in 6% (m/v) TCA, using the bead miller [Bead Ruptor 12 Homogenizer (Omni International Inc.)]. AsA (reduced form) was quantified spectrophotometrically (525 nm) based on the reduction of ferric ion to ferrous ion in the presence of 2,2′-bipyridine (BIP). Total ascorbate was quantified after a complete reduction of the total ascorbate pool [AsA + dehydroascorbate (DHA)] using 10 mM 1,4-dithiothreitol (DTT). The concentrations of total and reduced ascorbate were calculated from a calibration curve previously prepared with solutions of known AsA concentration. The levels of DHA were calculated by subtracting the levels of its reduced form from the total ascorbate level. Results were expressed in μmol $g^{-1}$ f.w.

#### 2.7.5. Estimation of the Total Antioxidant Capacity (TAC)

The determination of the TAC was carried out following the adapted methods of Zafar et al. [45]. Frozen aliquots of shoots (ca. 200 mg) were homogenized on ice in 80% (*v/v*) methanol, using a mortar and pestle with quartz sand. Then, 900 μL of a reaction solution containing 0.6 M sulfuric acid ($H_2SO_4$), 4 mM ammonium molybdate, and 28 mM sodium phosphate were added to 100 μL of diluted SN (1:3; in methanol). Tubes were kept at 95 °C for 90 min and then chilled on ice for 10 min. Abs readings were performed at 695 nm, and results were expressed in μg of AsA equivalents $g^{-1}$ f.w., using a calibration curve previously obtained from different AsA solutions of known concentration.

#### 2.7.6. Measurement of AOX Enzymes' Activity

The total activity of SOD was spectrophotometrically quantified based on the inhibition of the photochemical reduction of NBT at 560 nm [49]. SOD activity was expressed according to Beauchamp and Fridovich [50] as units of SOD $mg^{-1}$ protein, in which one unit of SOD corresponds to the amount of enzyme needed to inhibit the photochemical reduction of NBT by 50%. The activities of CAT, APX, DHAR, and GR were also spectrophotometrically assayed using Multiskan GO (Thermo Fisher Scientific) in a 96-well UV microplate by enzyme kinetics at 240, 290, 265, and 340 nm, respectively, as described by Soares et al. [51], following the procedures of Aebi [52] for CAT and of Murshed et al. [53] for enzymes involved in the AsA–GSH cycle. The activity of CAT was expressed in nmol of $H_2O_2$ $min^{-1}$ $mg^{-1}$ of protein, considering an ε value of 39.4 $mM^{-1}$ $cm^{-1}$. The activity of APX, measured by the decomposition of AsA into DHA, was expressed in μmol of AsA $min^{-1}$ $mg^{-1}$ of protein, using an ε value of 0.49 $mM^{-1}$ $cm^{-1}$. DHAR activity was measured by its potential to reduce DHA to AsA and was expressed in nmol AsA $min^{-1}$ $mg^{-1}$ protein, using an ε value of 14 $mM^{-1}$ $cm^{-1}$. Lastly, GR activity was measured in terms of NADPH degradation and was expressed in nmol NADPH $min^{-1}$ $mg^{-1}$ protein, using an ε value of 6.22 $mM^{-1}$ $cm^{-1}$.

### *2.8. Statistical Analysis*

The growth trial was set up as a randomized factorial block design, considering four experimental conditions (CTL; AsA; nano-NiO; nano-NiO + AsA). All eight biological replicates of each treatment were used in biometrical assessments, while for the biochemical parameters, at least three random biological replicates of each treatment were used, and in

microscopy detections, four. Results were expressed as mean ± standard error of the mean (SEM). After checking the homogeneity of variances (Levene's test), data from biometric and biochemical analyses were subjected to a one-way analysis of variance (ANOVA). Whenever significant differences were found ($p \leq 0.05$), Tukey's post hoc test was used to compare individual means. All statistical procedures were performed in GraphPad Prism 8 (version 8.0.2 (263), GraphPad Software Inc., San Diego, CA, USA).

## 3. Results

### *3.1. AsA Supply Affected Plant Growth and Redox Homeostasis but Alleviated the Phytotoxicity Induced by Nano-NiO*

Nano-NiO application caused a decrease in all biometric parameters of the tomato plantlet's growth (root length by 19%, root biomass by 47%, and shoot biomass by 23%), in relation to the CTL (Figure 1). Plants under the combined treatment of nano-NiO and AsA supplementation also showed a reduction in root and shoot biomass (by 59% and 36%, respectively) and in shoot length (by 22%), but not in terms of root elongation. Surprisingly, the treatment with AsA alone increased root length by 23% in comparison with the CTL, but diminished shoot length by 17% and biomass production in roots and shoots by 70%, and 32%, respectively. In terms of macroscopic symptoms, which is also possible to observe in Figure 2, the exposure to nano-NiO, besides affecting the growth of plants, also induced strong phytotoxic symptoms including leaf chlorosis and necrosis and a reduction in leaf area, which were markedly neutralized under the supplementation with AsA. Thus, these initial observations suggest that, despite the negative effects of AsA in unstressed plants, the co-treatment of nano-NiO-stressed plants with AsA alleviated the inhibition in root elongation and the macroscopic phytotoxicity effects induced by the NM.

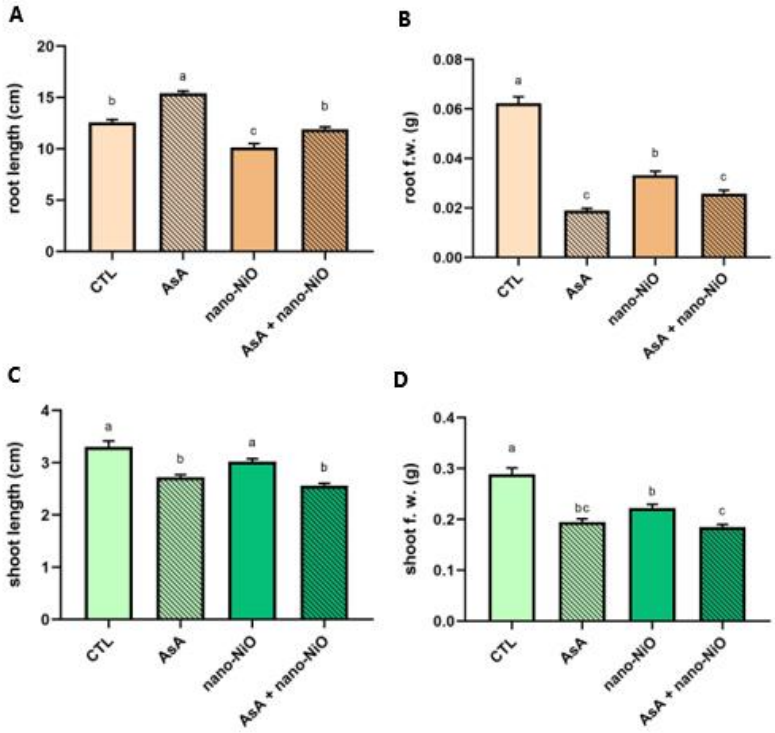


**Figure 1.** Effects of nickel oxide nanomaterials (nano-NiO; 30 mg $L^{-1}$) and/or ascorbate (AsA; 150 mg $L^{-1}$) on the length and biomass, expressed as fresh weight (f.w.), of roots (**A**,**B**) and shoots (**C**,**D**) of in vitro grown tomato plantlets. Data presented are mean ± SEM (n ≥ 3). Different letters above bars indicate significant statistical differences between treatments at $p \leq 0.05$.

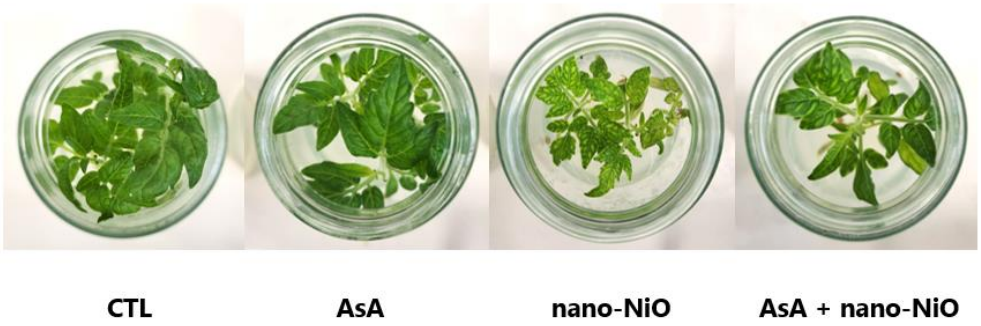


**Figure 2.** Macroscopic effects of nickel oxide nanomaterials (nano-NiO; 30 mg $L^{-1}$) and/or ascorbate (AsA; 150 mg $L^{-1}$) on the growth and development of in vitro grown tomato plantlets.

As can be seen in Figure 3, exposure to nano-NiO caused a slight reduction in the content of chlorophylls and carotenoids by 23% and 15%, respectively, which complies with the overall chlorosis observed in the leaves of these plants. Concerning AsA administration, its presence per se was rather positive in the production of photosynthetic pigments; under the combined treatment, the AsA supply seemed to alleviate the negative effect caused by nano-NiO, bringing the values closer to those observed in CTL plants.

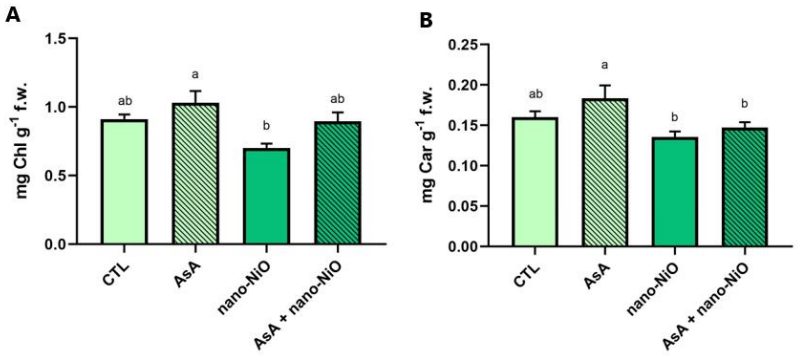


**Figure 3.** Effects of nickel oxide nanomaterials (nano-NiO; 30 mg $L^{-1}$) and/or ascorbate (AsA; 150 mg $L^{-1}$) on the content of chlorophylls (Chl; **A**) and carotenoids (Car; **B**) in the shoots of in vitro grown tomato plantlets. Data presented are mean ± SEM (n ≥ 3) (f.w.: fresh weight). Different letters above bars indicate significant statistical differences between treatments at $p \leq 0.05$.

There was no significant evidence of the occurrence of oxidative stress in the aerial organs of tomato plantlets under nano-NiO treatment, both in terms of membrane damage (Figure 4A), as well as regarding the accumulation of $H_2O_2$ (Figure 4B). Furthermore, a 40% increase in the degree of LP was noted in the shoots of plants treated with AsA alone, which is possibly related to the growth inhibitory effects observed in these plants. This effect was not sustained in plants under the combined treatment (Figure 4A). On the contrary, in the root tissues, a 33-fold accumulation of $H_2O_2$ was detected in plants exposed to nano-NiO (Figure 4C,D), which was successfully counteracted in plants treated with AsA.

### *3.2. AsA Supplementation Stimulated the AOX System*

The assessment of the TAC (Figure 5A) revealed an increase in the shoot tissues of tomato plantlets in response to all treatments: by 52% for AsA application alone, 54% for the nano-NiO treatment, and 63% for the co-treatment situation.

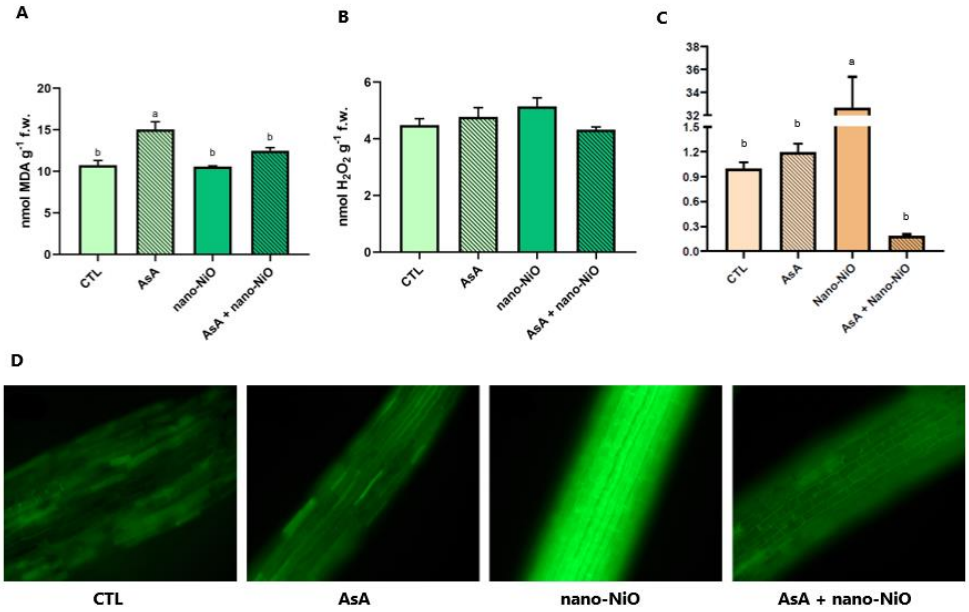


**Figure 4.** Effects of nickel oxide nanomaterials (nano-NiO; 30 mg $L^{-1}$) and/or ascorbate (AsA; 150 mg $L^{-1}$) on the content of MDA (**A**), an indicator of lipid peroxidation and of the reactive oxygen species hydrogen peroxide ($H_2O_2$) in the shoots (**B**) and roots (**C**) of in vitro grown tomato plantlets. Data presented are mean ± SEM (n ≥ 3) (f.w.: fresh weight). Different letters above bars indicate significant statistical differences between treatments at $p \leq 0.05$. Distribution of $H_2O_2$ in the roots of in vitro grown tomato plantlets (**D**). The presence of $H_2O_2$ was detected and quantified in vivo through confocal laser scanning microscopy in cells from the primary root elongation zone stained with 2′,7′-dichlorofluorescein diacetate (DCF-DA). $H_2O_2$ quantification is presented in normalized values of the calculated average signal intensity in relation to the control (**C**).

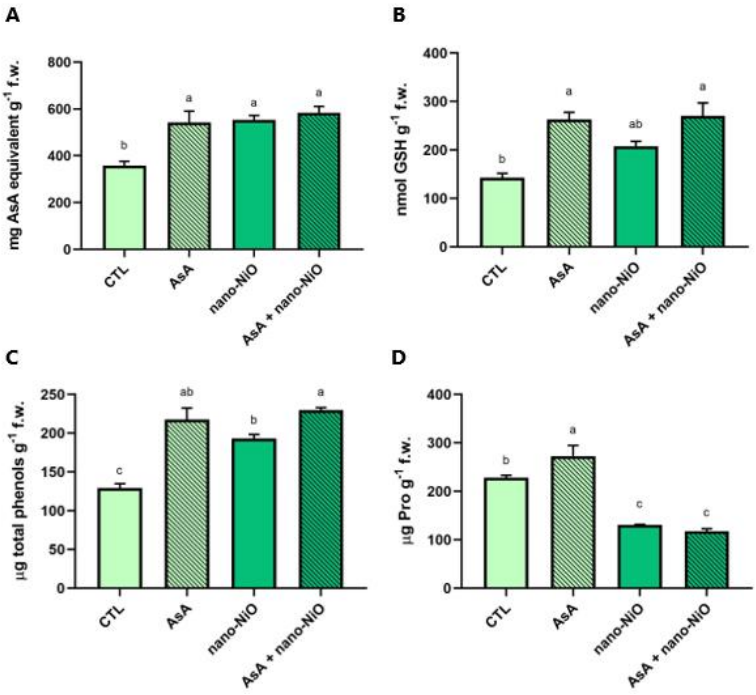


**Figure 5.** Effects of nickel oxide nanomaterials (nano-NiO; 30 mg $L^{-1}$) and/or ascorbate (AsA; 150 mg $L^{-1}$) on the total antioxidant capacity (TAC; **A**), content of glutathione (GSH, **B**), content of total phenols (**C**), and content of proline (Pro; **D**) in the shoots of in vitro grown tomato plantlets. Data presented are mean ± SEM (n ≥ 3) (f.w.: fresh weight). Different letters above bars indicate significant statistical differences between treatments at $p \leq 0.05$.

An increased accumulation of both GSH (Figure 5B) and phenols (Figure 5C) was noticed in shoots under exposure to nano-NiO, by 45% and 49%, respectively, in relation to the CTL. The treatment with AsA alone also led to an accentuated increase in the content of these AOXs, by 84% for GSH and 68% for phenols. Shoots from plantlets grown under combined supplementation of nano-NiO and AsA showed the highest levels of GSH and phenols, around 88% and 77% higher than in the CTL plants, respectively.

While the treatment with AsA alone caused the levels of Pro to increase by 19% in the shoot tissues of tomato plantlets, this compatible osmolyte decreased in response to nano-NiO, both individually (by 43%) and in the combined treatment (by 48%) (Figure 5D).

The endogenous levels of total AsA increased considerably in the shoots of plants grown in the culture medium supplemented with this AOX (by 90%; Figure 6A). From Figure 6B,C, it is possible to see that, in all treatments, the reduced form of AsA was less abundant than its oxidized form—DHA, but both partial levels of its reduced and oxidized forms increased in plants treated with AsA (by 63% and 88%, respectively, Figure 6B,C). Yet, the increase in DHA levels was not sustained in plants under the combined supplementation treatment in comparison to the CTL (Figure 6C), despite the supplementation with AsA at a similar concentration. Consequently, in these plants, the total buildup of AsA was much more tenue (by 35%; Figure 6A), being explained by the 47% increase in its reduced fraction. The levels of AsA in plants facing nano-NiO alone did not change in relation to the CTL plants.

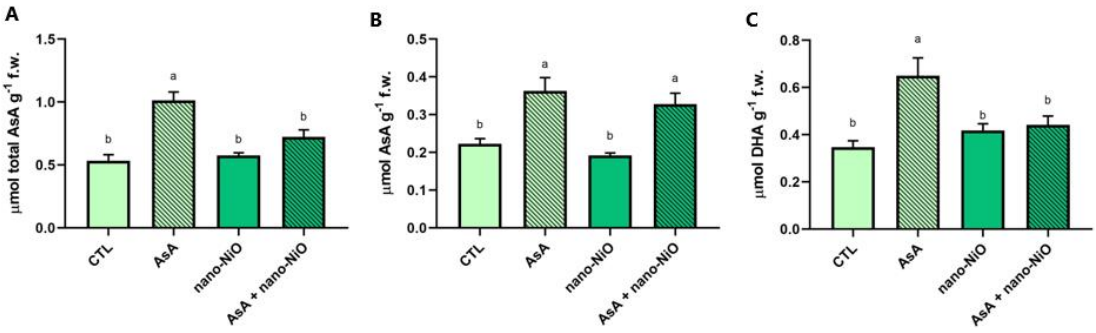


**Figure 6.** Effects of nickel oxide nanomaterials (nano-NiO; 30 mg $L^{-1}$) and/or ascorbate (AsA; 150 mg $L^{-1}$) on the content of total ascorbate (total AsA; **A**), reduced ascorbate (AsA; **B**), and oxidized ascorbate (DHA; **C**) in the shoots of in vitro grown tomato plantlets. Data presented are mean ± SEM (n ≥ 3) (f.w.: fresh weight). Different letters above bars indicate significant statistical differences between treatments at $p \leq 0.05$.

Regarding the enzymatic component of the AOX system, the treatment with AsA, despite increasing the accumulation of proteins by 56% (Figure 7F), did not lead to an overall enhancement in the activities of the assessed AOX enzymes (Figure 7A–E). In fact, single treatment with AsA only showed a positive effect on the activity of CAT (by 69%, Figure 7B), causing significant inhibition of DHAR by 53% and GR by 35% (Figure 7D,E). Exposure to nano-NiO did not affect the concentration of protein (Figure 7F), but stimulated the activities of CAT, APX, and GR, by 128%, 42%, and 63%, respectively (Figure 7B–D), while inhibiting those of SOD and DHAR by 38% and 37%, respectively (Figure 7A,E).

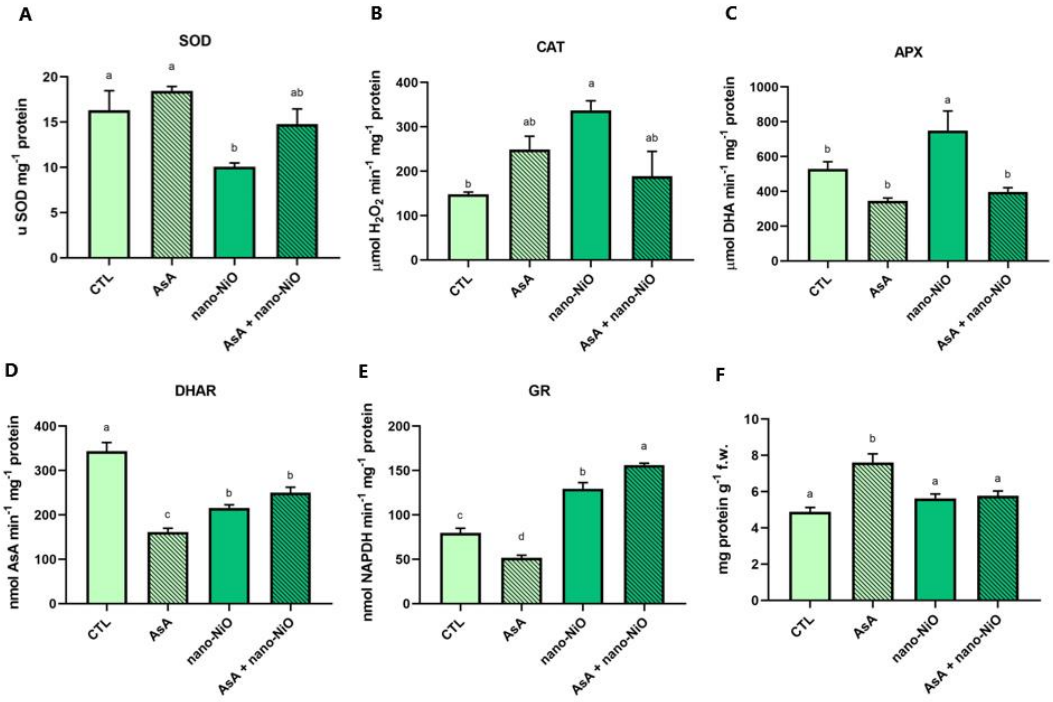


**Figure 7.** Effects of nickel oxide nanomaterials (nano-NiO; 30 mg $L^{-1}$) and/or ascorbate (AsA, 150 mg $L^{-1}$) on the activity of SOD (**A**), CAT (**B**), APX (**C**), DHAR (**D**), and GR (**E**) and on the content of soluble proteins (**F**) in the shoots of in vitro grown tomato plantlets. Data presented are mean ± SEM (n ≥ 3) (f.w.: fresh weight). Different letters above bars indicate significant statistical differences between treatments at $p \leq 0.05$.

## 4. Discussion

### *4.1. In Vitro Administration of Nano-NiO Causes Phytotoxic Effects on Tomato Seedlings*

Exposure to contaminant levels of Ni is well-known to cause phytotoxicity, affecting growth, photosynthesis, nutrient uptake, redox homeostasis, and enzymatic activity, among other essential processes [18–21]. The toxicity of Ni to higher plants is a species, age-, concentration-, and exposure-time-dependent response [21]. However, Ni-induced effects also vary according to the form, structure, and size in which Ni is present in the environment [17]. In recent years, the occurrence of metal-NMs has increased, adding even more risk to nontarget species such as crop plants. It has been demonstrated that nano-formulations of Ni can release more ions than their bulk material, which may aggravate the effects of Ni-induced phytotoxicity (see Pinto et al. [23], Soares et al. [15], and references therein). Besides, the small size, high surface area, and the ability to generate ROS allows NMs to be more reactive than their bulk counterparts, and, therefore, to be capable of inducing stronger effects at lower concentrations [4,17]. As is commonly observed in plants subjected to Ni exposure, in vitro grown Micro-Tom tomato plants exposed to nano-NiO showed clear symptoms of Ni-induced phytotoxicity, including evident chlorotic and necrotic spots on the leaves, which were accompanied by a reduced foliar area and decreased levels of pigments, as well as significant reductions in root growth and general biomass production. Concerning photosynthesis, the observed negative effects in response to the nano-NiO treatment were expected outputs, given that Ni is known to destroy mesophyll cells and epidermal tissues and compromise the membrane structure of the thylakoids and the grain, thus affecting Chl content and impacting photosynthesis [21]. In addition, Ni can alter the structure and/or activity of the Chl molecule by replacing important magnesium (Mg) ions. This mechanism may also negatively impact the structure and activity of key enzymes, affecting the photosynthetic efficiency [24]. The observed symptoms fall in line with results reported by Faisal et al. [54], Pinto et al. [23], and Soares et al. [37,55], also in regards to nano-NiO. In these studies, the authors observed significant

decreases in growth-related parameters and negative responses from the photosynthetic apparatus. Curiously, and in opposition to these previous works, in the present study, the overaccumulation of $H_2O_2$ seemed to have been confined to the root tissues, with no significant changes in the shoots. Even though several reports have shown that nano-NiO can induce severe oxidative damage in cells by excessive production of ROS [11,23], in the shoot tissues of Micro-Tom plantlets, the enhanced activity of the enzymes CAT and APX and the enhanced TAC in response to stress (namely due to the enhanced content of GSH and phenols, for instance) may have played a role in preventing $H_2O_2$ accumulation and LP. The inhibition of SOD in response to nano-NiO might have also been a consequence of the direct interference of Ni in the secondary structure of proteins, which often leads to the inactivation of enzymes or indirect action through competition with Fe cations [18]. In fact, it has been shown that the activity of AOX enzymes depends upon the concentration of Ni in the growth medium as well as the time of exposure and plant species, evidencing that lower amounts of Ni usually lead to the activation of AOX enzymes, while higher concentrations often have an opposite effect [18]. However, regarding nano-NiO, Pinto et al. [23] demonstrated that 15 mg $L^{-1}$ nano-NiO was able to significantly enhance SOD and APX activities, contrarily to bulk Ni at the same concentration. Other studies have also reported the activation of SOD and CAT in response to nano-NiO [11,54].

### *4.2. AsA Supply Helps to Alleviate the Phytotoxicity Induced by Nano-NiO*

The supplementation of the growth medium with AsA had a positive effect on the content of Chl and Car in plants exposed to nano-NiO. Similar results were obtained by Alayafi [56] in tomato plants exposed to heat stress, by Hussain [33] in okra plants, and by Hanafy and Ahmed [57] in tomato plants, where the exogenous application of AsA re-established the levels of Chl and improved photosynthesis. In the present study, the stress-attenuating effect of this potent AOX was further noted in reversing root growth inhibition and foliar damage and in mitigating the accumulation of $H_2O_2$ in the roots. These results point towards an AsA-induced stress-attenuating mechanism that likely encompasses the removal of excessive $H_2O_2$, given the high AOX potential of this molecule not only as a ROS scavenger but also as a stimulator of other AOX defenses. In sum, AsA may have helped avoid the induction of oxidative stress in nano-NiO stressed plants, safeguarding the removal of ROS, the relative water content, membrane stability, and osmotic adjustment. Moreover, the simultaneous stimulation of GR and inhibition of DHAR in co-treated plants were possibly responsible for the accumulation of GSH, proving that exogenous AsA has positive indirect effects on enhancing other AOX defenses in stressed plants. Additionally, AsA may have also played a role in stimulating metal detoxification mechanisms to avoid the accumulation of nano-NiO in tissues, as has been described by Shabana et al. [58] and Hanafy and Ahmed [57]. A very curious outcome from the present study was that the quantification of total AsA levels in the shoot tissues revealed a significant increase in plants that were supplemented with AsA under normal conditions, but not in plants that were also co-exposed to nano-NiO, with levels of total AsA that remained similar to the CTL. The solo treatment with nano-NiO did also not affect the total levels of endogenous AsA. This inability of plants to accumulate AsA when treated with nano-NiO and AsA goes against most reports on the subject [59–62]. However, it is important to note that although the total AsA levels were not maintained under the co-exposure to nano-NiO, the levels of the reduced form were almost as high as those found in plants treated with AsA alone, which suggests that in the presence of nano-NiO the uptake and/or metabolism of this exogenously applied AsA could be affected, possibly lowering its accumulation and mostly its oxidation rate, leading to lower levels of DHA and, therefore, lower levels of total AsA. This is further supported by the observed increases in the activities of DHAR and GR in plants from the combined treatment, which work together in the AsA-GSH cycle to keep AsA in its reduced state.

According to several authors, applying AsA to plants, either supplemented in the culture media, via seed priming, by foliar spraying, or through irrigation, is an effective

strategy to alleviate the effects of abiotic stresses. Athar et al. [59], Hamada and Al-Hakimi [63], and Sakr et al. [64] observed that exogenous AsA counteracted the adverse effects of salinity stress in wheat, sunflower, maize, and pepper plants, respectively. While Sakr et al. [64] suggested that AsA acted by improving Chl and Car contents, enhancing Pro accumulation and decreasing $H_2O_2$ content to counteract stress, Athar et al. [59] reported that the response of wheat plants to AsA was cultivar-specific and that the beneficial effects seemed to be mainly associated with increased levels of endogenous AsA, which possibly triggered the AOX system and enhanced photosynthetic capacity. Similarly, Dolatabadian et al. [65,66], Malik et al. [67], Noman et al. [68], and Hafez and Gharib [69] have also reported that AsA supplementation could help plants overcome water stress, mainly due to the potent AOX activity. Malik et al. [67] added that AsA was also responsible for maintaining growth, relative water content, cell membrane stability, and osmotic adjustment through Pro accumulation in wheat plants, and its application in the rooting medium was the most effective means of administration for counteracting drought-induced stress. Accordingly, Hafez and Gharib [69] stated that AsA application improved yield and Chl content, stimulated the activities of CAT and SOD, and was associated with the maintenance of leaf water status under water stress. Evidence for the effect of exogenous AsA on increasing tolerance to other types of abiotic stress has also been reported, including heat and chilling stresses [56,70]. In addition to these, there are also numerous examples of the beneficial effects of AsA on plants subjected to HM stress. For example, in okra plants, a seed priming treatment with 50 mg $L^{-1}$ AsA was effective in conferring tolerance to lead (Pb), restoring growth, levels of Chl, Pro, total proteins, amino acids, and endogenous AsA, and the activities of AOX enzymes [33]. Regarding copper (Cu) stress, studies with red cabbage seedlings [71], tomato plants [57], and onion plants [72] showed that exogenous AsA alleviated metal-induced oxidative stress, by decreasing the accumulation of ROS, peroxidase activity, and LP, increasing the endogenous levels of AsA, and improving membrane stability index, which shows a protective effect on growth, pigments, and enzymatic activity. Moreover, some studies have proven that AsA also has an important role in regulating the uptake and translocation of toxic HMs, decreasing their accumulation in the cytoplasm of plant cells. Zhou et al. [32] have recently discussed a few mechanisms that could be behind this protective role of AsA: an improvement in the uptake of essential nutrients such as N, P, K, Ca, and Mg could be one of the reasons for the AsA-mediated decrease in the uptake of HMs, by supporting plants in the development of physical barriers against the uptake of HMs. Besides, AsA supplementation has been shown to increase the content of cell wall pectin and cellulose, which have functional groups that can bind to HMs and prevent their translocation. Furthermore, by enhancing the reduced levels of GSH, AsA could also be responsible for enhancing the formation of HM complexes, not only with GSH itself, but also with phytochelatins, since their biosynthesis requires GSH. These complexes are transferred into the vacuole of cells, increasing the detoxification ability of HMs.

### *4.3. Unstressed Tomato Seedlings Show a Negative Response to AsA Supplementation*

In contrast, and different from what would be expected, the solo administration of this well-known stress attenuator and AOX agent showed detrimental effects on the growth of tomato plants that were not exposed to nano-NiO, clashing with most reports that refer to exogenous AsA as a potent stimulator of growth and germination [30,64,66]. In the present study, tomato plants treated with AsA under nonstressful conditions suffered strong inhibitions in the biomass production of roots and shoots, accompanied by a visible reduction in leaf area, albeit a slight increase in the length of roots and protein content. AsA-treated plants also showed a higher degree of LP, which does not agree with most findings on the subject [33,57,69]. Single AsA supplementation to the culture media inhibited the activity of APX, DHAR, and GR, but not of SOD under nano-NiO stress, which could be due to the role of AsA in preserving enzymes' activities that contain prosthetic transition metal ions [57]. As a matter of fact, although some authors have stated that AsA has beneficial

effects on the growth and physiology of plants even under a nonstressful environment [66], it has already been pointed out that the effects of AsA on plants under normal growth conditions remain little explored [62], as opposed to the numerous studies of AsA in stressed plants. Qian et al. [62] reported that the application of 2 and 8 mM AsA for 5 days decreased the fresh weight of *Arabidopsis thaliana* seedlings. In the previously mentioned study, Hussain et al. [33] also noticed that a seed-priming treatment with AsA at 50 and 100 mg $L^{-1}$ under nonstressful conditions caused a decline in dry and fresh masses in two okra cultivars. Moreover, in a comparative assessment of two canola cultivars that are different in salt resistance, Shafiq et al. [73] also noticed that the exogenously applied AsA at concentrations between 50 and 150 mg $L^{-1}$ decreased all growth attributes of the salt-tolerant cultivar Dunkeld, particularly root dry weight, under well-watered conditions, while acting as a growth stimulator for the salt-sensitive cultivar Cyclone under the same conditions [73]. AsA-induced inhibition of germination and seedling growth have also been observed in other plant species at much lower concentrations (100 μg $L^{-1}$) (M. P. Gomes, personal communication, 29 August 2021). Moreover, Henschel et al. [74] recently reported that 2 mM AsA affected biomass allocation in radish plants, causing a lower leaf mass fraction and affecting the instantaneous carboxylation efficiency in these plants. The same authors also noticed that these deleterious effects of AsA did not appear at concentrations below 2 mM (D. Batista, personal communication, 24 August 2021). In this sense, AsA-induced negative effects on biomass production are most likely species-dependent and, even, cultivar-dependent responses, as well as concentration-dependent and means of exposure-dependent, given the contrasting effects of AsA observed in different studies.

The stimulation of root elongation induced by AsA, followed by an increase in protein levels, comparable to findings by Chaparzadeh and Chagharlou [72], may have occurred as a consequence of the interference of AsA in the cell cycle, such as in the biosynthesis of hydroxyproline-rich proteins [72]. Thishastens the progression from G1 to the S phase, which may have translated into a higher cell division rate at the quiescent center of the root apical cap [38,75]. Furthermore, AsA may have stimulated root cells' expansion by controlling the crosslinking of cell wall glycoproteins [75,76]. Li et al. [77] suggested that low levels of AsA could stimulate root elongation in *Arabidopsis thaliana* by regulating the GSH pool and the production of $^{\bullet}OH$ and the $Ca^{2+}$ influx. Common regulation of these mechanisms would explain the slight enhancement in root elongation despite the stronger reductions in biomass production in response to the single AsA treatment. These negative effects observed on biomass production and LP were most likely caused by a combination of several factors: (1) the presence of high levels of this potent ROS-scavenger molecule may have induced imbalances in the redox homeostasis, for instance, through an exaggerated scavenge of ROS, or their unbalanced distribution in the cell compartments, namely in the apoplast, which has a redox state that is essential in the modulation of plant growth responses [38]; (2) the interference or inhibition of enzymatic AOX activity, as has been reported by Qian et al. [62]; (3) given that AsA is cofactor for enzymes involved in the biosynthesis of phytohormones, exogenous AsA may have interfered with the biosynthesis and signaling of interconnected phytohormone networks, which could have affected the development and growth of tomato plantlets [29]; and (4) AsA may have interfered with the transcription and/or activity of aquaporins, particularly plasma membrane intrinsic proteins (PIPs), leading to increased water losses and decreased $CO_2$ supply and fixation, which ultimately delays plant growth in terms of fresh weight [62].

## 5. Conclusions

Based on the results obtained, it was possible to conclude that the exposure of *Solanum lycopersicum* L. cv. Micro-Tom to nano-NiO 30 mg $L^{-1}$ affected the normal growth and metabolism of the plants, inducing significant reductions in organ elongation and biomass production as well as macroscopic symptoms of toxicity in the leaves and decreased levels of photosynthetic pigments. Additionally, the presence of nano-NiO induced the accumulation of $H_2O_2$ in the roots, suggesting the occurrence of oxidative stress in this

organ. Although the addition of AsA 150 mg $L^{-1}$ to the culture medium led to negative effects on the growth and development of tomato seedlings under control conditions, its supplementation to plants under co-exposure to nano-NiO was efficient in counteracting phytotoxicity symptoms and growth inhibition, increasing the tolerance of tomato plants to this NM. The role of AsA in plants under stress seemed to have been related to a rise in the total AOX capacity, particularly regarding GSH and total phenols contents, with possible implications for the avoidance of oxidative stress and the activation of detoxification mechanisms.

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