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Chitosan microparticles mitigate nitrogen deficiency in tomato plants

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ABSTRACT

Nitrogen (N) deficiency is one of the most prevalent nutrient deficiencies in plants, and has a significant impact on crop yields. In this work we aimed to develop and evaluate innovative strategies to mitigate N deficiency. We studied the effect of supplementing tomato plants grown under suboptimal N nutrition with chitosan microparticles (CS-MPs) during short- and long-term periods. We observed that the supplementation with CS-MPs prevented the reduction of aerial biomass and the elongation of lateral roots (LR) triggered by N deficiency in tomato plantlets. In addition, levels of nitrates, amino acids and chlorophyll, which decreased drastically upon N deficiency, were either partial or totally restored upon CS-MPs addition to N deficient media. Finally, we showed that CS-MPs treatments increased nitric oxide (NO) levels in root tips and caused the up-regulation of genes involved in N metabolism. Altogether, we suggest that CS-MPs enhance the growth and development of tomato plants under N deficiency through the induction of biochemical and transcriptional responses that lead to increased N metabolism. We propose treatments with CS-MPs as an efficient practice focused to mitigate the nutritional deficiencies in N impoverished soils.

1. Introduction

Nitrogen (N) is a major limiting macronutrient in agricultural systems. Given its significant impact on crop productivity, large quantities of N fertilizers have been historically employed to enhance crop yields. However, nearly half of the applied N is lost to the ecosystem via mechanisms such as leaching, run-off or volatilization, thereby exacerbating environmental pollution and negatively affecting biodiversity (Martínez-Dalmau et al., 2021).

Nowadays there is an increasing pressure to safeguard the environment through the adoption of more sustainable agricultural practices worldwide. In line with this, chitosan (CS)-based systems have been applied as protective or biostimulant compounds for plants, demonstrating the potential to minimize environmental harm (Faqir et al., 2021). CS is biopolymer highly biocompatible due to its properties of biodegradability, adhesion and bioactivity (Jiménez-Gómez and Cecilia, 2020). The effect of the application of bulk CS promoting plant growth and development has been extensively studied in various crops, including potato (Falcón-Rodríguez et al., 2017), pepper (Mahmood et al., 2017), radish (Katiyar et al., 2015) and strawberry (Mukta et al., 2017), among other plant species. CS has also been widely studied as an antimicrobial and plant elicitor compound (Bautista-Baños et al., 2016; Lopez-Moya et al., 2019). In plants exposed to abiotic stresses (drought, salt, and or heat) pre-treating with CS, enhances growth, antioxidant enzyme production, and the synthesis of secondary metabolites and abscisic acid (ABA) (Rhaman et al., 2022). CS also induces changes in plant cell physiology, biochemistry, and molecular biology (Hidangmayum et al., 2019; Pongprayoon et al., 2022). However, it is also well known that responses to CS treatments may vary based on CS structures, concentrations, plant species, and developmental stages (Malerba and Cerana, 2016).

More recently, CS-based nanoparticles (NPs) and microparticles (MPs) have gained preference over bulk CS applications due to their

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Abbreviations

CS	chitosan
MP	microparticle
NP	nanoparticle
N	nitrogen
DM	deficient media
CM	complete media
DW	dry weight
SE	standard error
LR	lateral roots
PR	primary root
NO	nitric oxide
NO_3^-	nitrate

characteristics, such as enhanced biodegradability and sustained release into water and soil, which render them beneficial for agricultural purposes (Angelo et al., 2021). However, despite its promising perspectives, CS- based particles had yet to be tested on most vegetable crops.

Following the principles of the circular economy model, we produced CS-MPs by first preparing CS from waste generated by the shrimp fishing industry in Argentinean Patagonia, and then processing it to obtain the CS-MPs. These CS-MPs have shown beneficial effects on plant growth and root development in lettuce, tomato and *Arabidopsis thaliana* plants (Colman et al., 2019; Iglesias et al., 2019; Martin-Saldaña et al., 2018). Nevertheless, the impact of CS-MPs on plants cultivated in nutrient-deficient substrates has not been thoroughly assessed yet.

In this work, we used CS-MPs to supplement tomato plants grown in N deficient media. Tomato is a species which has a high N demand and constitute a very useful model for studying plant responses to nutritional deficits (Machado et al., 2022). Tomato also holds a significant position as a key vegetable crop on a global scale, surpassing 185 million tons in production in 2020 for both fresh consumption and processing purposes (FAO, 2022). Our hypothesis proposes that CS-MPs are capable to enhance the growth and development of tomato plants under N deficiency through the induction of biochemical and transcriptional responses associated with N metabolism.

We observed that CS-MPs treatments mitigated the effects of N deficiency in both, short-term *in vitro* assays and long-term experiments. Parameters as aerial biomass, root architecture and levels of nitrate, amino acids and chlorophyll were either partial or totally restored upon CS-MPs addition to N deficient media. In addition, we identified the involvement of nitric oxide (NO) and the regulation of genes involved in N uptake, signaling and assimilation as part of the response triggered by CS-MPs. Altogether, we suggest that CS-MPs supplementation mitigates N deficiency by promoting growth and development through the enhancing of N metabolism in tomato plants.

2. Materials and Methods

2.1. Plant material

Tomato (*Solanum lycopersicum* cv. Platense) seeds were commercially obtained from FECOAGRO Ltda., San Juan, Argentina.

2.2. Chemical materials

CS-MPs used for plant treatments were recently described and characterized by Martin-Saldaña et al. (2018). CS was acquired from Gihon Laboratorios Químicos SRL (Mar del Plata, Argentina) where it was obtained by the processing of wastes of the shrimp fishing industry. The deacetylation degree of CS was higher than 87%. The CS-MPs were prepared by the ionic gelation method (Cerchiara et al., 2015) with modifications using TPP as crosslinker. The mean diameter of CS-MPs was 2.10 ± 0.78 μm with a polydispersity index of 0.14 (Martin-Saldaña et al., 2018). Most CS-MPs were spherical with some showing irregular shapes. In this study, CS-MPs were re-suspended and assayed in sterile aqueous suspension (pH 5.5–6) at 10 and 100 μg mL^{-1} according to our previous reports (Colman et al., 2019; Iglesias et al., 2019). For *in vitro* treatments, culture media was sterilized before adding the CS-MPs.

2.3. Experimental designs and treatments

2.3.1. Composition of complete media (CM) and deficient media (DM)

Different nutritional conditions were established: a complete nutritional media (CM), which corresponded to *Arabidopsis thaliana* salts (ATS) solution (Wilson et al., 1990) which was considered as positive control; and a deficient media (DM), which contained ATS with the following modifications: 5 mM KNO_3 and 2 mM $Ca(NO_3)_2$ were replaced with 0.5 mM KNO_3, 4.5 mM KCl and 2 mM $CaCl_2$.

2.3.2. In vitro assays with plantlets

For plate assays, tomato seeds were surface-sterilized in 30% (v v^{-1}) hypochlorite and 0.1% (v v^{-1}) Tween-20 solution during 10 min followed by three washing steps in sterilized distilled H_2O. Sterilized seeds were placed on Petri dishes containing 1% (p v^{-1}) agar at 25 °C in the dark during four days. Germinated seeds were transferred to square Petri dishes (120 × 120 mm) containing CM or DM with 1% (p v^{-1}) agar supplemented or not with 10 μg mL^{-1} CS-MPs. Square Petri dishes were incubated at 25 °C under 250 μmol photons m^{-2} s^{-1} with 16:8 h light: dark cycles during five days and harvested for determinations. For each treatment, seven germinated seeds were used per plate with three replicates.

2.3.3. Pot assays with plants

For long-term assays, tomato seeds were germinated in Petri dishes on filter paper soaked in sterile water and incubated in a growth chamber for 4 day at 25 °C in the dark. Germinated seeds were transferred to plastic pots (0.18 L) containing vermiculite and grown at 25 °C under 250 μmol photons m^{-2} s^{-1} with 16:8 h light: dark cycles during 26 days. Plants were watered three times with CM or DM at 1-, 8- and 17-days post-sowing; distilled water was added when necessary. In these assays, we applied the particles through irrigation using a concentration determined based on our previous experiments conducted with 26-day-old tomato plants (Colman et al., 2019). For treatments, 5 mL of 100 μg mL^{-1} CS-MPs or H_2O used as control were applied by soil drench at 3-, 11- and 19-days post sowing. After 26 days, plants were harvested for analysis. Ten plants were used for each treatment. All experiments were performed at least, in duplicated. Two or three independent experiments were performed in each case.

2.4. Measurements of growth parameters and pigment contents

Five-day old plantlets were photographed with a camera (Nikon Coolpix T80, Indonesia). Root area, lateral root (LR) length and LR number were analyzed by ImageJ image-analysis software (U.S. National Institutes of Health, http://rsb.info.nih.gov/ij/). At least seven roots were analyzed per plate.

Twenty six-day-old plants were cut on the surface of the substrate, and fresh weight (FW) of shoots (stem and leaves) was measured on a laboratory scale (Sartorius, Germany). Dry weights (DW) were obtained after samples were dried in a drying oven for 3 day at a constant temperature of approximately 75 °C (Hernández-Hernández et al., 2018).

The levels of pigments chlorophyll and anthocyanin were measured in 26-day-old plants. The chlorophyll content in leaves was measured with a SPAD-502 Chlorophyll Meter (SPAD-502, Minolta, Japan). For anthocyanins, 0.1 g of fresh leaf tissue was ground with liquid N_2 in a mortar and incubated with 1% (v v^{-1}) HCl in methanol, stirring for 1

min and incubated at 4 °C for 2 h in the dark. The total monomeric anthocyanin content was determined using a pH-differential method (Giusti and Wrolstad, 2001). A microplate reader (ELX 800, Biotek, USA) was used for spectral measurements at 530 and 700 nm. Pigment content was expressed as mg cyanidin-3-glucoside g^{-1} FW, using an extinction coefficient of 34300 L cm^{-1} mol and molecular weight (MW) of 449.2 g mol^{-1}.

2.5. Analysis of endogenous N-associated metabolites and NO level

Endogenous metabolites, NO_3^- and total free amino acids were analyzed in 5-day-old plantlets grown in CM or DM and supplemented or not with 10 μg mL^{-1} CS-MPs. To measure NO_3^-, approximately 100 mg of fresh leaf tissue was ground in liquid N_2 and suspended in 200 μL of 100 mM sodium phosphate, pH 7.4. After centrifugation at 12,000 g for 5 min at 4 °C, supernatants were used for quantifications. The spectrophotometric analysis of NO_3^- was determined by the sulphamic/perchloric acid method (Carvalho et al., 1998). Samples with a volume of 1.5 mL were added to 0.1 mL of 20% (v v^{-1}) sulphamic acid and vortex shaken, allowed to rest for 2 min and vortex shaken once again; 0.4 mL of 10% (w v^{-1}) perchloric acid was then added and the solution was again vortex shaken. Absorbance was read at 210 nm. For each treatment, a pool of leaves and shoots from three plantlets was analyzed. Each treatment was performed in three independent replicates.

Total amino acids were measured by ninhydrin colorimetric analysis according to Rosen (1957). A pool of three entire plantlets was analyzed in each treatment. Experiments were done in three independent replicates.

Endogenous NO was detected using the NO-sensitive fluorescent probe DAF-FM DA (Invitrogen, USA) according to (Kojima et al., 1999). Roots from 5-day old plantlets were observed by fluorescence microscopy and bright-field microscopy using an Eclipse E200 microscopy (Nikon). The fluorescence was quantified using the ImageJ image-analysis software (U.S. National Institutes of Health, http://rsb.info.nih.gov/ij/). Fluorescence of primary root tips from five independent plantlets were analyzed in each treatment.

The N content was determined by the Kjeldahl method (AOAC, 240.27; 1990). Experiments were done in three independent replicates.

2.6. RNA isolation and RT-qPCR

Total RNA from 5-day-old plantlets grown in CM or DM, supplemented or not with 10 μg mL^{-1} CS-MPs, was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's recommendations. Samples were incubated with RQ1 RNase-free DNase (Promega, USA) to remove DNA contamination. The cDNA was synthesized from 1 μg of total RNA using IMPROM II (Thermo Fisher Scientific, USA) with random primers (Biodynamics SRL, Argentina). The expression of a subset of N-related metabolism genes coding for two NO_3^- transporters, *NTR1.1* and *NRT2.1*; an ammonium transporter, *AMT1.1*; *nitrate reductase* (*NR)* and *glutamine synthetase 1 (GS1*) was analyzed by RT-qPCR. The primers used were those designed by Abouelsaad et al. (2016) and are listed in Table S1. RT-qPCR reactions were performed in triplicates (40 cycles at 95 °C for 10 min and 1 min at 60 °C) employing a Step One Real-time PCR system (Applied Biosystems, USA) using SYBR green PCR master mix (Applied Biosystems, USA) following manufacturer's instructions. Primers were tested for specificity and for amplification efficiency with a cDNA dilution curve. Real time data was analyzed using the StepOne™ Software v2.3 Tool (Applied Biosystem) and LinRegPCR v11.0 (Ruijter et al., 2009). The expression of each gene was determined using the comparative threshold cycle method (Pfaffl, 2001) and data were normalized to the expression level of the control gene actin and to positive control (CM). The expression values were standardized by the media of each experiment. Three independent experiments were performed.

2.7. Statistical analysis

The values shown are mean values ± standard errors (SE). The data were subjected to ANOVA with post hoc Dunnett's method comparisons. For analysis of growth parameters and pigment content, data were processed with a linear mixed model using the lme function from the nlme library in R software (version 4.2; R Foundation for Statistical Computing). Fixed effects were treatments, experiments were treated as a random effect.

3. Results

3.1. CS-MPs relieved the N deficiency consequences on aerial biomass and root architecture in tomato plantlets

As expected, 5-day-old plantlets grown with N deficiency showed a significant decrease in aerial biomass compared to plantlets growing on CM in *in vitro* assays (Fig. 1). The addition of 10 μg mL^{-1} CS-MPs to the DM caused a positive effect on the plantlets performance, as the values of aerial biomass of these plantlets did not differ significantly from the CM grown plantlets (Fig. 1A and B).

Interestingly, CS-MPs treatment increased aerial fresh weight (FW) when plants were grown under sufficient nitrogen conditions (CM) in *in vitro* assays (Supplementary Table S1).

Considering that changes of root system have been associated with N deficiency in plants (Sun et al., 2020), we measured the length of LR as an output of the treatment. In concordance with previous findings, plantlets from DM showed an increment of 45% in LR length compared with CM plantlets (Fig. 1A–C). CS-MPs supplementation to DM was able to partially restore the root phenotype displayed by CM grown plantlets (Fig. 1A–C).

When we measured the number of LR, no differences were observed between plants growing on CM and DM, however, CS-MPs-supplemented DM grown plantlets showed an increase of 36% in the number of LR compared with non-supplemented DM grown plantlets (Supplementary Fig. S1).

No significant differences in LR length were observed when CS-MPs were added to plantlets grown on CM (Supplementary Table S2).

3.2. CS-MPs increased NO_3^- root and free amino acids of N deficient plantlets

We evaluated if the N content in the plants resulted affected by CS-MPs supplementation. We measured the levels of two major N compounds: NO_3^- (in leaves and in roots) and free amino acids in 5-day old plantlets grown on CM, DM and DM supplemented with CS-MPs.

We observed that N deficiency caused in roots a significant reduction of NO_3^- level compared with plantlets grown on CM (Fig. 2A). However, CS-MPs supplementation of DM caused a partial recovery, as root NO_3^- levels resulted significantly higher than in DM plantlets (about. 25%), although still significantly lower than root NO_3^- levels of CM grown plantlets (Fig. 2A). On the other hand, no differences were observed between plantlets from these treatments when NO_3^- content was measured in leaves (Fig. 2B).

The content of free amino acids lowered significantly in plantlets grown in DM compared to CM grown plantlets (Fig. 2C). CS-MPs supplementation to plantlets grown under N deficit was able to maintain the free amino acid levels recorded in CM grown plantlets (Fig. 2C).

There were no differences in NO_3^- (either in root or in leaves) or in free amino acids levels when CS-MPs were applied to plantlets cultivated on CM (Supplementary Table S2).

3.3. CS-MPs supplementation mitigated N-deficiency in tomato plants

Next, we validated the results presented above, obtained from 5-day-old plantlets in *in vitro* assays, in a study performed with 26-day-old

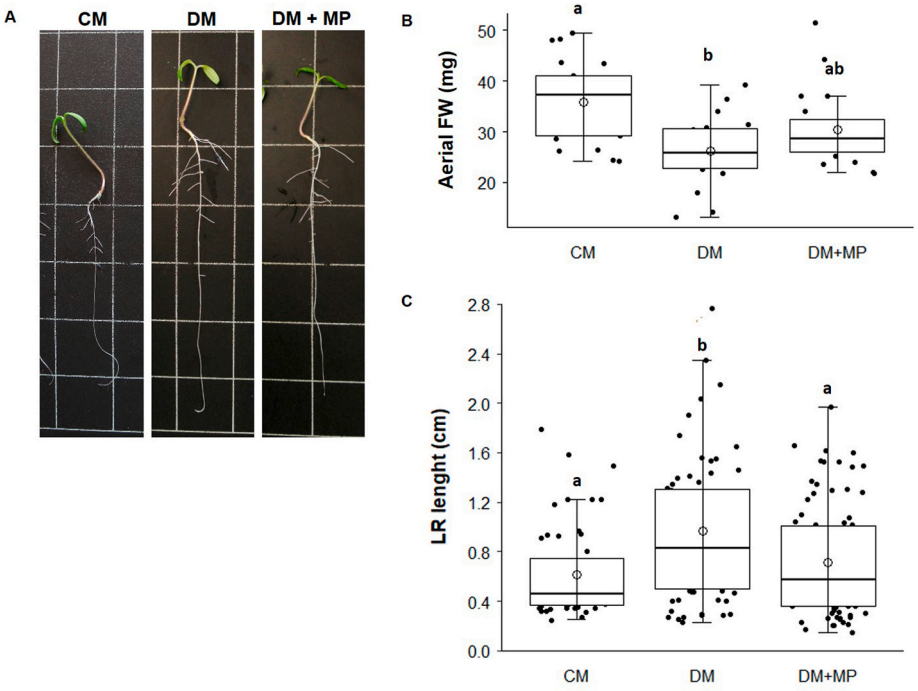

Fig. 1. Effect of CS-MPs on aerial FW and root architecture in N deficiency. Germinated seeds were placed on CM or DM media, supplemented or not with 10 μg mL^{-1}. (A) Representative images of 5-day old plantlets. (B) Aerial FW and (C) LR length were measured. CS-MPs have been named as MP. Three independent experiments were performed (n = 21). Square scale: 3 × 3 cm.

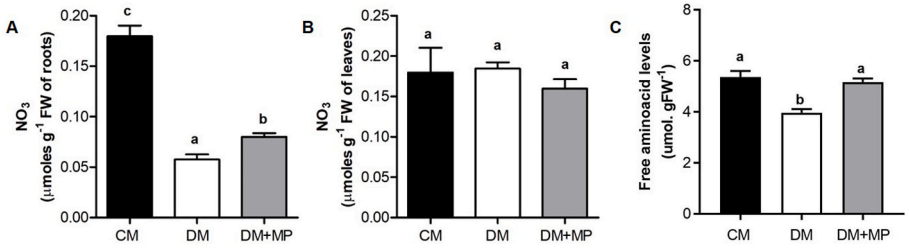

Fig. 2. Effect of CS-MPs supplementation on the levels of NO_3^- and free amino acids in N deficiency. Content of NO_3^- was individually measured in (A) roots and in (B) leaves from plantlets grown on CM, DM and DM + MP. (C) Total amino acids were measured in plantlets grown under these same conditions. CS-MPs have been named as MP. For each treatment, a pool of three samples was analyzed from three independent replicates.

plants grown in pots.

Again, plants grown in DM shown a significant decrease (27%) in aerial DW compared to CM plants (Fig. 3A and B). The supplementation with CS-MPs resulted in a complete maintenance of aerial biomass, resulting in similar values to CM treatment and 46% higher than DM grown plants (Fig. 3A and B).

Analysis of root growth parameters (root DW and root area) are presented in Supplementary Fig. S2. Root DW behaved similarly to aerial biomass: a significant decrease (60%) was recorded in DM compared to CM grown plants, but it was fully maintained by CS-MPs supplementation (Supplementary Fig. S3A). On the other hand, root area values were comparable in DM and CM grown plants, but a significant increase (66%) was reported upon CS-MPs addition (Supplementary Fig. S2B).

Chlorophyll levels responded also in a similar fashion. Plants grown in DM had lower chlorophyll content (ca. 15%) than those grown on CM (Fig. 3A–C). However, plants from DM supplemented with CS-MPs showed the same level of chlorophyll than plants from CM (Fig. 3C). On the other hand, anthocyanin content showed an approximately 15-fold increase in leaves of plants grown in DM compared with those grown in the presence CM. CS-MPs supplementation of DM caused a downward trend, although not significant, in the level of anthocyanin (Fig. 3D).

In contrast, the addition of CS-MPs had no effect on the aerial DW of plants growing in CM neither affected the levels of chlorophyll or anthocyanins (data not shown).

The level of total nitrogen was measured in whole 26-day-old plants, and no changes were observed after CS-MPs supplementation in either CM or DM plants (Supplementary Fig. S3).

3.4. CS-MPs increased the endogenous NO level in tomato roots

In order to get insight into the mode of action of the CS-MPs, we analyzed the levels of endogenous NO, a reactive nitrogen species, in roots of 5-day old tomato plantlets grown on CM or DM supplemented or not with CS-MPs.

No significant differences in NO levels were observed between CM and DM treatments (Fig. 4). However, CS-MPs supplementation to plantlets growing on DM significantly increased the NO level in root tips (Fig. 4).

In addition, CS-MPs treatment also incremented NO level in root tips from plantlets grown on CM (Supplementary Fig. S4), suggesting that CS-MPs are able to modify the level of NO in tomato plantlets independently of the N nutritional condition.

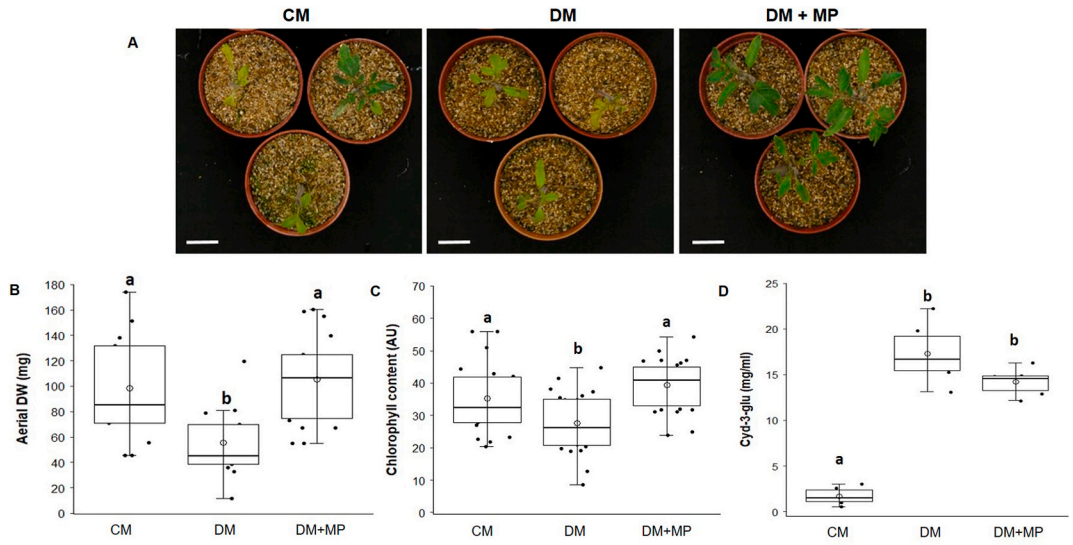

Fig. 3. CS-MPs effect on growth parameters and anthocyanins content in 26-day old tomato plants grown under N deficiency. A) Representative images of plants grown under different treatments are indicated, B) Aerial DW, C) chlorophyll and D) anthocyanins expressed as cyanidin-3-glucoside. Ten plantlets were analyzed in each treatment. The experiment was performed by duplicate (n = 20 for Aerial DW and chlorophyll and n = 6 for anthocyanins). Bar: 3 cm. CS-MPs have been named as MP.

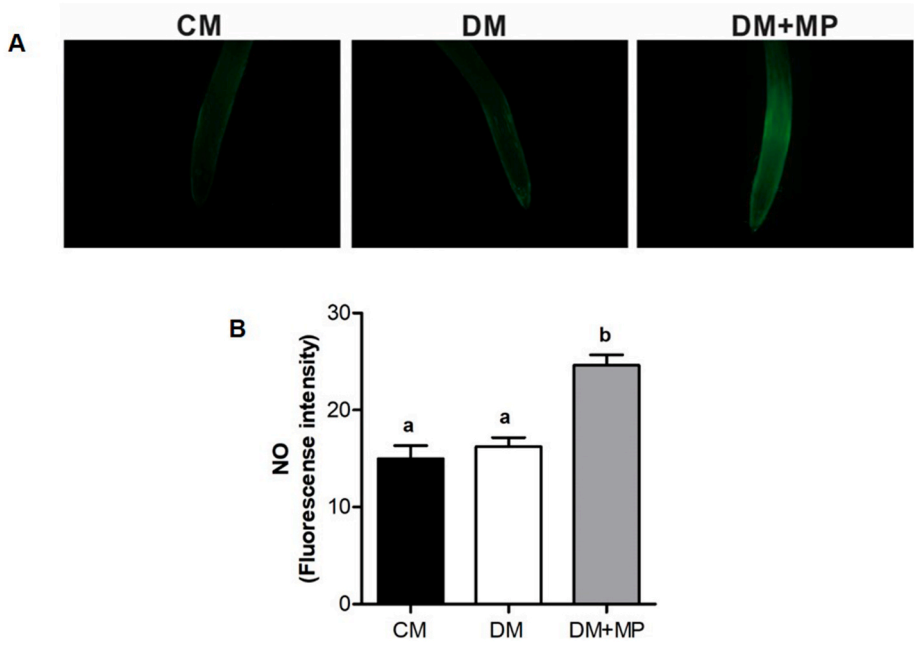

Fig. 4. CS-MPs effect on endogenous NO in primary root tips from 5-day old plantlets grown under N deficiency. NO was detected by NO-sensitive dye DAF-FM DA and observed by fluorescent microscopy. A) Representative images of primary root tips of plantlets grown on CM, DM or DM + MP. B) Quantification of fluorescence values using the ImageJ image-analysis as described in Materials and Methods. Data are expressed as arbitrary units (AU). Three independent experiments were performed (n ≥ 5). CS-MPs have been named as MP.

3.5. CS-MPs supplementation enhanced the expression of primary N-associated uptake and assimilation genes

We studied the effect of CS-MPs supplementation on the expression of primary N metabolism genes through RT-qPCR analysis. Two sets of genes, one related to N uptake: *NRT1.1*, *NRT2.1* and *AMT1.1*, and the other related to NO_3^- assimilation: *NR* and *GS1* were analyzed in 5-day-old plantlets grown on CM or DM supplemented or not with CS-MPs.

Under N deficiency condition, genes related to N uptake *(NRT1.1, NTR2.1* and *AMT1.1)* resulted up-regulated while assimilation genes behaved differentially (*NR* was down-regulated and *GS1* did not change) (Fig. 5). CS-MPs supplementation of plantlets growing under N deficiency significantly increased the expression of *AMT1.1, NRT1.1*, *NRT2.1*, and *GS1,* intensifying the N acclimation response. This result suggests that a modulation of N metabolism could be induced by CS-MPs.

4. Discussion

N is a macro-element essential in the nutrition of plants and its deficiency significantly affects plant productivity, as it directly impacts on photosynthesis rate, leaf area, and the lifespan of green leaves (Mu

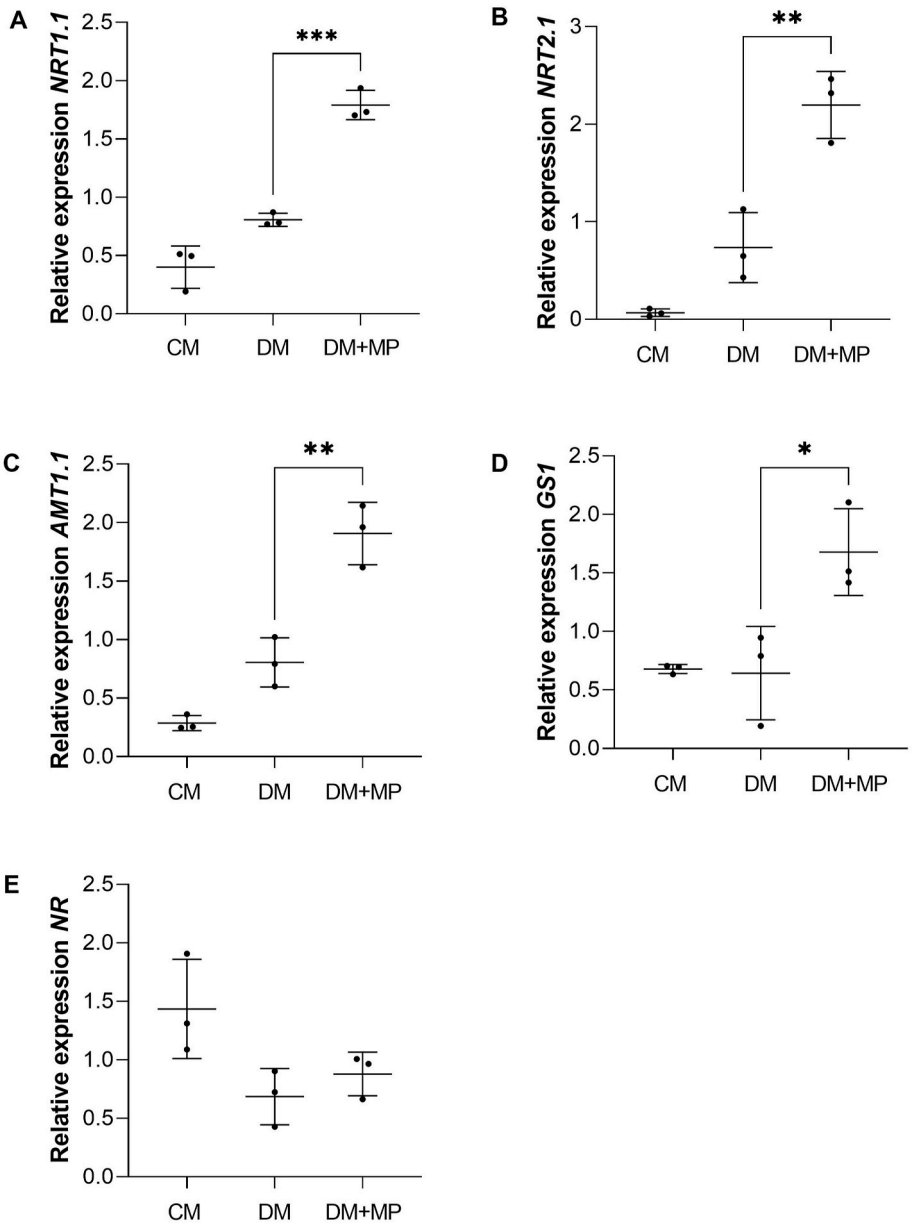

Fig. 5. CS-MPs effect on the expression of genes associated to N uptake and assimilation in 5-day old plantlets grown under N deficiency. The expression of N metabolism genes: *NRT1.1* (A); *NRT2.1* (B); *AMT1.1* (C); *GS1* (D); *NR* (E) was analyzed in 5-day old tomato plantlets grown on CM, DM and DM + MP. Scatter-plot of relative gene expression normalized to actin. Data were normalized to the expression of positive control (CM). The expression values were standardized by the media of each experiment. For each treatment, a pool of three samples was analyzed from three independent replicates. CS-MPs have been named as MP. (*$p < 0.05$, **$p < 0.01$, *t*-test).

and Chen, 2021). In order to meet N needs of the crops, there has been a widespread promotion of using nitrogen fertilizers extensively in croplands to increase crop yields (Lammerts van Bueren and Struik, 2017). Specifically, in the context of tomato cultivation, recommendations have advised applications ranging from 300 to 400 kg ha^{-1} in intensive cropping systems. However, over-fertilization has become a prevalent strategy in recent decades to mitigate the risk of nutrient deficiency (Llanderal et al., 2018). It is noteworthy that only half of the N applied as fertilizer is effectively absorbed, attributable to the low N use efficiency inherent in high-yielding crop varieties under such conditions (Truffault et al., 2019). The surplus of N fertilizer not utilized by the plant finds its way into the environment, contributing to N pollution. Nitrates easily leach into waterways, resulting in groundwater contamination and the eutrophication of aquatic ecosystems. Additionally, N in the soil may contribute to troposphere pollution as gaseous reduced N compounds, thereby playing a role in global warming (Beatty and Good, 2018).

Consequently, the agricultural industry is actively pursuing sustainability and environmental friendliness, all while striving to meet the demands for food and biomass.

In this work, we showed the beneficial action of CS-MPs supplementation in plants grown under suboptimal N nutrition. We observed the positive effect of CS-MPs supplementation as an increase in aerial biomass and changes in the architecture of the root system in *in vitro* assays with 5-days-old plantlets (Fig. 1). The supplementation of tomato plantlets with CS-MPs rescued the elongation phenotype of LR associated to N deficiency (Fig. 1C) and additionally, increased the number of LR (Supplementary Fig. S1). The architecture of the root system occurs as a principal adaptive response under N deficiency thereby, promoting an increased access into soil space and N resources (Sun et al., 2020).

Later, we showed that CS-MPs supplementation of plants grown

under suboptimal N nutrition contained higher levels of major N compounds: NO_3^- (in roots) and free amino acids (Fig. 2), suggesting that CS-MPs were able to promote either the uptake, assimilation or remobilization of N. Considering the synthesis methodology and the nature of the components of the CS-MPs utilized, CS-MPs are unlikely to act as N source (Martin-Saldaña et al., 2018). Although chitin and CS have been reported to serve as a source of carbon, oxygen, nitrogen and phosphorus for plants (Riseh et al., 2023), chitin- or CS-based materials are unable to release readily available sources of nitrogen such as NO_3^- and NH_4^+ (Egusa et al., 2020).

In addition, we showed that the beneficial action of CS-MPs supplementation in plants grown under suboptimal N nutrition continued to be significant after 26 days of plant development. This was evidenced by the maintenance in aerial biomass and chlorophyll levels in plants grown in DM supplemented with CS-MPs, which resembled values from plants grown in CM (Fig. 3). The chlorophyll content is an indicator of photosynthetic performance and it is greatly affected by N availability in tomato (Sun et al., 2020). Anthocyanins have been documented to accumulate under low N stress, serving a protective function (Liang and He, 2018). CS-MP treatment tended to decrease anthocyanin accumulation, indicating that plants would be experiencing a milder N deficiency.

When we measured total N levels in the whole plant after CS-NPs supplementation in 26-day-old plants, no changes were observed (Supplementary Fig. S3). This suggests that the increases observed in levels of amino acids and NO_3^- upon CS-NPs supplementation (Fig. 2), were mainly caused by increases in the remobilization of N, rather than in N uptake. This response could increase the availability of N compounds as a N source, as well as the signaling to improve the nutrient status of plants.

Then, we investigated a potential mechanism of action of CS-MPs. We measured NO levels in primary root tips of plantlets and we observed that CS-MPs supplementation was able to trigger NO generation independently of the nutritional condition. NO levels increased both in CM and DM grown plants upon CS-MPs addition (Fig. 4, Supplementary Fig. S4). These results are in agreement with Iglesias et al. (2019) and Chandra et al. (2015), who demonstrated that CS-MPs and CS-NPs improved NO production in *Arabidopsis thaliana* and *Camellia sinensis*, respectively. In Arabidopsis NO, a byproduct of NO_3^- metabolism and a well-characterized signal molecule plays a critical role in the primary response to NO_3^-. Recently, Nejamkin et al. (2023) established that NO is essential for the activation required for the expression of several nitrate-regulated genes including *NRT1.1* and *NRT2.1*.

To study the effect of CS-MPs in the regulation of the N metabolism, we analyzed the expression of genes involved in N uptake and assimilation. We observed that CS-MPs supplementation was able to enhance the transcriptional response of these genes to N deficiency. Relative abundance of transcripts of genes *AMT1.1, NRT1.1*, *NRT2.1*, and *GS1,* which were already increased by DM treatments, were further enhanced by a factor of approximately three when CS-MPs were added to the growth media (Fig. 5). This result suggests that CS-MPs induced a transcriptional regulation of N metabolism genes, likely affecting the machinery involved in N uptake and effectively promoting N assimilation, strengthening the nutritional status and helping the plant survive. In line with this, it has been shown that, *AtAMT1.1* ammonia transporter gene is highly expressed in roots where restructures its architecture under limited N conditions (Engineer and Kranz, 2007). *NRT1.1* and *NRT1.2* encode for high-capacity, low-affinity nitrate transporters in tomato (Ono et al., 2000) and it was reported that increases in the expression in both genes improved nitrate uptake in tomato plants under high N demand (Albornoz et al., 2018). In addition, considering that *NTR1.1* has the capacity to switch to a high-affinity nitrate transporters and also acts in nitrate signaling (Fang et al., 2021), the transcriptional response observed by CS-NPs treatment could not only affect N uptake but also N signaling. In Arabidopsis *NRT2.1* plays a major role in NO_3^- uptake and determines root architecture by controlling LR formation (Engineer and Kranz, 2007). Overexpression of *GS1* has been already demonstrated to result in increases in biomass and grain yield in *N. tabacum* (Oliveira et al., 2002) and *Phaseolus vulgaris* (Habash et al., 2001).

The impact of CS treatment on N metabolism was previously studied in wheat seedlings (Zhang et al., 2017). Through a comprehensive analysis of metabolite profiles, enzyme activities and transcript expression of primary carbon and N metabolisms, Zhang et al. (2017), demonstrated the ability of CS to enhance N reduction and assimilation processes. CS-treated plants exhibited elevated levels of glutamate, aspartate, and other amino acids, coinciding with the activation of key enzymes involved in N reduction and the glutamine cycle, such as glutamine synthetase/glutamate synthase. Nevertheless, the specific regulatory mechanism by which CS influences these responses remains undiscovered. In addition, pathways of entrance of CS (or CS-MPs or CS-NPs) are not yet clearly understood, but it is known that could be influenced the size and charge of the type of bioparticle (Mujtaba et al., 2020). The use CS, CS-MPs or CS-NPs could result in similar as well as specific responses, considering the physicochemical parameters associated to uptake and translocation into plant tissues, and it must be further investigated. Our work provides useful information in this regard.

In previous studies, we demonstrated that CS-MPs enhanced growth parameters by modulating auxin and cytokinin signaling pathways in tomato (Colman et al., 2019), and in *A. thaliana* plants (Iglesias et al., 2019). Besides, other studies have highlighted a close correlation between N nutrition and cytokinin content in different plant species such as tobacco, *Urtica dioica*, barley and maize (Sakakibara et al., 2006). We hypothesized that CS-MPs might trigger growth and developmental responses throughout the activation of NO, NO_3^-, auxin and cytokinin mediated regulation during tomato acclimatization to the nutritional condition.

Overall, we view CS-based microparticles as a promising biostimulant applicable to vegetable crops grown in low-nitrogen soils. Future research should focus on elucidating CS-MPs entry pathways into plants, examining related physicochemical factors governing uptake and translocation, and identifying optimal application methods consistent with sustainable agricultural practices.

CRediT authorship contribution statement

Silvana Lorena Colman: Writing – original draft, Methodology, Investigation, Conceptualization. **María Florencia Salcedo:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **María José Iglesias:** Writing – original draft, Formal analysis. **Vera Alejandra Alvarez:** Supervision, Funding acquisition. **Diego Fernando Fiol:** Writing – review & editing, Writing – original draft. **Claudia Anahí Casalongué:** Writing – review & editing, Supervision, Funding acquisition. **Noelia Pamela Foresi:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.108728.

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