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Enhanced properties of chitosan microparticles over bulk chitosan on the modulation of auxin signaling pathway with beneficial impacts on root architecture in plants

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ABSTRACT

Improving the root system architecture (RSA) under adverse environmental conditions by using biostimulants is emerging as a new trait to boost crop productivity. Recently, we have reported the characterization of novel chitosan-based microparticles (CS-MPs) with promising biological properties as rooting agent in lettuce. In this work, we demonstrated that in contrast to bulk chitosan (CS) which exerts root growth inhibition, CS-MPs promoted root growth and development from 1 to 10 µg. ml\(^{-1}\) without cytotoxicity effects at higher doses in Arabidopsis and lettuce seedlings. In addition, we studied the mechanistic mode of action of CS-MPs in the development of early RSA in the Arabidopsis model. CS-MPs unchained an accurate and sustained spatio-temporal activation of the nuclear auxin signaling pathway. Our findings validated a promising scenario for the application of CS-MPs in the modulation of RSA to respond to changing soil environment and improved crop performance.

Key words: Arabidopsis, auxin, chitosan microparticles, lettuce, root system architecture.

INTRODUCTION

The root system architecture (RSA) involves the coordinated growth and development of primary root (PR), lateral root (LR) and adventitious roots in order to improve soil exploration and resource acquisition, being pivotal for plant fitness and crop productivity.\(^1\) Since the development of RSA is a crucial factor in determining plant survival particularly under adverse environmental conditions, its modulation is emerging as a strategy to generate improvement in crop yields.\(^2,3\) In this context, the development of new bioactive materials with emerging properties fits with the actual challenge of augmenting crop productivity with reduced environment impact.\(^4,5\) Chitosan (CS) are composed by \(\beta-1,4\)-linked glucosamine and N-acetyl glucosamine residues and are generated by the partial deacetylation of chitin polymer. Due to its
unique properties such as biodegradability, biocompatibility, ubiquity and low cost, CS has several applications in several fields including agriculture.\(^6\),\(^7\) CS action in the protection of plants against biotic stress by inhibiting microorganism growth and eliciting plant innate immunity has been extensively studied in multiple species.\(^8\)-\(^10\) Although CS has been suggested as biostimulant promoting plant growth in several horticultural plants, a delicate unbalance from optimal concentrations leads to growth inhibition with high impact on root development.\(^11\),\(^12\) The patterns of plant development are consolidated by the action of hormonal regulation mechanisms. The dynamic and versatile modulation of auxin biosynthesis, transport and signaling has been found to be required for RSA development under changing environmental conditions.\(^13\),\(^14\) Auxin regulates root development mainly through the nuclear signaling pathway mediated by the TIR1/AFBs receptors.\(^15\) Auxin binding to TIR1/AFBs receptors triggers the degradation of Aux/IAAs repressors with the consequent activation of auxin response genes.\(^16\) The early response genes involved Aux/IAAs, SAUR and GH3 gene families.\(^17\) The inhibition of auxin-induced growth and the repression of auxin gene expression required for root development has been evidenced in CS-treated wheat coleoptiles and sweet orange plants, respectively.\(^18\),\(^19\) Recently, Lopez-Moya, et al. reported the inhibition of PR and LR development in barley and tomato plants.\(^20\) The same authores demonstrated that CS modulates RSA in Arabidopsis plants through the repression of the transcription factor \textit{WUSCHEL RELATED HOMEobox 5 (WOX5)} which is a major regulator gene of root stem cell activity. \textit{WOX5} repression was associated to alterations in auxin biosynthesis and transport leading to an over accumulation of auxin in the root tip with detrimental impact on root development, suggesting that doses, frequency and formulation of CS should be adjusted to prevent negative effects on plant development. Another point to take into account for the application of CS in the field is its limited solubility in water. The complex behavior of bulk CS on plant physiology as a
consequence of chemically heterogeneous copolymers preparations has slowed down its promissory potential use in agriculture.\textsuperscript{21,22} Therefore, the development of CS particulated systems is an emerging alternative to the complex problems of bulk CS. They are easy to obtain, and also to modify their water solubility and interactive biological ability.\textsuperscript{23} Nevertheless, it is necessary to demonstrate how type, concentration and particle size impact in organs and tissues of plants. We have previously reported the characterization of CS-MPs developed with high molecular weight CS obtained from \textit{Pleoticus mulleri} fishing industry waste from Argentine Sea (Scheme 1). Preliminary assays have shown that CS-MPs stimulate PR elongation in lettuce seedlings suggesting a novelty potential use as rooting agents.\textsuperscript{24} In this work we studied CS-MPs properties as biostimulant of root development compared to bulk CS and the hormonal mechanism by which CS-MPs impact on the modulation of RSA in Arabidopsis. Arabidopsis is suggested as an adequate model for dicot plant root research since its root system fits with the typical eudicot root topography.\textsuperscript{25}

\begin{center}
\textbf{Scheme 1:} Synthesis of CS-MPs.
\end{center}

\section*{MATERIALS AND METHODS}

\subsection*{Plant materials and growth conditions}
\textit{Arabidopsis thaliana} (Arabidopsis) wild-type (WT), \textit{pMSG2/IAA19:GUS}, \textit{DR5:GUS}, \textit{BA3:GUS} and \textit{DII-VENUS} are in the Columbia (Col-0) ecotype.\textsuperscript{26-29} Butterhead lettuce
(Lactuca sativa L) cv. Reina de Mayo seeds were purchased from “El Colono” local seed market, Mar del Plata, Argentina. Arabidopsis and lettuce seeds were surface-sterilized in 30% sodium hypochlorite and 0.2% Tween-20 solution for 10 min, followed by 3 washing steps in sterilized distilled water and stratified at 4 °C for 2-3 d in the dark. Seeds were placed on half-strength Murashige and Skoog medium (½ MS) (SIGMA-Aldrich, USA) plus 0.8% agar in Petri plates and grown vertically at 23 °C under 250 µmol photons m⁻² s⁻¹ with 16:8 h light:dark cycles until analysis.

Chitosan-based materials and treatments

CS-MPs and CS used in this study were described and characterized by Martín-Saldaña, et al.\textsuperscript{24} CS exhibited a Mass average molecular weight (Mw) of 1531 ± 372 kDa, a number average molecular weight (Mn) of 559 ± 95 kDa, a polydispersity index (PI= Mw/ Mn) of 1.95 ± 0.32 determined by gel permeation chromatography and a deacetylation degree (DD) higher than 87% determined by Fourier-transform infrared (FTIR) spectroscopy.\textsuperscript{30} CS-MPs were prepared by the gelation method with modifications using sodium tripolyphosphate (TPP) as crosslinker and have a mean diameter of 2.10 ± 0.78 µm and a PDI of 0.14 determined by scanning electron microscopy (SEM;JEOL JSM-6100) with 15 kV. Samples were previously coated with metallic gold for 30 s with an Auto Sputter Coater 108 (Cressington, England). Micrographs were analyzed with ImageJ software (USA National Institutes of Health, (http://rsb.info.nih.gov/ij/)).\textsuperscript{24,31} CS-MP also present a zeta potential value (ζ) of 27.65 ± 1.22 mV at pH 6.8 determined by a laser particle sizer (Z-sizer 3000 HS, Malvern, UK). Materials were developed and characterized by Gihon Laboratorios Químicos SRL, Argentina. Figure S1 a and b show the morphology of CS-MP by SEM and FTIR spectra and relevant peaks assigned to CS-MP and bulk CS, respectively. FTIR was performed on an IRAffinity-1S FTIR spectrophotometer (Shimadzu, Japan) in the attenuated total reflection mode (ATR-FTIR). To analyze the efficacy of CS-MPs on root growth parameters, the dry CS-MPs were resuspended in water from 0.1 to 100 µg
mL⁻¹. Bulk CS was diluted in 0.1% acetic acid. The pH of each assayed dilution of both bulk CS and CS-MP was in the range of 6.0-6.5.

**Fresh weight, primary root and lateral root measurements**

Five days post-germination (dpg) Arabidopsis and lettuce seedlings were transferred to ½ MS medium supplemented with CS-MPs or CS and grown vertically in a growth chamber at 25 °C under 250 µmol photons m⁻² s⁻¹ with 16:8 h light:dark cycles until analysis. Root and aerial Fresh weights (FWs) were weighed on a laboratory scale (Sartorius, Germany). Seedlings were photographs after 3 d for PR length and after 5 d for LR number and LR length. PR and LR lengths were quantified using the ImageJ image-analysis software (USA National Institutes of Health, http://rsb.info.nih.gov/ij/).

**Measurements of root hair length and density**

Five dpg seedlings were transferred to liquid ½ MS medium supplemented with CS-MPs or CS for 48 h. Bright-field images from Arabidopsis roots were taken using a Zeiss Axioplan imaging 2 microscope with an Axiocam HRC CCD camera (Zeiss, USA) using the Axiovision program (version 4.2). Root hair (RH) density and length were analyzed in a 5 mm section from the beginning of the PR differentiation zone. RH length was analyzed using Image-analysis software (USA National Institutes of Health, http://rsb.info.nih.gov/ij/).

**Root gravitropic assay**

Three dpg seedlings were transferred to fresh ½ MS medium supplemented with 10 µg mL⁻¹ CS-MPs. To ensure homogeneous absorption and action, liquid medium was also poured at the surface of each root. The plates were mounted vertically on a scanner (Epson Perfection V600) and let sit for 60 min. After root gravistimulation, images were taken every 15 min for 8 h. Root growth and tip angle were measured by using FIJI software bundle.

**Treatment of DII-VENUS transgenic sensor plants with CS-MPs**

DII-VENUS Arabidopsis transgenic sensor seedlings were designed to map auxin signaling response at a high resolution in plant cells. Five dpg seedlings were
transferred to fresh plates with the addition of 10 µg mL\(^{-1}\) CS-MPs. Liquid solution of CS-MPs (200 µL) was poured at the surface of each root to ensure homogeneous absorption. Seedlings were grown for 24 h. Fluorescence from VENUS protein was detected in root cells using a 20 x objective, a 0.5 numerical aperture; and 470/40-525/50 nm as excitation and detection in a Zeiss Axioplan imaging 2 microscope with an Axiocam HRC CCD camera (Zeiss, USA). Images were analyzed by using FIJI software bundle.\(^{33}\)

**Glucuronidase (GUS) staining**

Five dpg transgenic \(BA3:GUS\), \(DR5:GUS\) and \(pMSG2/IAA19:GUS\) seedlings were transferred into liquid \(\frac{1}{2}\) MS medium containing 1, 10 or 100 µg mL\(^{-1}\) CS-MPs and then incubated with mild shaking for 24, 48, 72 or 96 h at 23 °C. For \(BA3:GUS\) line, CS-MPs particles were applied together with 100 nM indole acetic acid (IAA) and incubated for 6 h. After treatment, \(BA3:GUS\), \(DR5:GUS\) and \(pMSG2/IAA19:GUS\) seedlings were fixed in 90% acetone for 1 h at 20 °C, washed twice in 50 mM sodium phosphate buffer pH 7.0 and incubated in staining buffer \([50 \text{ mM Na phosphate (pH 7.0), 5 mM EDTA, 0.1% Triton X-100, 5 mM K}_4\text{Fe (CN)}_6, 0.5 \text{ mM K}_3\text{Fe (CN)}_6 \text{ and 1 mg mL}^{-1} \text{ X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) (Gold Biotechnology, USA)}]\) from 2 h to overnight at 37 °C. Bright-field images were taken using a Zeiss Axioplan imaging 2 microscope (Zeiss, USA).

**Measurement of nitric oxide (NO) production.** Five dpg Arabidopsis seedlings were loaded in the dark with 5 mM of the specific NO dye DAF-FM-DA (4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate; Calbiochem, USA) in 20 mM HEPES–NaOH Buffer at pH 7.5 for 30 min. After three washes, seedlings were examined by epi-fluorescence by using a Nikon DS-Fi 1 digital camera coupled to a Nikon Eclipse Ti (Nikon, Japan) epifluorescence microscope (excitation 495 nm; emission 515–555 nm).

**RNA extraction and quantitative real-time RT-qPCR**

Five dpg seedlings were transferred to liquid \(\frac{1}{2}\) MS medium supplemented with increasing concentrations of CS-MPs or 10 µg mL\(^{-1}\) CS and H\(_2\)O as controls. After 24
h, total RNA from Arabidopsis seedlings was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s recommendations. Samples were treated with RQ1 RNase-free DNase (Promega, USA) for DNA contamination removal. For cDNA synthesis, 1 µg of total RNA was reverse transcribed by IMPROM II (Thermo Fisher Scientific, USA) using random primers (Biodynamics, Argentine). The expression of a subset of early auxin response genes was analyzed by Real Time (qPCR), using the following primers: IAA5F: 5´-CCGGAGAAAGAACAGTCTCG-3´; IAA5R: 5´-AGCATCCGAACAGAATTGC-3´; IAA14F: 5´-GAAGCAGAGGAGGCAATGAG-3´; IAA14R: 5´-CCCATGGTAAAGGAGCTGAA-3´; GH3.5F: 5´-CCATCTCTGAGTTCCTCACAAGC-3´; GH3.5R: 5´-TCCTCTGATTGTTGGCATTAGC-3´; GH3.17F: 5´-ACGCAGACCGCTCACATCCAATCCC-3´; GH3.17R: 5´-TGCTGTGACGGCTTTAGC-3´; ACTINF: 5´-GCCATCCAAGCTTCTCTCTC-3´; ACTINR: 5´-GAAACCCTCGTAGATTGGCA-3´. qPCR reactions were conducted in triplicates (95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s) in a Step One real-time PCR system (Applied Biosystems, USA) using FastStart Universal SYBR Green Master Rox (Roche, Germany) following manufacturer’s instructions. Results were normalized to the expression level of the gene actin and expressed as fold-change over controls using the comparative cycle threshold (CT) method. PCR products were analyzed by melting curve analysis to confirm the presence of a single product.

**Statistical Analysis**

The values shown in figures are mean values +/- standard error (SE) of at least 3 experiments. The data were subjected to analysis of t-Test or ANOVA with Dunnet post hoc comparisons against control by Graphpad Prism version 5.01 software (*p<0.05 **p<0.01 *** p<0.001).
CS-MPs modulate root architecture in Arabidopsis and lettuce plants

To deep on the potential action of CS-MPs on RSA, we performed a complete analysis of root growth parameters. PR elongation, LR and RH development were studied in 5 dpg Arabidopsis seedlings transferred to ½ MS medium supplemented with 0.1, 1, 10 and 100 µg mL\(^{-1}\) CS-MPs, or CS as control. After 3 d, seedlings grown in 1 µg mL\(^{-1}\) CS-MPs supplemented medium evidenced the higher and significant increment in PR length compared to untreated seedlings (Figure 1 a). In contrast to CS which at higher doses (10 and 100 µg mL\(^{-1}\)) severely arrested PR elongation, MP-CSs did not show a detrimental effect on PR length in a wide range of assayed doses (Figures 1a and e). In addition, supplementation of the growing medium with 1 and 10 µg mL\(^{-1}\) CS-MPs resulted after 5 days of treatment in a 40% and 60% of increment in the number and length of LRs, respectively compared with control (Figures 1b and c). The promotion of LR development was accompanied by slight reduction in PR elongation (Figure 1e). Again, CS-MPs showed a better performance on LR development compared with bulk CS treatment (Figures 1b and c). In addition to PR inhibition, seedlings exposed to the highest doses of CS evidenced a reduce number of LR, while no cytotoxicity was detected under CS-MPs treatments at the studied concentrations (Figures 1b and e). The reduced cytotoxicity of CS-MPs could be explained by the fact that CS-MPs present a reduce number of exposed -NH\(^3+\) positive charges which modified the interactive ability with cell membranes.\(^{35}\)

In concordance, the rearrangement of RSA triggered by 1 and 10 µg mL\(^{-1}\) CS-MPs resulted in approximately 25% improvement on root and aerial FW after 9 d of treatment suggesting a positive effect on plant biomass (Figure 1d). Next, we also analyzed the effect of selected CS-MPs concentrations on RH development (Figure 2). Again, due to its emerging physico-chemical properties, CS-MPs at 1 and 10 µg mL\(^{-1}\) resulted in an increment of Arabidopsis RH density (Figure 2a), and RH length (Figure 2b), while no positive effect was detected under 1 µg mL\(^{-1}\) CS treatment. To analyze if the gravitropism as a key root growth process is affected by CS-MPs, the root tip angle
was quantified after turning the 3 dpg Arabidopsis seedling 90 degrees as described in Paris, et al. However, compared with control, CS-MPs treated seedlings did not show changes in root bending, suggesting that CS-MP has specific cell-tissue action (Figure S2). In addition, CS-MPs also promote root development in lettuce (Figure S3). These findings demonstrate that compared with bulk CS, CS-MPs resulted in an improved material to enhance early root growth in Arabidopsis and lettuce seedlings (Figures 1, 2 and S3).

Figure 1. CS-MPs promote root development in Arabidopsis. Five dpg Col-0 Arabidopsis seedlings grown in 1/2 MS medium were treated with increasing concentrations of CS-MPs or CS as control. PR elongation (a) was quantified 3 d post-treatment. LR number (b) and LR length (c) were analyzed 5 d post treatment. Seedlings FW was quantified 9 d post-treatment (d). Representative images 9 d post...
treatments are shown in (e). Data are mean values of 5 independent experiments (n=60; ANOVA, Dunnet post-hoc test against control,* p<0.05 ** p<0.01 ***p<0.001).

Figure 2. Promotion of RH upon CS-MPs exposure. Five dpg Col-0 Arabidopsis seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of CS-MPS for 48h. RH density (a) and RH length (b) were analyzed in a 5 mm section from the beginning of the differentiation zone. Representative images are shown in (c). Data are mean values of 4 independent experiments (n= 30; ANOVA, Dunnet post-hoc test against control, * p< 0.05).

**Auxin response is activated by CS-MPs in Arabidopsis roots**

Since auxin is a key regulator of root growth and development, we studied if the modulation of auxin signaling constitutes a mechanism of action downstream MP-CSs application in Arabidopsis plants by using the auxin reporter transgenic seedlings, _BA3:GUS_ and _DR5:GUS_. These lines consist of artificial promoters based on auxin response elements which drive the expression of GUS gene. The activation or repression of auxin response is correlated with GUS activity levels.27,28
Figure 3. Differential effect of CS-MPs and CS on auxin BA3:GUS reporter gene activity. Five dpg Arabidopsis BA3:GUS seedlings grown in 1/2 MS medium were treated with 100 nM IAA and increasing concentrations of CS-MPs or CS. Seedlings were subjected to GUS staining after 5 h of treatment. Representative images of PR tips are shown.

We studied the effects of CS and CS-MPs on the early activation of auxin response analyzing BA3:GUS activity on the tip of PR of 5-dpg seedlings treated with the natural auxin IAA, in combination with increasing concentrations of CS or CS-MPs for 6 h. While bulk CS repressed BA3 auxin-responsive promoter activity in a dose-dependent manner, CS-MPs did not show an effect on auxin response at all analyzed doses (0.1, 1, 10 and 100 µg mL⁻¹) in an early period of treatment (Figure 3). Repression of auxin response by CS correlated with the cytotoxicity effects on root growth shown in Figure 1e. However, analyzing auxin response after 24 h of treatment, 1 and 10 µg mL⁻¹ CS-MPs triggered the activation of DR5 auxin responsive promoter in the tip of PR of 5 dpg DR5:GUS seedlings (Figures 4a and b). In concomitance with CS-MPs action on LR (Figure 1), an increment in the number of LR primordia showing DR5 activity was detected in 1 and 10 µg mL⁻¹ CS-MP treated seedlings after 48 h of treatment (Figures 4c and d). In order to evaluate the dynamics of LR induction, a time-course analysis of stained DR5:GUS roots was performed. Figure 4e shows statistically higher and faster induction of lateral root development by CS-MPs since CS-MPs treated seedlings...
showed an increased number of GUS-stained LR primordia compared with control after 48 h and 72 h treatment. However, after 96 h no significant difference between treatments was found. This temporarily advance in auxin activation was also observed in PR where CS-MPs treated seedlings reached similar activation of DR5 promoter than control 48 h post-treatment (Figure 4b). The early and sustained activation of auxin response activity in DR5 reporter line fits with the promotion of root growth and development described in Figure 1.

**Figure 4. CS-MPs promote an early activation of auxin response in Arabidopsis roots.** Five dpg *DR5:GUS* seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of MP-CS. GUS activity was revealed after incubation with X-Gluc at 37 °C. GUS activation in PR was analyzed 24 h post-treatment (a). GUS staining in representative root tip segments after 24 h and 48 h is shown in b. Stained LR primordia were quantified after 48h (c). GUS staining in representative root segments of the differentiation zone are shown in (d). Time-course analysis of stained primordia following 48, 72 and 96 h of treatment with 10 μg mL\(^{-1}\) is shown in (e). Data are mean values of 5 independent experiments (n= 60; ANOVA, Dunnet post-hoc test,* p< 0.05 ** p<0.01).
CS-MPs trigger the activation of nuclear auxin signaling pathway

To add evidence on the activation of the nuclear auxin signaling pathway, we analyzed the level of fluorescence emitted by the auxin sensor DII-VENUS in root cells from control and CS-MPs- treated plants. This auxin reporter line has been engineered to allow the detection of dynamic changes in the levels of Aux/IAA auxin repressor associated to a sensitive activation of nuclear TIR1/AFBs dependent auxin pathway. DII-VENUS sensor is rapidly degraded resulting in a decrease of the fluorescence when the auxin pathway is activated. A decrease in the DII-VENUS fluorescence signal was detected in the nucleus of epidermic cells in PR and LR of 10 µg mL⁻¹ CS-MPs-treated seedlings compared to control (Figures 5a and b).

Figure 5. CS-MPs enhance auxin sensitive in DII-VENUS Arabidopsis seedlings.

Five dpg DII-VENUS seedlings were transferred to liquid ½ MS medium supplemented with 10 µg mL⁻¹ CS-MPs (a) DII-VENUS expression (green) in PR of control and CS-MPs treated seedlings. Bright field images (BF). (b) Quantification of total DII-VENUS signal and DII-VENUS positive nuclei number per root. Box plot showing median, minimum and maximum values of 2 independent experiments (n= 24). P-values in
comparison to control were calculated with two-tailed Student's t-test, \(*p \leq 0.05\) \(**p \leq 0.01\). Scale bars, 50 μm.

Next, the expression of a set of auxin-response genes, _Aux/IAAs_ (IAA5 and IAA14) and _GH3s_ (GH3.5 and GH3.17) was analyzed upon 1, 10 and 100 μg mL\(^{-1}\) CS-MPs treatment by quantitative qPCR (Figure 6). It is known that auxin response genes have differential patterns of expression under auxin stimuli.\(^{37}\) Although each gene evidenced a particular expression pattern, most of them were up-regulated in seedlings exposed to 1 and 10 μg mL\(^{-1}\) CS-MPs 24 h post treatment (Figures 6a-d). _MSG2/IAA19_ constitutes an early auxin response gene associated to root development.\(^{26}\)

Therefore, the activation of IAA19 promoter in roots of _pMSG2/IAA19:GUS_ reporter line was analyzed in 1 and 10 μg mL\(^{-1}\) CS-MPs treated seedlings (Figure 6e). Both concentrations of CS-MPs led to a significant increment of GUS staining in the PR (Figure 6f).
Figure 6. CS-MPs promote the activation of early auxin response genes in Arabidopsis seedlings. Five dpg Col-0 seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of CS-MPs or CS as control. (a-d) show the expression of a subset of early auxin response genes analyzed by qPCR after 24 h of CS-MPs treatment. (e) GUS activity in representative PR of 5 dpg MSG2/IAA19:GUS Arabidopsis seedlings treated with 1 and 10 μg mL⁻¹ CS-MPs for 24 h. Quantification of GUS staining in representative root tip segments is shown in (f). Data are mean values of 3 independent experiments (n= 30; ANOVA, Dunnet post-hoc test against control,* p< 0.05 ** p<0.01).

DISCUSSION

CS action seems to be complex in the dynamic and versatile modulation of plant developmental programs since a narrow change from optimal concentrations can lead from a promotion of growth to detrimental effects on plant biomass. CS-MPs
modulate RAS inducing an early promotion of PR elongation, and a subsequent increment in the number and elongation of LRs and RHs compared to untreated seedlings root architecture in Arabidopsis and lettuce plants (Figures 1, 2 and S3). CS-MPs exert its positive effect in a wide range of concentrations (1-10 µg mL⁻¹), resulting in a very beneficial property to recommend doses of application in the field. This fact also represents improved properties compared to bulk CS and CS-nanoparticles recently described which inhibit root growth or alternatively induce root growth at specific doses showing cytotoxicity at higher concentration in several plant species including Arabidopsis thaliana, Solanum lycopersicum, Hordeum vulgare, Capsicum annuum and Ipomoea purpurea treated with the same range of concentrations than in our study. Main differences in our results could be attributed to physico-chemical properties of CS that become drastically changeable according to the biological sources and the synthesis methods. In our study CS exhibited a Mw of 1531 ± 372 kDa which is significantly higher than CS used in other published papers. Interestingly, the interaction between TPP⁻ and -NH₃⁺ groups of CS during the formation of the microstructure organization by the gelation method, confers new properties to the CS-MPs if compared to bulk CS macrostructure. CS present biological properties associated to its cationic nature under acidic pH. The protonated amino groups of the glucosamine could interact by electrostatic interactions with anionic groups of the lipids of cell membrane, causing impairment on its physicochemical equilibrium. In CS-MP, these charged groups are partially neutralized and allow reorganization of the polymeric chains providing new properties to the CS-MP, which might have different interactions between the remaining free cationic groups and the cells. Assuming that the crosslinking efficiency is not 100% (TPP ratio was only 10% in the CS-MP), these remaining positively charge amino groups could interact with plasma membrane phospholipids as well as chelation of metal elements. Therefore, this new microstructure increases the surface of contact reducing the exposed -NH₃⁺ positive charges of CS which could disrupt cell membrane potential. In addition, CS-
MPs present a regular shape and medium size around 2.10 μm which favors a better interaction with cells if compared with bulk CS. Then, the polymeric microstructure properties of CS-MPs make them an improved material for root growth promotion in a wider range of application doses compared with bulk CS.

In addition, multiple evidences from genetic, molecular, and cellular approaches demonstrate the relevance of maintaining auxin gradients which ensure a proper activation of TIR1/AFBs-dependent auxin signaling during root development in Arabidopsis. It was recently reported that Arabidopsis seedlings react to sensible changes in auxin concentrations by extremely rapid adaptation of root growth rate. Therefore, compounds which exert an effect on auxin metabolism should be well characterized prior application. The fact that CS is able to induce a rapid and strong accumulation of auxin in Arabidopsis fits with the reports where plant growth is affected by it application. Although we used a CS of higher Mw than these authors, our results also demonstrated that CS triggers auxin signaling repression (Figure 3). However, CS-MPs unchain an accurate and coordinated spatio-temporal induction of the nuclear auxin signaling in CS-MPs-treated seedlings evidenced by the activation of DR5 promoter, the repression of DII-VENUS activity, and the expression of early auxin response genes, Aux/IAAs and GH3s (Figures 4, 5 and 6). A counter balance of nitric oxide (NO) concentrations appears to be essential for the control of the auxin action during root growth and development. The induction of NO levels by CS-MP (Figure S4) could contribute to the enhancement in auxin sensitivity which promotes root growth. NO exert its action, in part, through the S-nitrosylation of multiple components of the nuclear TIR1-dependent auxin signaling. Despite the differences in the dimensions of the particles with an average diameter of 90 ± 5 nm compared to 2.10 ± 0.78 μm exhibited by the particles described in this work and in concordance with our results, Chandra, et al. demonstrated that CS nanoparticles also induce NO accumulation in addition to antioxidant enzymes as part of the defense response mechanism in Camellia sinensis tea plants suggesting that particles might mediate
different physiological processes sharing, at least partially, the same signaling
mechanisms.

In addition, main differences in our results and previously reported papers could be
attributed to physicochemical properties of CS including the molecular weight since it
has been suggested that it has more influence on the biological activity than the DD. 46

The electrostatic interaction between TPP and -NH3+ groups of CS during the
formation of the MP allow to a new organization of the molecules of the polymer and
also a new way to exhibit the cationic charges reflected in the \( \zeta \) of the CS-MP. This
new conformation confers new properties to the material if compared to bulk CS
macrostructure. This microstructure increases the surface of contact when compared to
bulk CS reducing the exposed -NH3+ positive charges of CS which could disrupt cell
membrane potential. 24

Although auxin is considered an omnipotent regulator of root development cytokinin
and jasmonate hormonal pathways and the crosstalk auxin-ethylene have been
extensively described in the regulation of LR initiation, emergence and positioning in
Arabidopsis. 47,48 The fact that the application of CS-MPs to the double mutant in the
auxin receptors TIR1 and AFB2, \( \text{tir1afb2} \) was able to promote root FW and the number
and length of LRs (Figure S5) suggests that CS-MPs enhance the sensitivity of
remaining auxin receptors of TIR1/AFBs family or alternatively that CS-MPs exert its
action through additional pathways.

Curiously, CS-MPs-induced phenotype resembles RSA of plants exposed to soil with
low phosphate (Pi) availability where modulation of auxin sensitivity leads to
augmented density and length of LRs and RHs.49 However, CS-MPs enhanced root
branching without a drastic effect on aerial organs in contrast to plants grown under low
Pi which allocate more carbon to roots increasing their root-to-shoot ratio.50 Due to the
relevance of soil Pi level for crop yield, Ham, et al. proposed the bio-engineering of
agricultural species for improved Pi acquisition and utilization in plants. 51 Although the
mode of action of CS has not been completely deciphered yet, CS exhibits several reactive amino side groups which enhance its applicability. For instance, it has been demonstrated that CS stimulates the activity of plant symbiotic microbes affecting the homeostasis of microbial rhizosphere and also promoting the nutrient uptake by plant. Then, CS-MPs could participate in the modulation of Arabidopsis root interphase and/or the microbiome and associated mineral nutritional compounds. In this context, new CS biomaterials with improve biological performance like CS-MPs may constitute an overcome alternative to transgenic plants for the promotion of plant growth under soil with nutrient deficiency. However, further studies are necessary in order to decipher the cellular uptake and biodistribution of CS-MPs in root cells.

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ABBREVIATIONS
RSA, root system architecture; CS, chitosan; CS-MPs, chitosan microparticles; DPG, days post-germination; FW, fresh weight; IAA, Indole acetic acid; NO, nitric oxide; LR, lateral root; PR, primary root; RH, root hair; SE, standard error; TPP, sodium tripolyphosphate;

SUPPORTING INFORMATION CONTENT

Figure S1. Characterization of CS-MP by SEM and FTIR.

Figure S2. Analysis of CS-MPs effect on root gravitropism in Arabidopsis.

Figure S3. Analysis of CS-MPs effect on root development in lettuce.
Figure S4. Analysis of CS-MPs effect on nitric oxide accumulation in Arabidopsis roots.

Figure S5. Analysis of CS-MPs effect on root development in tir1afb2 double mutant.

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Figure 1. CS-MPs promote root development in Arabidopsis. Five dpg Col-0 Arabidopsis seedlings grown in ½ MS medium were treated with increasing concentrations of CS-MPs or CS as control. PR elongation (a) was quantified 3 d post-treatment. LR number (b) and LR length (c) were analyzed 5 d post treatment. Seedlings FW was quantified 9 d post-treatment (d). Representative images 9 d post treatments are shown in (e). Data are mean values of 5 independent experiments (n= 60; ANOVA, Dunnet post-hoc test against control,* p< 0.05 ** p<0.01 ***p<0.001).

252x236mm (150 x 150 DPI)
Figure 2. Promotion of RH upon CS-MPs exposure. Five dpg Col-0 Arabidopsis seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of CS-MPS for 48h. RH density (a) and RH length (b) were analyzed in a 5 mm section from the beginning of the differentiation zone. Representative images are shown in (c). Data are mean values of 4 independent experiments (n= 30; ANOVA, Dunnet post-hoc test against control, * p< 0.05).
Figure 3. Differential effect of CS-MPs and CS on auxin BA3:GUS reporter gene activity. Five dpg Arabidopsis BA3:GUS seedlings grown in ½ MS medium were treated with 100 nM IAA and increasing concentrations of CS-MPs or CS. Seedlings were subjected to GUS staining after 5 h of treatment. Representative images of PR tips are shown.
Figure 4. CS-MPs promote an early activation of auxin response in Arabidopsis roots. Five dpg DR5:GUS seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of MP-CS. GUS activity was revealed after incubation with X-Gluc at 37°C. GUS activation in PR was analyzed 24 h post-treatment (a). GUS staining in representative root tip segments after 24 h and 48 h is shown in b. Stained LR primordia were quantified after 48h (c). GUS staining in representative root segments of the differentiation zone are shown in (d). Time-course analysis of stained primordia following 48, 72 and 96 h of treatment with 10 μg mL⁻¹ is shown in (e). Data are mean values of 5 independent experiments (n= 60; ANOVA, Dunnet post-hoc test, * p< 0.05 ** p< 0.01).
Figure 5. CS-MPs enhance auxin sensitive in DII-VENUS Arabidopsis seedlings. Five dpg DII-VENUS seedlings were transferred to liquid ½ MS medium supplemented with 10 μg mL⁻¹ CS-MPs (a) DII-VENUS expression (green) in PR of control and CS-MPs treated seedlings. Bright field images (BF). (b) Quantification of total DII-VENUS signal and DII-VENUS positive nuclei number per root. Box plot showing median, minimum and maximum values of 2 independent experiments (n= 24). P-values in comparison to control were calculated with two-tailed Student's t-test, *p ≤ 0.05 **p ≤ 0.01. Scale bars, 50 μm.
Figure 6. CS-MPs promote the activation of early auxin response genes in Arabidopsis seedlings. Five dpg Col-0 seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of CS-MPs or CS as control. (a-d) show the expression of a subset of early auxin response genes analyzed by qPCR after 24 h of CS-MPs treatment. (e) GUS activity in representative PR of 5 dpg MSG2/IAA19:GUS Arabidopsis seedlings treated with 1 and 10 μg mL⁻¹ CS-MPs for 24 h. Quantification of GUS staining in representative root tip segments is shown in (f). Data are mean values of 3 independent experiments (n=30; ANOVA, Dunnet post-hoc test against control, * p<0.05 ** p<0.01).

251x235mm (150 x 150 DPI)
Scheme 1: Synthesis of CS-MPs

219x137mm (150 x 150 DPI)