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Putrescine biosynthetic pathways modulate root growth differently in tomato seedlings grown under different N sources

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ABSTRACT

The biosynthesis of putrescine is mainly driven by arginine decarboxylase (ADC) and ornithine decarboxylase (ODC). Hence, in this study, we generated independent *ADC* and *ODC* transgenic silenced tomato lines (*SilADC* and *SilODC*, respectively) to test the effect of defective *ADC* and *ODC* gene expression on root development under nitrate (NN) or ammonium (NA) conditions. The results showed that *SilODC* seedlings displayed an increase in *ADC* expression that led to polyamine accumulation, suggesting a compensatory effect of ADC. However, this effect was not observed in *SilADC* seedlings. These pathways are involved in different growth processes. The *SilADC* seedlings showed an increase in fresh weight, shoot length, lateral root number and shoot:root ratio under the NN source and an enhancement in fresh weight, and shoot and root length under NA conditions. However, *SilODC* seedlings displayed greater weight and shoot length under the NN source, whereas a decrease in lateral root density was found under NA conditions. Moreover, two overexpressed *ODC* lines were generated to check the relevance of the compensatory effect of the ADC pathway when *ODC* was silenced. These overexpressed lines showed not only an enhancement of almost all the studied growth parameters under both N sources but also an amelioration of *ammonium syndrome* under NA conditions. Together, these results reflect the importance of both pathways in plant growth, particularly *ODC* silencing, which requires compensation by *ADC* induction.

1. Introduction

Nitrogen (N) is an essential plant nutrient. The accumulation of N-containing compounds inside the cell is determined by the uptake of N mainly as nitrate (NO_3^-) and/or ammonium (NH_4^+), N assimilation, the transport of N-containing molecules throughout the plant and their recycling and remobilization (Tegeder and Masclaux-Daubresse, 2018). In addition, NH_4^+ could be better absorbed to the soil exchange complex, but a high NH_4^+ concentration induces the so-called *ammonium syndrome*, which is characterized by biomass suppression, alterations in the root:shoot ratio, leaf chlorosis, rhizosphere acidification and changes in the root system architecture (Britto and Kronzucker, 2002; Da Silva et al., 2016; Esteban et al., 2016; Liu and von Wirén, 2017; Xuan et al., 2017). Regarding root architecture, it is already known that NH_4^+ nutrition leads to an inhibitory effect on primary root (PR) and shoot growth and induces branching of short lateral roots (LRs) in *Arabidopsis thaliana* (Li et al., 2010; Lima et al., 2010; Liu et al., 2013; Yuan et al., 2017), whereas an increase in PR length has been observed in tomato plants (Liu et al., 2013). In addition, NH_4^+ nutrition also leads to a high level of basal responses that allow plants to activate systemic acquired acclimation and trigger defence responses against biotic and abiotic stresses (Fernández-Crespo et al., 2012; González-Hernández et al., 2019). One of the pathways involved in this response appears to be the putrescine biosynthetic routes, because tomato plants treated with NH_4^+ as the sole N source display higher arginine (Arg), ornithine (Orn) and putrescine (Put) levels than control plants (Fernández-Crespo et al., 2015; González-Hernández et al., 2019).

The starting point of N assimilation is glutamate, which is responsible for Arg and Orn biosynthesis, among other compounds. Both Arg and Orn are precursors of polyamine (PA) biosynthesis, a process which produces the ubiquitous polycationic and aliphatic amines of low molecular weights found in living cells (Alcázar et al., 2006; Groppa and Benavides, 2008). The most abundant PAs in plants are Put, spermidine (Spd) and spermine (Spm), and they are considered the major sinks of

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assimilated N, as they are found at high concentrations in plant cells. As previously mentioned, Put is synthesized via two different pathways: from ornithine (Orn) or arginine (Arg) by the action of the enzymes ornithine decarboxylase (ODC) or arginine decarboxylase (ADC), respectively (Walters, 2003). Moreover, these compounds are involved in a set of plant growth and developmental processes, because they are involved in cellular processes such as cell proliferation, differentiation, root formation, apoptosis, senescence, and fruit development and ripening as well as in plant tolerance or resistance against abiotic or biotic stresses (Bagni and Tassoni, 2001; Couée et al., 2003; Fernández-Crespo et al., 2015; Hussain et al., 2011; Kusano et al., 2008; Liu et al., 2015; Pang et al., 2007; Walters, 2003). In addition, plant architecture, specifically root development, is a crucial parameter of nutrient and water use efficiencies in plants, which are controlled by these compounds and phytohormones, among others (Killiny and Nehela, 2020). For example, a reduction in ADC activity produced a depletion of PA levels that led to an inhibition of root length in common bean plants (Palavan-Ünsal, 1987). Moreover, excised roots from *Nicotiana tabacum* plants treated with difluoromethylornithine (DFMO), an ODC inhibitor, displayed an increase in root length and putrescine depletion (Ben--Hayyim et al., 1996). This phenotype was reversed when putrescine was added to the DFMO treatment. However, these effects appear to be species-dependent, as Lee (1997) showed that DFMO inhibited root elongation and polyamine levels in rice roots. For this reason, more studies are required to determine the modulation of tomato root system architecture through PA metabolism.

In abiotic stress interactions, PAs play a role in tolerance against low temperature, drought, oxidative stress, salinity, nutrient deficiencies such as N or B or metal toxicity, among other stressors (Alcázar et al., 2011; Camacho-Cristóbal et al., 2005; Geny and Broquedis, 2002; Kuznetsov and Shevyakova, 2010; Rider et al., 2007; Sequera-Mutiozabal et al., 2016; Shevyakova et al., 2010). *Arabidopsis thaliana* lacks the ODC enzyme, but it possesses two ADC isoforms (ADC1 and ADC2). *ADC1* is mainly induced by cold (Hummel et al., 2004), whereas *ADC2* gene expression is intensely induced by drought, salinity, wounds or K^+ deficiency (Alcázar et al., 2006; Hummel et al., 2004; Perez-Amador et al., 2002; Urano et al., 2003; Armengaud et al., 2009). Moreover, when *ADC* genes from *Avena sativa* and *Datura stramonium* are overexpressed, Put accumulation is induced, conferring drought tolerance (Capell et al., 2004). In addition, overexpressed ODC activity produces tobacco plants that are more tolerant of salinity (Kumria and Rajam, 2002).

As mentioned above, PA involvement in the plant stress response has mostly been studied in *Arabidopsis* plants or through modifications of levels by exogenous PA application or chemical inhibition of biosynthetic enzymes (Ben-Hayyim et al., 1996; Fernández-Crespo et al., 2012; Lee, 1997). These applications display several limitations such as the capacity of the cells to uptake the chemical compounds supplied exogenously or the possible production of oxidation compounds by several degradation enzymes such as PAOs. Thus, the effects are variable depending on the plant system or the existence of compensatory mechanisms (Navakouidis et al., 2003). An alternative is to produce genetically modified plants with altered levels of PAs due to a loss or gain of function in the genes encoding the enzymes involved in the biosynthesis of PAs.

Given this background, the main objective of this work was to generate independent *ADC* and *ODC* transgenic silenced tomato plants to test the effect of defective *ADC* and *ODC* gene expression on the root architecture development of ten-day-old tomato plants under NO_3^- and NH_4^+ sources. Moreover, based on the results, two overexpressed ODC lines were generated to check the relevance of the compensatory effect of the ADC pathway when ODC was silenced.

2. Materials and methods

2.1. Plant material

Tomato seeds (*Solanum lycopersicum* Mill. cv. Moneymaker) were surface-sterilized in 75% v/v commercial NaClO (3%) with 0.1% Tween-20 for 8 min and then rinsed 5 times for 5 min each with sterile distilled water.

For tomato transformation, the seeds were germinated *in vitro* in culture jars (Thermo Scientific) containing solid MS medium (pH = 6.2) supplemented with SH vitamins, 10 g L^{-1} sucrose and 8 g L^{-1} agar. The plants were grown for 10 days in axenic conditions in a growth chamber at 18/24 °C with a 16/8 h photoperiod (day/night) of 120 μE/m^2·s intensity. The cotyledons were then collected before the appearance of the first leaf and were used as explants for *Agrobacterium tumefaciens*–mediated transformation.

2.2. Generation of the SlADC-RNAi and SlODC-RNAi constructs for the silenced plants

The nucleotide sequences were searched in NCBI (https://www.ncbi.nlm.nih.gov/), and the primers were designed. A 314-bp fragment of *SlADC* and a 265-bp fragment of *SlODC* were amplified from genomic DNA with the primer pairs *SlADC* forward, 5′-gggcttggaatcgactatga-3′, and *SlADC* reverse, 5′-aactgatcagaatagatgagacatg-3′ or *SlODC* forward, 5′-ggttttcgaaacagcagctc-3′, and *SlODC* reverse, 5′-cgtcgcatggtcgtaaagt-3′ linked to attB sites for Gateway cloning. Amplification was conducted as follows: denaturation at 98 °C for 30 s; 35 cycles of denaturation at 98 °C for 10 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s; and a final extension at 72 °C for 10 min. The PCR product was cloned into pDONR207 using the BP recombination reaction following the manufacturer's instructions (Thermo Fisher) and then transferred using the LR recombination reaction to the destination vector pBIN19-RNAi. Transformation was carried out using *Agrobacterium tumefaciens* strain LBA4404 containing the *SlADC-RNAi* or the *SlODC-RNAi* construct. Moreover, the empty vector pBIN19-RNAi was also used to generate empty vector control plants (*EVs*).

2.3. Generation of the SlODC constructs for overexpressed plants

The nucleotide sequences were searched, and primers were designed (*SlODC* forward, 5′-atggccggccaaacagtc-3'; *SlODC* reverse, 5′-gtttggataagcataagcaag-3′) with the Gateway adapters included. The full-length *SlODC* cDNA was amplified with PCR using Phusion DNA Pol (Biolabs) and specific primers. The PCR product was cloned into pDONR207 using a BP ClonaseMixII kit (Thermo Fisher). After sequencing, all constructs were recombined into the pEarleyGate101 destination vector using an LR ClonaseMixII kit (Thermo Fisher). Transformation was carried out in the wild-type line using *Agrobacterium tumefaciens* strain LBA4404 containing the full-length *SlODC* constructs.

2.4. Agrobacterium-mediated transformation and selection of transgenic plants

Agrobacterium tumefaciens with *SlADC-RNAi*, *SlODC-RNAi* or the empty constructs were precultured independently in LB medium containing kanamycin and rifampicin. A culture grown overnight in LB medium with an optical density (λ = 600 nm) of 0.4–0.6 was used to inoculate 10-day-old tomato plant cotyledons, which had been previously cut into pieces and kept for two days in MS medium. For inoculation, the cotyledons were immersed into the *A. tumefaciens* bacterial suspension for 20 min. Explants were then slightly dried on sterile filter paper and placed on co-cultivation medium (MS basal medium) for 2 days at 26 °C in darkness. After cocultivation, the explants were washed with a washing solution (MS basal medium) containing timentin (100 mg mL^{-1}) to remove the remaining *A. tumefaciens*, dried on sterile filter

paper and transferred to shoot induction medium. The protocol followed for shoot and root induction has been previously described by Scalschi et al. (2014).

Rooted plantlets were transferred to pots containing a mixture of biopeat and perlite, maintained in a growth chamber, acclimated, and then transferred to a greenhouse for seed production. These plants were referred as T_0 transgenic lines. The presence of the T-DNA insertion in the T_0 primary transformants was assessed via PCR amplification of the kanamycin encoding gene (*NPTII*) with *NPTII* primers (*NPTII* forward, 5′-gacaagccgttttacgtt-3'; *NPTII* reverse, 5′-gatactttctcggcagga-3′). The DNA for PCR analysis was isolated from leaves using a method for plant genomic DNA extraction developed by Edwards et al. (1991). Once the *NPTII* gene presence was confirmed, fruits were harvested at the ripening stage from 4-month-old plants, and seeds were collected independently. Moreover, the expression of both genes was measured in one-month-old cuttings of T_1 tomato plants to choose transgenic lines with lower *ADC* or *ODC* expression at the basal level. Finally, T_2 homozygous tomato plants were selected in MS medium with kanamycin.

To obtain the ODC-overexpressing lines, transformation was performed as described above using the pEarlyGate 101 vector and *A. tumefaciens* containing the pEarlyGate 101 vector with the full-length *SlODC* constructs. We checked the insert of the full construct with the *35S* forward (5′-gacgcacaatcccactatc-3′) and *ODC* reverse primers. Transformed tomato seeds were selected by spraying with a 0.06% Basta solution, and T_2 homozygous plants were used for further experiments.

2.5. Shoot and root growth measurements

Seeds of the transgenic lines were surface sterilized as described above and transferred to 15 g L^{-1} agar plates. After three days, germinated seeds of similar size were placed into control NO_3^- (NN) or NH_4^+ (NA) sterilized agar medium for 7 days. The NN and NA plates were composed of Hoagland solution (Hoagland and Arnon, 1950) modified in the N regime and 15 g L^{-1} agar. The N concentration was 10 mM in both cases; the NN solution contained KNO_3, and $(NH_4)_2SO_4$ was the sole N source for the NA solution. Moreover, K_2SO_4 and $CaSO_4$ compounds were added to the NH_4^+ solution to avoid K^+ and Ca^{2+} deficiencies. To maintain a pH of approximately 5.8–6.0, the pH was adjusted with 1 mM KOH, and MES sodium salt was added as a buffer to both N solutions. The growth conditions were a 16/8 h light/dark photoperiod, 26/18 °C day/night temperature, 200 μmol $m^{-2}{\cdot}s^{-1}$ light intensity and 60% relative humidity.

Root growth parameters were measured after 7 days of NN or NA treatment. Total fresh weight (FW) was measured with an analytical balance. Moreover, root and shoot length and LR number were quantified with ImageJ software (National Institutes of Health, Maryland, USA). Root density was estimated as the LR number divided by the PR length. Each parameter was quantified in at least ten seedlings of three independent technical replicates.

2.6. Chromatographic analyses

Polyamine analysis was conducted according to the method described by Sánchez-López et al. (2009). Briefly, fresh material was frozen in liquid N and ground. Before extraction, a mixture of internal standards containing [$^{13}C_4$] putrescine and 1,7-diamineheptane (100 ppb final concentration) was added. The extraction was conducted with perchloric acid (2%). The extract was further mixed with methanol (10%) and heptafluorobutyric acid (HFBA, 25 mM). A 20 μl aliquot of this solution was directly injected into an HPLC system interfaced to a Quatro LC mass spectrometer. To process the quantitative data of samples, MASSLYNX NT software version 4.1 (Micromass) was used.

2.7. Quantitative RT–PCR

Leaves were ground in liquid N, and RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions; 1 μg of total RNA after RNase-free DNase digestion (Promega; Wisconsin, USA) was reverse transcribed into cDNA using an oligodT primer and PRIMESCRIPT reverse transcriptase, as described by the manufacturer (Takara). Quantitative real-time PCR was performed with SybrGreen Premix Ex Taq (Thermo Fisher) in a StepOne system with primers designed for *ADC* and *ODC*. *EF1α* was used as a control to normalize the gene expression in each sample. The primed primers used were *ADC* forward primer, 5′-gggtccttggatattgagca-3′; *ADC* reverse primer, 5′-atccagacggtgaattggaa-3′; *ODC* forward primer, 5′-ccacctgtgatgcacttgat-3′; and *ODC* reverse primer, 5′-gagtaacaatggcggatgtg-3′. The primers for the constitutive gene were *EF1α* reverse primer, 5′- gggtattcagcaaaggtctc-3′ and *EF1α* forward primer, 5′-gacaggcgttcaggtaagga-3'.

2.8. Statistical analyses

Statistical analyses were performed with a one-way analysis of variance in Statgraphics Centurion XVI. I software (Statistical Graphics Corp., Rockville, MD, USA). The results are expressed as the mean with standard error and were compared with the LSD test with a 95% confidence interval ($p < 0.05$).

3. Results

3.1. Generation of ADC and ODC silenced tomato lines

To investigate the roles that the ADC and ODC pathways play in tomato plant development, we generated transgenic lines in which the expression of *ADC* and *ODC* genes was knocked down using a double-stranded RNAi (dsRNAi) strategy. Two independent transformed T_2 homozygous lines were obtained for each gene, designated *SilADC-1* and *SilADC-2* and *SilODC-1* and *SilODC-2*.

We determined the expression levels of *ADC* and *ODC* genes in cotyledons of *SilADC*, *SilODC* and *EV* tomato seedlings grown under NN and NA treatments (Fig. 1). The basal level of *ADC* gene expression was not different from that of the *EV* seedlings grown under NN conditions (Fig. 1A), whereas A*DC* gene expression was reduced in the *SilADC* lines grown under NA (Fig. 1C). In addition, *SilODC-1* and *SilODC-2* showed a reduction in *ODC* expression compared with control plants (*EV*) under NN and NA nutrition (Fig. 1B and D).

Furthermore, *ODC* gene expression in *ADC*-silenced lines (Fig. 1E and G) and *ADC* gene expression in *ODC*-silenced plants (Fig. 1F and H) were analyzed. We observed a significant increase in *ADC* gene expression in *SilODC* seedlings grown in NN medium (Fig. 1F) and a slight increase in NA (Fig. 1H). However, ODC gene expression did not increase in any case (Fig. 1E and G), and even in the NA medium; a reduction was observed (Fig. 1G). Moreover, when comparing the two N sources, it was observed that under NA nutrition, *ADC* levels were higher only in the *EVs,* whereas *ODC* gene expression was slightly increased in *SilADC-1 and SilODC-2* plants (Fig. S1).

Moreover, consistent with the reduction in *ADC* gene expression under NA conditions, ADC-silenced lines showed a strong decrease in the accumulation of putrescine (Put) in leaves under NA (Fig. 2C), whereas no significant differences were observed under the NN treatment (Fig. 1A). However, this reduction was not detected in *ODC*-silenced lines, as an increase in Put levels was observed (Fig. 2B and D). Thus, this increase could be explained by the induction of *ADC* gene expression observed in these plants (Fig. 1F and H). Interestingly, the repression of *ADC* gene expression in *SilADC* seedlings was not balanced by an increase in *ODC* expression (Fig. 1E and G). When the two N sources were compared, the Put concentration was higher in NA-treated EV, *SilADC* and *SilODC* plants (Fig. S1C). Finally, it should be noted that no differences in spermidine (Spd) concentration were found in *SilADC* seedlings (Fig. 2E and G), whereas an increase was observed in *SilODC* seedlings under both N sources (Fig. 2F and H). Moreover, no differences were

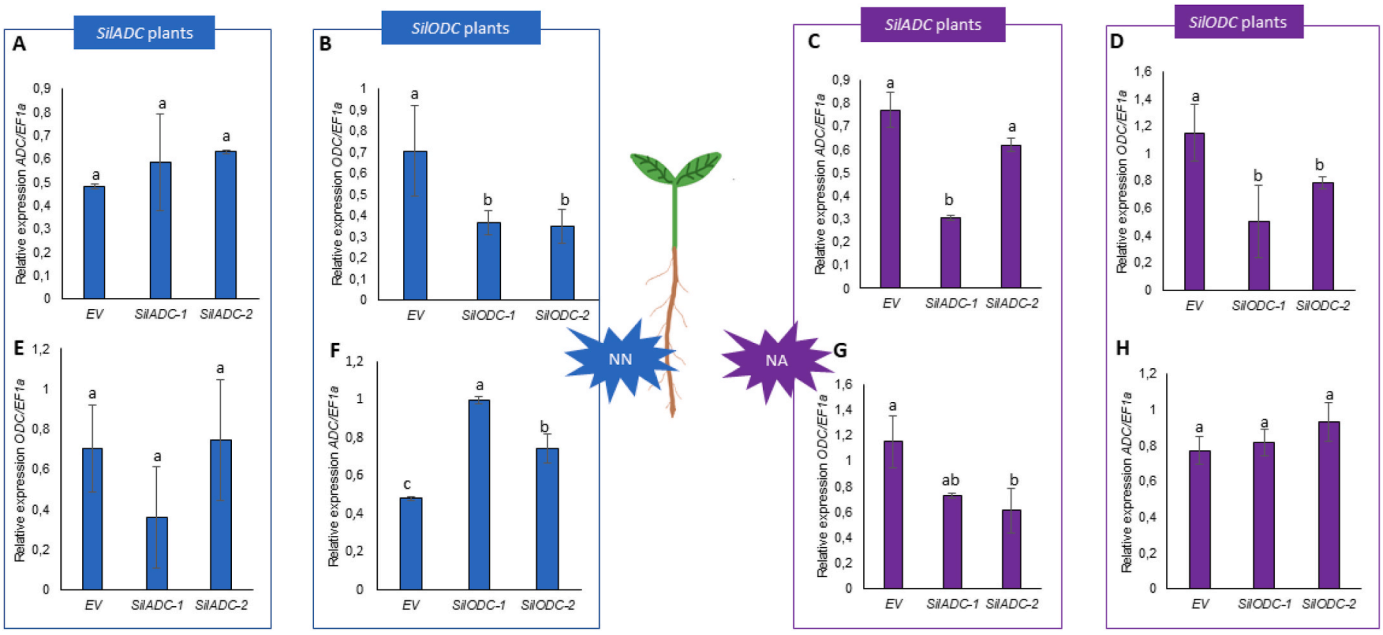

Fig. 1. Relative expression of *ADC* and *ODC* genes in cotyledons of *EV*, *SilADC* and *SilODC* tomato seedlings grown under NN or NA as the sole N source. The results are the mean of three independent experiments ± standard error (SE). Distinct letters represent statistically significant differences by LSD test ($p < 0.05$).

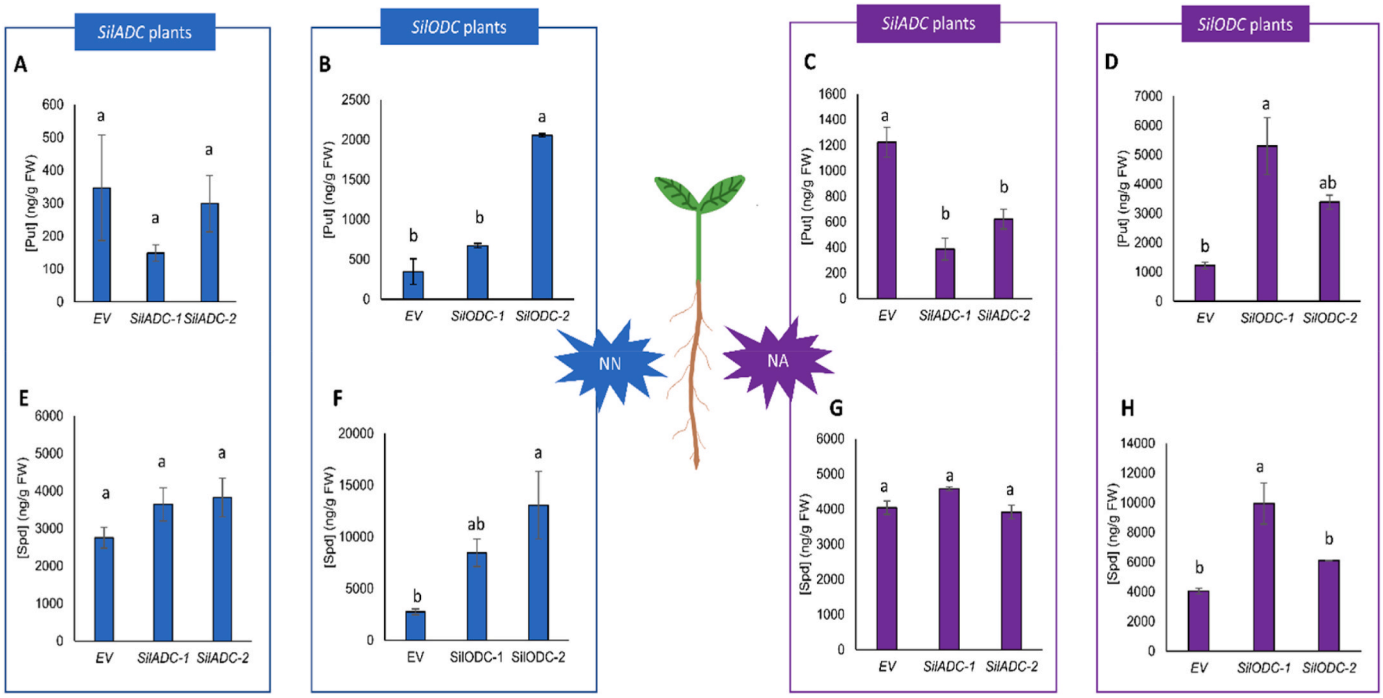

Fig. 2. Putrescine and spermidine concentrations in cotyledons of *EV*, *SilADC* and *SilODC* tomato seedlings grown under NN or NA as the sole N source. The results are the mean of three independent experiments ± standard error (SE). Distinct letters represent statistically significant differences by LSD test ($p < 0.05$).

found when the Spd concentration was compared between N sources except for the *EV* plants, which displayed a concentration enhancement under NA conditions (Fig. S1D).

3.2. Root and shoot development in SilADC and SilODC tomato seedlings

To study root and shoot development responses to the external N supply in *ADC*- and *ODC*-silenced lines, different N sources, NN and NA, were used to grow tomato seedlings (Fig. 3). The *SilADC* lines displayed higher FW and shoot length than those of the control plants (*EV*) under both N sources (Fig. 3A, B, G and H). However, under NN conditions, only *SilADC1* showed a decrease in PR length (Fig. 3C) and a large LR number (Fig. 3D), which corresponds to a high shoot/root ratio (Fig. 3E) and LR density (Fig. 3F) compared with *EV* seedlings. Regardless, shoot (Fig. 3H) and PR length (Fig. 3I) were higher in response to NA treatment in both ADC-silenced lines. When comparing NN and NA sources, all the studied parameters were greater in plants grown under NN sources except for PR length, which was higher in the silenced lines *SilADC-1* and *SilADC-2* under NA conditions (Fig. S2).

The *ODC*-silenced lines showed fewer changes in root and shoot

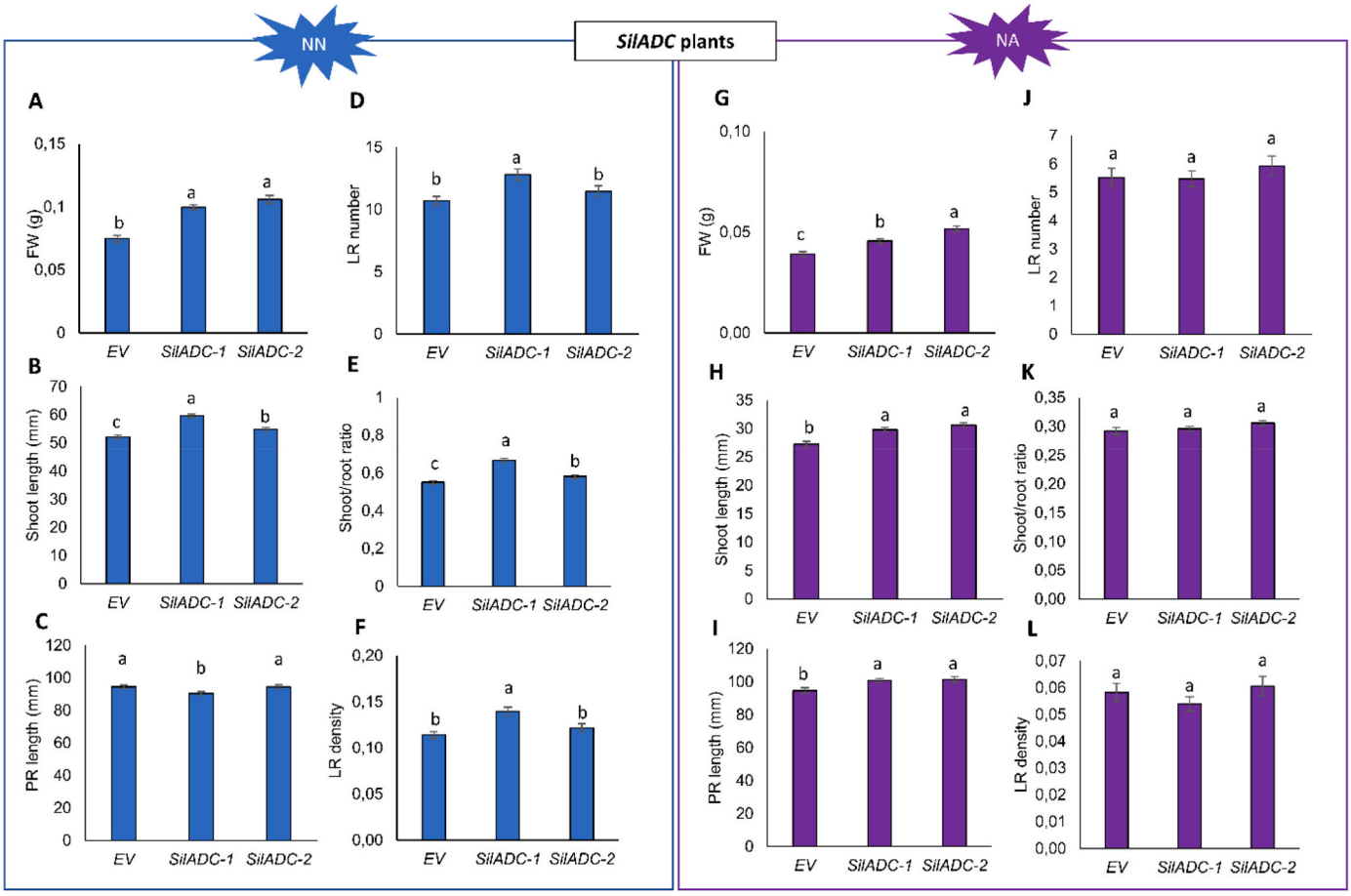

Fig. 3. Shoot length, PR length, LR number, shoot/root ratio, LR density (LR number/PR length) and fresh weight (FW) of *SilADC* tomato seedlings grown under NN and NA conditions compared with *EV* plants. The results are the mean of three independent experiments ± standard error (SE). Distinct letters represent statistically significant differences by LSD test ($p < 0.05$).

development than the *ADC*-silenced plants. Only FW (Fig. 4A) and shoot length (Fig. 4B) were higher in *SilODC lines* under NN conditions, but no significant differences were observed under the NA treatment (Fig. 4G-L). In this case, the comparison between the N sources indicated that the plants grown under NN conditions displayed higher values in all parameters except PR length than those under NA conditions (Fig. S2).

3.3. Generation of ODC overexpressed tomato lines

Following the previous results, we proposed to study the effect of overexpression of *ODC* to clarify the compensatory induction of the ADC pathway when *ODC* was silenced as well as to study its role in growth modulation. To accomplish this goal, we generated two overexpressed *ODC* lines (*OvODC-1* and *OvODC-2*). Generally, both overexpressed lines showed an increase in *ODC* gene expression (Fig. 5). This trend was also observed under NA conditions for *ODC* expression, but without significant differences. Interestingly, *OvODC* seedlings showed increased levels of Put when compared with *EV* seedlings under both N sources, although without significant differences (Fig. 6A and B). However, Spd levels were higher in *OvODC* lines grown under NN conditions (Fig. 6C), whereas there were no differences in seedlings treated with the NA source (Fig. 6D). Finally, it should be noted that NA-treated *EV* plants showed significant differences in ADC gene expression and Put and Spd concentrations compared with NN plants (Fig. S3). Moreover, a trend of increase in *ODC* expression and Put concentration was also observed in *EV* and *OvODC* plants under NA compared with NN (Fig. S3).

3.4. Root and shoot development in OvODC tomato seedlings

Variation in most of the growth parameters was found in the comparison between *EV* and *OvODC* seedlings (Fig. 7). Plant FW, shoot length, PR length and LR number were the major traits that increased under both N conditions (Fig. 7A–D, 7G-7J), whereas an increase in LR density was only observed under NN conditions (Fig. 7L). Moreover, under NA conditions, the growth parameters did not increase as much and did not reach NN-treated plant values (except for PR length) in *OvODC* plants, but an improvement in growth was observed (Fig. S4).

4. Discussion

Tomato plants have been widely used as a model system to study plant growth and development. However, despite several studies on the role of polyamines in plant growth, the effect of both biosynthetic putrescine pathways (ADC and ODC) on tomato shoot and root modulation remains unclear, particularly in plants grown under different N sources. The study of the root organ is essential, because the root is the main plant anchorage and nutrient and water absorption organ, and it defines viability and the subsequent production of crops. It is already known that tomato plants treated with NH_4^+ as the sole N source display higher Arg, Orn and Put levels than control plants (Fernández-Crespo et al., 2015; González-Hernández et al., 2019). Thus, to gain a better understanding of the role of both Put biosynthesis pathways in root development, we generated the transgenic tomato lines *SilADC-1*, *SilADC-2*, *SilODC-1* and *SilODC-2*, which showed reduced *ADC* and *ODC* expression upon NH_4^+ nutrition. This reduction is associated with a decrease in Put

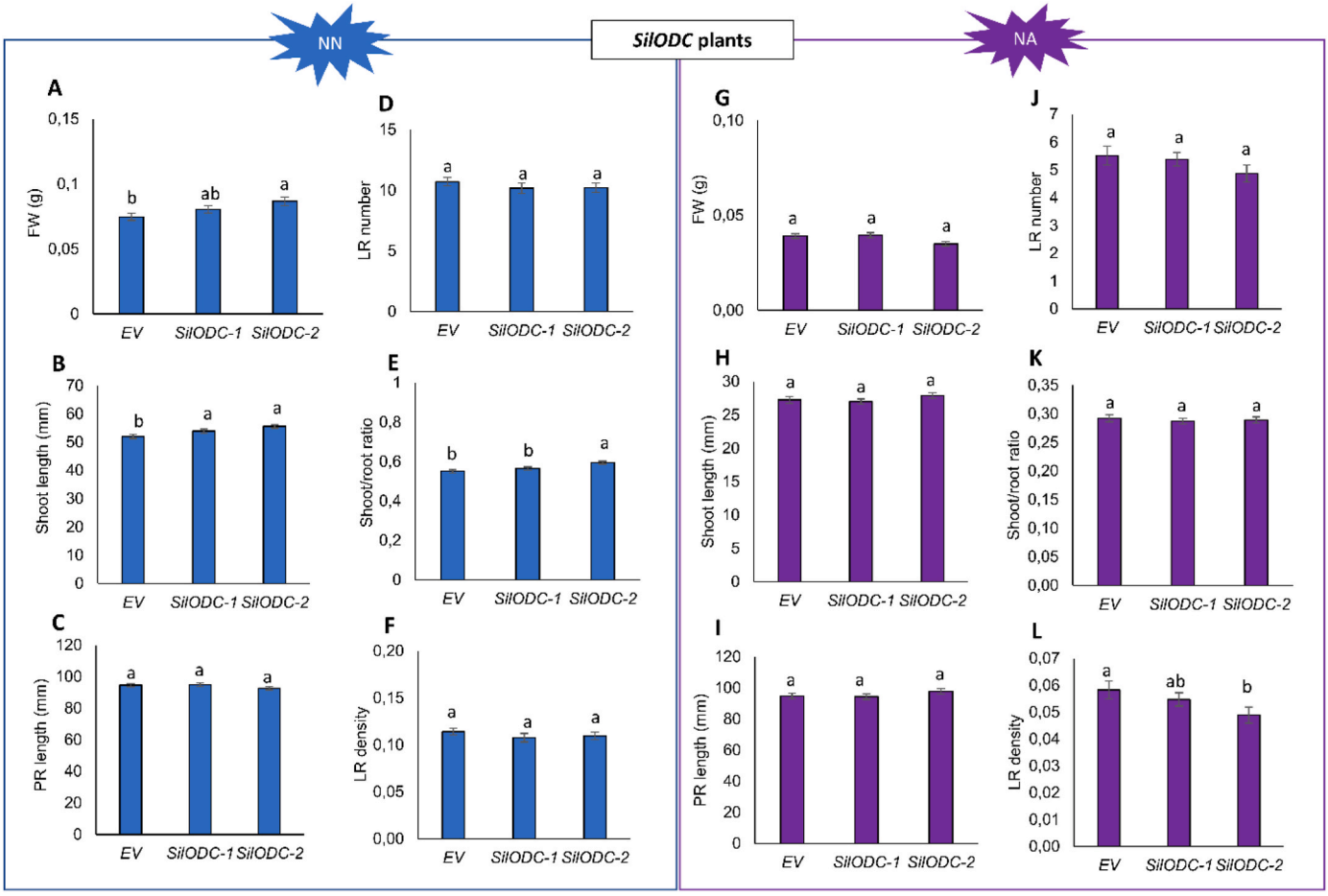

Fig. 4. Shoot length, PR length, LR number, shoot/root ratio, LR density and fresh weight (FW) of *SilODC* tomato seedlings grown under NN and NA conditions compared with *EV*. The results are the mean of three independent experiments ± standard error (SE). Distinct letters represent statistically significant differences by LSD test ($p < 0.05$).

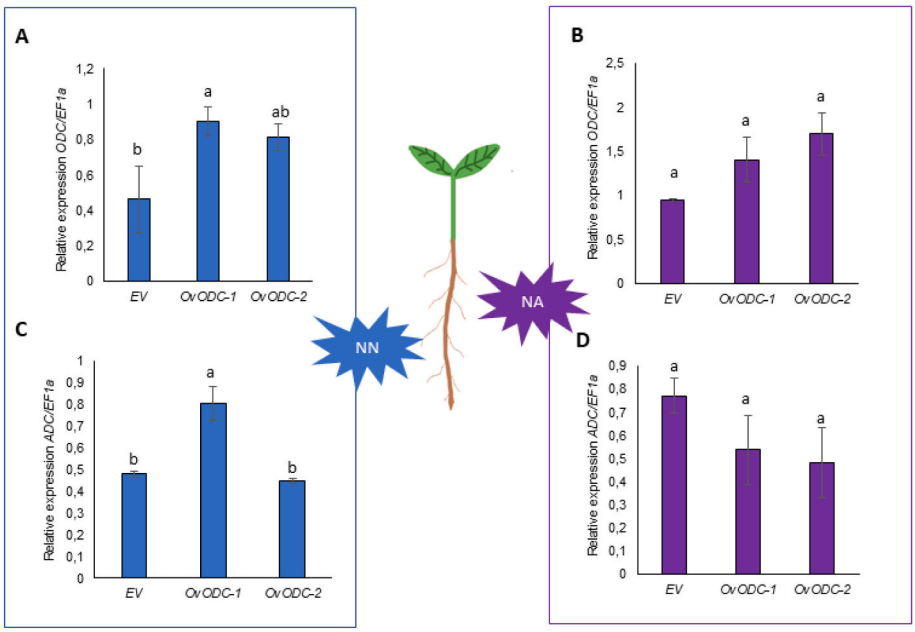

Fig. 5. Relative expression of *ADC* and *ODC* genes in cotyledons of *EV* and *OvODC* tomato seedlings grown under NN or NA as the sole N source. The results are the mean of three independent experiments ± standard error (SE). Distinct letters represent statistically significant differences by LSD test ($p < 0.05$).

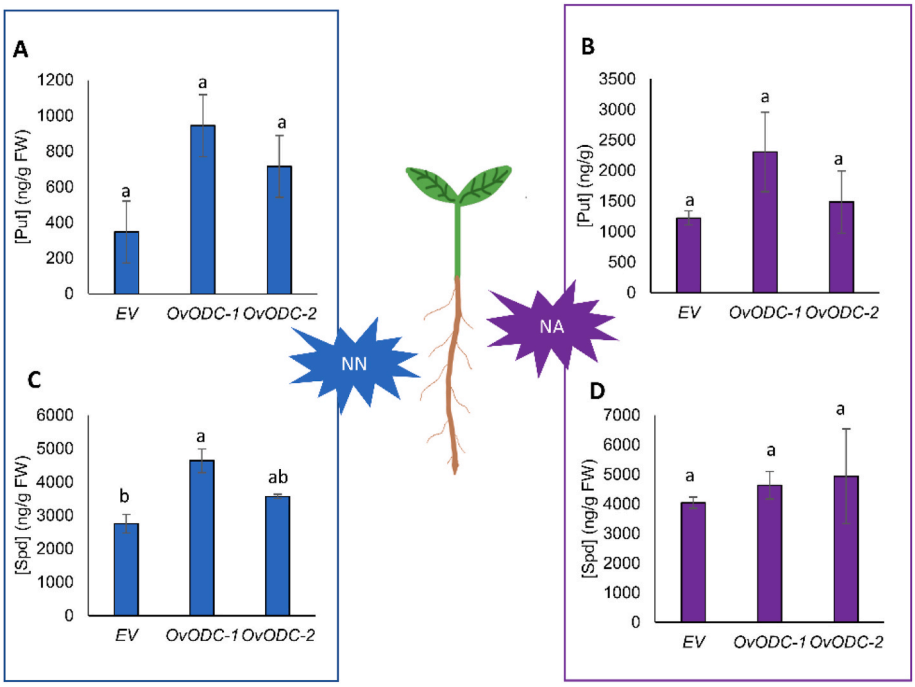

Fig. 6. Putrescine and spermidine concentrations in cotyledons of *EV* and *OvODC* tomato seedlings grown under NN or NA as the sole N source. The results are the mean of three independent experiments ± standard error (SE). Distinct letters represent statistically significant differences by LSD test ($p < 0.05$).

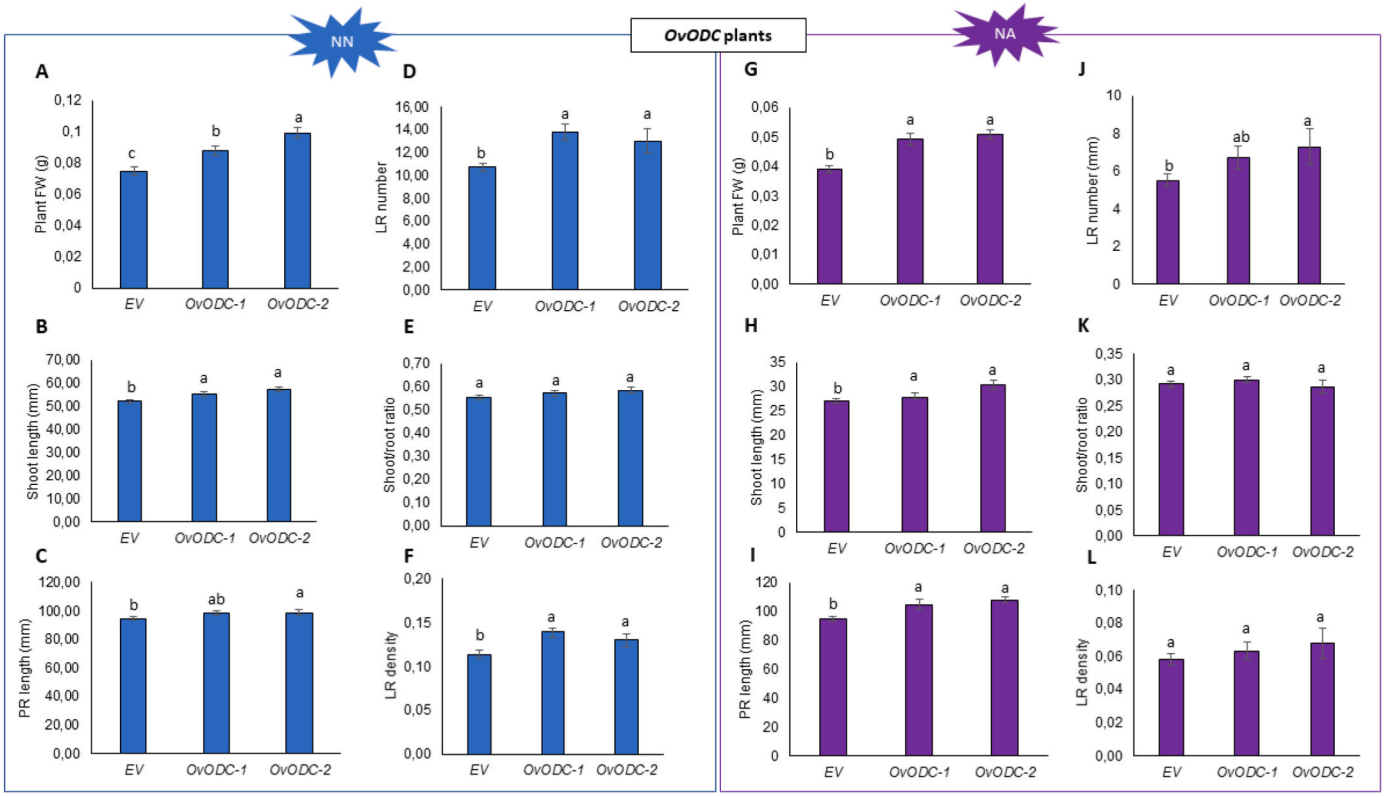

Fig. 7. Shoot length, PR length, LR number, shoot/root ratio, LR density and fresh weight (FW) of *OvODC* tomato seedlings grown under NN and NA conditions compared with *EV*. The results are the mean of three independent experiments ± standard error (SE). Distinct letters represent statistically significant differences by LSD test ($p < 0.05$).

concentration in *SilADC* plants under NA treatment and an increase in Put and Spd in *SilODC* plants under both N sources. This increase suggests that there is a compensatory induction of the ADC pathway in *SilODC* plants, which has been previously indicated by other authors (DeBoer et al., 2011; Nölke et al., 2005). Following this line of investigation, Dalton et al. (2016) showed that silenced ODC transgenic plants

of *Nicotiana tabacum* L. displayed elevated transcript levels of *ADC*, but in that case, they observed a decrease in Put concentration. As no increase in relative *ADC* expression and an enhancement in Put levels were observed in *SilODC* plants under NA nutrition in this study, the existence of other isoforms that are not yet characterized in tomato plants cannot be discarded.

It has been previously reported that the presence of free PAs, conjugated compounds and macromolecule-bound polyamines in roots and changes in their endogenous levels by different processes such as inhibitor treatment, gene manipulation, mutation, or exogenous treatment have different effects on root modulation (Couée et al., 2003). In this work, *SilADC* plants fed NN displayed an enhancement of plant FW, shoot length, LR number, LR density and shoot:root ratio, whereas a slight reduction was observed in PR length. Consistent with these results, Palavan-Ünsal (1987) showed that a reduction in ADC activity produced a depletion of PA levels that led to an inhibition of root length in common bean plants. This effect could be due to changes in auxin distribution due to the reduction of *ADC* expression, as suggested by Hashem et al. (2021). Moreover, Tonon et al. (2001) demonstrated the effect of ODC and ADC inhibitors (DFMO and DFMA) on endogenous IAA and Put concentrations and their close relationship in root induction, suggesting that polyamine catabolism has an important role in root formation and elongation. In addition, NA-treated *SilADC* plants only showed an increase in PR and shoot length as well as in plant FW, so the growth response of these seedlings appears to be dependent on the N source. Moreover, González-Hernández et al. (2020) showed that seedlings grown under NA treatment displayed a reduction in root and shoot FW and shoot length compared with NN treatment. These parameters were increased in our study when ADC was silenced, suggesting an effect of ADC silencing on NH_4^+ toxicity alleviation and root development.

Regardless, *SilODC* plants displayed an increase in shoot length, shoot:root ratio and plant FW under NN nutrition, whereas only a decrease in LR density was observed under the NA treatment. The action of ODC appears to be species-dependent, as several authors have described different modes of action. Previously, a reduction in *ODC* expression appeared to have negative effects on plant growth and vigour in *Nicotiana* plants (Dalton et al., 2016), whereas plants treated with DFMO did not change the growth or endogenous concentrations of polyamines in *Vicia faba* plants (Walters, 1986) or increase root length and Put depletion in excised roots from *Nicotiana tabacum* plants (Ben-Hayyim et al., 1996). In this work, the improvement of root development appears to be related to a putrescine increase. Pal Bais et al. (2001) suggested that Put influences plant root development and differentiation and provided insight into the morphological changes that occur in roots in response to an exogenous supply of polyamines. Exogenous Put may alleviate NaCl-induced growth inhibition through the promotion of carbohydrate metabolism, degradation of damaged proteins, and activation of stress defence responses (Yuan et al., 2016). We then generated two overexpressed ODC lines (*OvODC-1* and *OvODC-2*) to study the effect of the ODC pathway on root modulation and confirm the compensatory induction of the ADC pathway when ODC was silenced. The generated *OvODC-1* and *OvODC-2* seedlings displayed a clear increase in relative *ODC* expression under NN conditions. Furthermore, Put and Spd levels slightly increased in *OvODC* seedlings. The same trend was observed when *OvODC* seedlings were grown under NA conditions, although no significant differences were observed, which could be related to PA catabolism, as Fernández-Crespo et al. (2015) showed that NA-treated plants displayed higher *rboh1* and *CuAO* mRNA accumulation than those in control plants.

Hussein et al. (2019) showed that foliar application of L-ornithine ameliorated the negative effects on the root length and root and shoot weights caused by drought stress in sugar beet plants. This improvement in root and shoot parameters was also observed in *OvODC* plants, particularly when they were grown under NA conditions. This suggests a role for the ODC pathway in plant tolerance against NH_4^+ toxicity. Consistent with this idea, Kalamaki et al. (2009) generated transgenic *Arabidopsis* lines expressing the N-acetyl-L-glutamate synthase (*SlNAGS1*) gene that displayed a significant accumulation of Orn, which was accompanied by a higher germination and higher tolerance to salt and drought compared with WT plants. In addition, it was demonstrated that there is a close relationship between meristematic activity and polyamines in corn roots, as ODC is localized mainly in the meristematic zones (Schwartz et al., 1986). Furthermore, Acosta et al. (2005) indicated that ODC mRNA is localized to mitotically active cells in the shoot and root apical meristem of tomato plants, whereas ADC mRNA did not complement the location pattern obtained for ODC mRNA. Given these results, we can confirm the role of ODC in root development due to cell-type specialization during normal development.

5. Conclusions

Polyamines are considered the major sinks of assimilated N, and they play a role in a number of plant growth and developmental processes. Although the molecular and cellular processes associated with the polyamine pathways have been previously studied, much less is known about how polyamine homeostasis is maintained under different N sources. Putrescine is synthesized by two different pathways, from ornithine (Orn) or arginine (Arg), by the action of ornithine decarboxylase (ODC) and arginine decarboxylase (ADC), respectively. Using genomic approaches, this study revealed that the two putrescine biosynthetic pathways play different roles in tomato seedling growth, and are involved in different growth processes. The results suggest that *ODC* silencing appears to be compensated by *ADC* induction. Moreover, plants overexpressing *ODC* appear to show an amelioration of *ammonium syndrome*, as they displayed enhanced development. Thus, further studies are required to deepen the understanding of the molecular mechanisms of polyamines in plant tolerance.

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CRediT authorship contribution statement

Ana Isabel González-Hernández: Conceptualization, Methodology, Investigation, Writing – original draft, preparation. **Loredana Scalschi:** Conceptualization, Methodology, Investigation, Writing – original draft, preparation. **Pilar Troncho:** Methodology. **Pilar García-Agustín:** Writing – review & editing, Project administration, Funding acquisition. **Gemma Camañes:** Conceptualization, Writing – original draft, preparation, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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