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Research article

Chitosan microparticles improve tomato seedling biomass and modulate hormonal, redox and defense pathways

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ABSTRACT

Agrobiotechnology challenges involve the generation of new sustainable bioactives with emerging properties as plant biostimulants with reduced environment impact. We analyzed the potential use of recently developed chitosan microparticles (CS-MP) as growth promoters of tomato which constitutes one of the most consumed vegetable crops worldwide. Treatments of tomato seeds with CS-MP improved germination and vigor index. In addition, CS-MP sustained application triggered an improvement in root and shoot biomass reinforcing tomato performance before transplanting. The level of reactive oxygen species (ROS), antioxidant enzyme activities and defense protein markers were modulated by CS-MP treatment in tomato plantlets. Analyses of *ARR5:GUS* and *DR5:GUS* transgenic reporter tomato lines highlighted the participation of cytokinin and auxin signaling pathways during tomato root promotion mediated by CS-MP. Our findings claim a high commercial potential of CS-MP to be incorporated as a sustainable input for tomato production.

1. Introduction

Tomato (*Solanum lycopersicum*) constitutes a horticultural commodity cultivated at almost all latitudes being one of the most consumed vegetable crops due to its nutritional properties (http://www.fao.org/). Most tomatoes are firstly grown in greenhouse for approximately 5 weeks before being transplanted into the field in order to promote an adequate germination and root establishment. Frequently, biostimulants and starter solutions are applied to promote rapid root development and early plant growth in the soil (Maynard and Hochmuth, 2006). An improvement in the first stages of growth and development can also enhance the plant fitness improving later crop productivity (Servin et al., 2015). However, the type and doses of the starter solution as well as its application should be carefully controlled since it can also generate damages to the plant and even to the soil microbiota. In this context, there is great interest in the development of polymeric-based particulated biomaterials which fit with the actual challenge of augmenting the growth, yield and nutritional quality of crops with a reduced environment impact (Tilman et al., 2011; Tittonell, 2014).

Chitosan (CS) is a polysaccharide composed by β-1,4-linked glucosamine and N-acetylglucosamine residues derived from chitin with special characteristics including biocompatibility, biodegradability, ubiquity and relative low cost (Malerba and Cerana, 2018; Pirbalouti et al., 2017). In addition, CS represents the most widely used polymer for particulated drug delivery systems (Kashyap et al., 2015). Evidences in multiple horticulture species have suggested an active role of bulk CS in the protection of plants against stress due to its properties as biocide as well as plant elicitor (Iriti and Varoni, 2015; Mansilla et al., 2013; Yin et al., 2010). In many cases, CS mediated plant protection against environmental stresses involved the induction of ROS and reactive nitrogen species (RNS) through the modulation of enzymes from the ROS scavenging system including catalase (CAT), superoxide dismutase (SOD) and peroxidases (Kumaraswamy et al., 2018). However, ROS and

Abbreviations: CS, chitosan; CS-MP, chitosan microparticles; Mw, molecular weight; DD, deacetylation degree; DW, dry weight; FW, fresh weight; IAA, Indole acetic acid; SE, standard error; TPP, sodium tripolyphosphate; PRs, pathogenesis related proteins; ROS, reactive oxygen species; RNS, reactive nitrogen species

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RNS also act in a delicate balance as signal transduction molecules that crosstalk with hormonal pathways in order to modulate plant growth and development (Turkan, 2018). CS action on the dynamic and versatile regulation of plant growth seems to be delicate since small changes of concentrations can lead from a biostimulant effect on plant biomass to citotoxicity effects including plant growth arrest (Pichyangkura and Chadchawan, 2015). The fact that bulk CS induces a strong and rapid accumulation of different phythormones such as, abscisic acid, jasmonic acid, salicylic acid and auxin could explained at least in part, why plant growth is extremely senstive to it treatment (Asgari-Targhi et al., 2018; Lopez-Moya et al., 2017; Pichyangkura and Chadchawan, 2015). In turn, the heterogeneous response of bulk CS on plant physiology in addition to the limited solubility in water which difficult its application in the field has led to design CS-based micro and nanoparticles with emerging properties compared to CS on plant biological activity. CS- based particles properties are often affected by different factors including CS molecular weight (Mw), degree of deacetylation (DD), concentration and crosslinking to the matrix (Sreekumar et al., 2018). Previously, we characterized novel CS-based microparticles (CS-MP) prepared with CS derived of chitin of *Pleoticus mulleri* exoskeletons obtained from Argentine fishing industry (Martín-Saldaña et al., 2018). The particles obtained had a mean diameter of 2.10 ± 0.78 μM and a PDI 0.14 and were prepared with a CS of high Mw of 1531 ± 372 kDa and DD ≥ 85%. In the plant model Arabidopsis, CS-MP promoted *in vitro* root growth and development suggesting a novel potential use as root promoting agent (Iglesias et al., 2019). Supporting our findings, Asgari-Targhi et al. (2018) described that nano-chitosan/TPP particles promote root organogenesis in the micropropagation of *Capsicum annuum L* plantlets. In this work, we studied the efficacy of CS-MP as a growth promoter agent of tomato identified as one of the most consumed horticultural crop worldwide. Our findings highlighted a very promising scenario for the application of CS-MP for the improvement of tomato productivity.

2. Materials and methods

2.1. Biological materials

Seeds of *S. lycopersicum* L. cv. Platense which is one of the most traditional and commercially cultivated tomatoes in Argentina were obtained from FECOAGRO Ltda., San Juan, Argentina. Seeds of tomato (*S. lycopersicum*, cv. Micro-Tom) hormone transgenic lines *DR5:GUS* and *ARR5:GUS* were kindly donated by Dr. Agustin Zsögön (Universidade Federal de Viçosa, Brazil). *DR5:GUS* tomato plants express the reporter gene beta-glucuronidase (*GUS*) under a synthetic auxin-responsive promoter (Silva et al., 2018). *ARR5:GUS* tomato plants express GUS under an Arabidopsis cytokinin-responsive promoter. *ARR5:GUS* tomato plants were generated by the laboratory of Dr. Lázaro Eustáquio Pereira Peres (Universidade de São Paulo, Brazil).

2.2. CS-MP preparation

CS and CS-MP used for plant treatments were recently described and characterized by Martín-Saldaña et al. (2018). CS was obtained by Gihon Laboratorios Químicos SRL (Mar del Plata, Argentina) from shrimp fishing industry wastes through three step based method: demineralization, deproteinization and deacetylation.

The Mw and number-average molecular weight (Mn) of CS, determined by size exclusion chromatography, were 1531 ± 372 kDa and 559 ± 95 kDa, respectively. The polydispersity index (PDI) was 1.95 ± 0.32 (Martín-Saldaña et al., 2018).

The DD, determined by Fourier-transform infrared spectroscopy, was higher than 87%. The CS-MP were prepared by the ionic gelation method (Cerchiara et al., 2015) with modifications using TPP as crosslinker. The size and morphology of CS-MP were studied by Scanning Electronic Microscopy and the mean diameter was 2.10 ± 0.78 μm with a PDI 0.14.

To analyze the effect of CS-MP on tomato seedlings the dry CS-MP were solubilized in sterile water from 0.01 to 1 mg mL^{-1}. Bulk CS was dissolved in 0.1% acetic acid and then diluted at final concentrations. Final dilutions of CS-MP and CS evidenced a pH the range of 6.0–6.5.

2.3. Measurements of germination and vigor index in seedlings

Tomato seeds from cv. Platense were incubated in 0.01, 0.1 and 1 mg mL^{-1} CS-MP and CS or H_2O as control at 25 °C for 2 h. After treatments, seeds were placed in Petri dishes on filter paper soaked in sterile water and incubated in growth chamber at 25 °C with 16:8 h light:dark cycles. Seed germination percentage (% germination) and vigor index were calculated after 6 and 10 days, respectively. Vigor index was calculated on percentage of germination and mean of root and shoot lengths according to the following equation: Vigor index = (mean root length + mean shoot length) * % germination (Siddaiah et al., 2018).

2.4. Experimental design and treatments

Tomato seeds from cv. Platense were sowed in cell flats (10 × 20) containing Grow Mix Multipro (Terrafertil, Argentina) as substrate and incubating at 25 °C for germination. Then, seedlings were cultivated at 25 °C under 250 μmol photons m^{-2} s^{-1} with 16:8 h light:dark cycles daily watered until harvested. All leaves from 11 day old tomato seedlings were sprayed on the upper surface with different concentrations of CS-MP and CS (0.01, 0.1 and 1 mg mL^{-1}) or H_2O as control. Acetic acid control is included in Supplemental Fig. 1. The commercial starter solution Inicium (Bioiberica, Spain) was used as a positive control. According to our experimental design, each mentioned solution was applied in 3 times and at indicated time random samples of entire seedlings were harvested (Fig. 1).

2.5. Measurements of growth parameters: biomass and leaf area

Seedlings were photographed with a camera (Nikon Coolpix T80, Indonesia) and area of the second leaf of each seedling was measured using the ImageJ image-analysis software (National Institutes of Health, USA). Chlorophyll content was measured with a chlorophyll meter (SPAD-502, Minolta, Japan). Seedlings were cut on the surface of the substrate, and fresh weight (FW) of roots and shoots (stem and leaves) were measured on a laboratory scale (Sartorius, Germany). Dry weight (DW) of roots and shoots were obtained after samples were dried in a drying oven for 3 days at a constant temperature of approximately 75 °C (Hernández-Hernández et al., 2018).

2.6. H_2O_2 and anthocyanins measurements

Sixteen day old seedlings were sprayed with 0.1 mg mL^{-1} CS-MP, CS and H_2O and after 24 h approximately 0.5 g of tissue from first and second leaf were harvested. To quantify H_2O_2, leaf tissue was ground with liquid N_2 and extracted with H_2O for 30 min in the dark, followed by centrifugation at 10,000 g for 20 min. The H_2O_2 content was quantified in each supernatant according to Bellincampi et al. (2000). For anthocyanins, tissue was extracted with 1% v/v HCl in methanol, stirring for 1 min and incubated at 4 °C for 2 h in the dark. Monomeric anthocyanins 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 34,300 cm^{-1} mol^{-1} and Mw of 449.2 g mol^{-1}.

2.7. Protein extraction and quantification of enzymatic activities

In the same assay performed to quantify redox compounds, SOD and

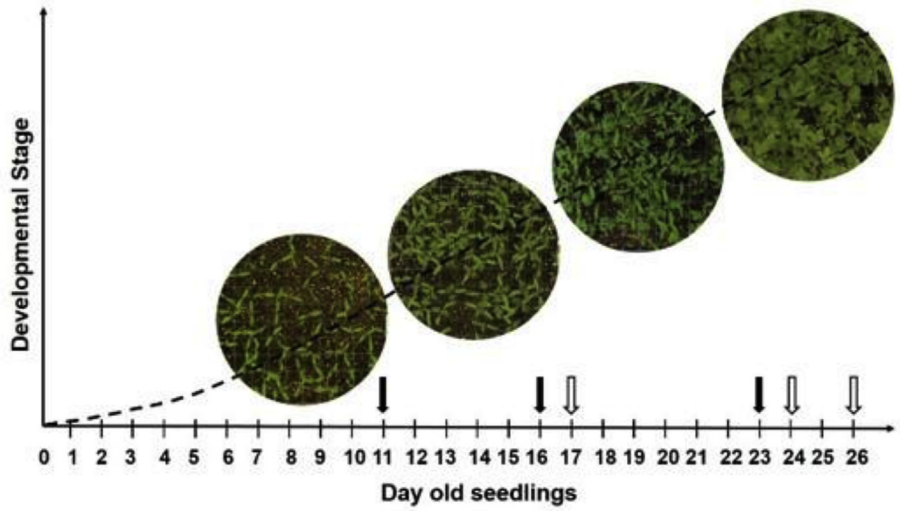

Fig. 1. Experimental design. Tomato seedlings were sprayed with 0.01, 0.1 and 1 mg mL^{-1} of CS-MP, bulk CS and H_2O or 0.1% Inicium as negative and positive controls, respectively, in three times (black arrows) along 26 days during the growth of tomato seedlings. White arrows indicate the time when random samples of entire seedlings were taken for determinations.

CAT activities were measured in leaf tissue samples extracted with 3 vol of buffer (50 mM Tris-HCl buffer, pH 7.5, 3 mM $MgCl_2$, 1 mM ethylene diamine tetra acetic acid, and 1.5% polyvinylpyrrolidone). The homogenate was then centrifuged at 25,000 g for 20 min, and the supernatant was used to measure the antioxidant enzymes.

CAT activity was quantified according to Beers and Sizer (1952) with modifications. The reaction mixture contained 600 µl of 50 mM phosphate buffer pH 7.4, 100 µl of 1% v/v H_2O_2 and 25 µl of enzyme extract diluted to keep measurements within the linear range of the analysis. A blank was used for each sample, which contained 50 mM phosphate buffer and 25 µl of enzyme extract. The decrease in H_2O_2 was followed as a decline in absorbance at 240 nm in a spectrophotometer (GeneQuant 1300, GE Healthcare, USA).

SOD activity was measured according to Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM phosphate buffer pH 7.8, 75 µM Nitro blue tetrazolium chloride (NBT), 2 µM riboflavin, 13 mM methionine, 0.1 mM EDTA and 2 µl of protein sample. Enzymatic reactions were carried out at 37 °C for 15 min in a water-bath fitted with a 22 W Phillips fluorescent lamp. The absorbance was measured at 550 nm using a microplate reader (ELX 800, Biotek, USA). One unit of SOD activity was defined as the amount of enzyme that produced a 50% decrease, with respect the control, in the absorbance at 550 nm and it was expressed as Activity of SOD (U mL^{-1}) = ($A_{control}$ * $A_{assay}/A_{control}$) * (1/50%) * (V_{total}/V_{SOD}). Where $A_{control}$ and A_{assay} are the absorbance units at 550 nm of control and sample, respectively; V_{total} is the total volume; and V_{SOD} is the enzyme volume.

2.8. Protein gel blots and densitometry

Random samples of all leaves were taken 16 h after the third spray with treatments (0.01, 0.1 and 1 mg mL^{-1} of CS-MP and CS or H_2O as control) to evaluated the abundance of PR2 and PR3 proteins. For western blots, total proteins were extracted from 100 mg of tissue in protein extraction buffer (Agrisera, Sweden) supplemented with the protease inhibitor cocktail (Nº P9599 Sigma Aldrich, USA). After centrifugation at 12,000 g for 15 min, supernatant was collected and boiled for 5 min in SDS-PAGE Laemmli sample buffer. Protein content was determined by Bicinchoninic acid (Smith et al., 1985) using a bovine serum albumin calibration curve.

Total proteins were run on 12% SDS-PAGE according to Laemmli (1970) using the Miniprotean 185 II vertical gel system (BioRad, USA) and then electrotransfered onto nitrocellulose membranes (BioRad, USA). Membranes were incubated with 1:5000 of AS07 208 and AS07 207 (Agrisera, Sweden) overnight followed by incubation with 1:7000 anti-rabbit secondary antibody conjugated to alkaline phosphatase (Invitrogen, USA). Each antibody specifically detects PR2 and PR3 proteins, respectively. Both PR2 and PR3 are pathogenesis related proteins (PR) currently used as plant defense markers (Sudisha et al., 2012). The abundance of PR2 was measured at 16 h and 40 h after treatment. Reactions were developed with tetrazolium blue 5-Bromo-4-Chloro-3-indolyl phosphate as substrates according to the supplier's instructions (Sigma-Aldrich, USA). All immunoblotting experiments were performed in triplicate. Densitometry analysis of protein abundance was performed using ImageJ software (USA National Institutes of Health) and protein expression levels were normalized to the median of each experiment. Ponceau staining was used as equivalent loading control in each lane of the gel.

2.9. Analyses of ARR5:GUS and DR5:GUS tomato reporter seedlings

ARR5:GUS and *DR5:GUS* Micro-Tom seeds were surface-sterilized in 30% v v^{-1} hypochlorite and 0.2% v v^{-1} Tween-20 solution for 10 min followed by 3 washing steps in sterilized distilled H_2O. Sterilized seeds were placed on Petri dishes containing half-strength Murashige and Skoog medium (½ MS medium) at 25 °C under 250 µmol photons m^{-2} s^{-1} with 16:8 h light:dark cycles. Five day old *ARR5:GUS* and *DR5:GUS* seedlings were transferred to liquid ½ MS medium supplemented with 0.1 and 1 mg mL^{-1} CS-MP for 24 h under same growth conditions. Seedlings were removed and fixed in 90% acetone v v^{-1} for 1 h at 20 °C, washed twice with 50 mM sodium phosphate buffer pH 7.0 and incubated in staining buffer [50 mM Na phosphate (pH 7.0), 0.1% Triton X-100, 5 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 1 mg mL^{-1} X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt, Gold Biotechnology, USA)] as substrate at 37 °C for 2 h to overnight. Images were taken using a scanner (Epson Perfection V600 Photo, Indonesia). GUS staining levels were normalized to the median of each experiment.

2.10. Statistical analysis

The values shown in Figures are mean values ± standard error (SE) of at least 3 experiments. The experimental design consisted in three trials in randomized blocks with two replications. The data were subjected to analysis ANOVA with Dunnet or Tukey post hoc comparisons against control by R Studio Team (2015). Germination percentage was evaluated by generalized linear mixed model with binomial distribution.

Table 1
Effect of MP-CS and bulk CS on seed germination percent and vigor index in tomato.

Treatment	Concentration (mg mL^{-1})	Germination (%)	Seedling vigor
Control	Untreated	73 ± 2.9 [a]	3713 ± 136 [a]
MP-CS	0.01	76 ± 1.9 [a]	4802.27 ± 289 [b]
	0.1	87 ± 2.2 [b]	5127.6 ± 271 [b]
	1	80 ± 3.4 [a]	4255.8 ± 134 [a]
CS	0.01	72 ± 3.7 [a]	5581 ± 125 [b]
	0.1	68 ± 2.1 [a]	5077 ± 180 [b]
	1	72 ± 2.4 [a]	4511 ± 68 [a]

3. Results

3.1. Promotion of seed germination, vigor index seedlings, and biomass in CS-MP treated seedlings

Since rapid germination and uniform emergence are desirable for horticulture production crops we initially evaluated the effect of CS-MP on % germination and vigor index in tomato seedlings. Pre-treatment of seeds with 0.1 mg mL^{-1} CS-MP promoted a statistically significant increase in the % germination compared to control seeds pre-treated with H_2O, 87% and 73%, respectively. Moreover, 0.01 and 1 mg mL^{-1} also evidenced an increment on germination efficiency compared with bulk CS which did not promote the % germination in any concentration assayed. CS-MP also improved the vigor index in 11 day old seedlings. Pre-treatment with 0.01 and 0.1 mg mL^{-1} CS-MP resulted in index vigor of 4802 and 5127, respectively which were significantly higher than the control (3713). In this case, the same concentrations of bulk CS showed similar improvement in seedlings vigor index (Table 1).

The values are mean from 3 independent experiments. Means designated with the same letter in each column are not significantly different to the control according to Dunnett test ($p < 0.05$; $n = 240$).

To study the effect of CS-MP in early stages of seedlings during soil establishment, comparative analyses of the action between CS-MP, bulk CS and the starter solution Inicium as positive control were carried out along 3–4 weeks according to experimental design shown in Fig. 1. Thus, after 3 applications made at the indicated times, physiological and growth parameters were analyzed in 26 day old seedlings. In this stage, seedlings had an average length of 6 cm and showed the first pair of true leaves (Fig. 2A). Particularly, 0.01 and 0.1 mg mL^{-1} CS-MP treated seedlings showed a significant increase in the leaf area average similarly to the action of 0.1% Inicium. Similar results were obtained with 0.01 mg mL^{-1} CS treatment. The treatment of seedlings with 0.1 mg mL^{-1} CS-MP also improved aerial and root DWs in a 44% and 59%, respectively, compared to control. Bulk CS and Inicium produced a reduced increase in biomass compared to CS-MP (Fig. 2). Chlorophyll content was also determined in this stage of tomato seedlings but this parameter was not altered significantly by all treatments analyzed (Supplemental Fig. 2). The effect of CS-MP on growth parameters was also analyzed earlier after 17 days of treatment following second application but no significant differences in biomass were still detected (Supplemental Fig. 3).

3.2. CS-MP effect: a focus on biochemical and phytohormone mechanisms

It is well documented that plants treated with hormone-like compounds or bioactives trigger early hormonal and biochemistry responses, which next unchained physiological responses impacting on root and shoot architecture (Shah et al., 2018; Xia et al., 2015). To get further insights on the early mechanism of action downstream CS-MP, biochemical and hormonal parameters were analyzed in seedlings treated with 0.1 mg mL^{-1} CS-MP which was the doses that exerted beneficial effect as biostimulant. The level of H_2O_2 was measured in CS-MP and bulk CS treated plants and harvested at 24 h upon the second foliar spray (Fig. 1), based on our previous works describing the timing and crosstalk between ROS metabolism and hormonal signalings (Iglesias et al., 2010, 2014). The H_2O_2 content was diminished in leaves from treated plants compared with H_2O control (Fig. 3A). Content of anthocyanins in CS-MP and CS-treated seedlings were almost unchangeable (Fig. 3B). A reduction in H_2O_2 level could be the consequence of the activation of antioxidant enzymes. Compared with bulk CS and H_2O as controls, CAT activity increased 22% in CS-MP treated seedlings (Fig. 3C). However, in the case of SOD activity we observed an increase of approximately 14% and 11% for CS-MP and bulk CS-treated leaves, respectively.

Since exist an interlink between redox state and phytohormone signaling in the regulation of plant growth and development (Considine and Foyer, 2014) we analyze if CS-MP activate auxin and cytokinin pathways in seedlings of the *DR5:GUS* and *ARR5:GUS* transgenic Micro-Tom reporters of auxin and cytokinin signalings, respectively. Taking into account that plant tissues react to very sensible changes of exogenous hormone-like compounds (Fendrych et al., 2018) two different concentrations of CS-MP (0.1 and 1 mg mL^{-1}) were assayed in an *in vitro* system set up by Iglesias et al. (2019). A strong activation of auxin signaling was detected upon 24 h of CS-MP treatment in the root tip of tomato seedlings. Cytokinin promoter was also induced in a dose-dependent manner by the application of CS-MP in root tips. However, neither the auxin nor cytokinin-dependent signalings were activated in control seedlings (Fig. 4A and B) suggesting that CS-MP specifically activate growth hormone responses.

3.3. CS-MP induce plant innate defense proteins

CS and derivatives are known to act as potent inducers, enhancing a battery of plant responses events as well as the accumulation of defense-related proteins such as PR-proteins (El Hadrami et al., 2010). Both β-1,3-glucanases and chitinases are well known as PR proteins, belonging to the PR2 and PR3 families, respectively (van Loon et al., 2006). To analyze the accumulation pattern of PR2 (Glucanase I) and PR3 (Chitinase I) immunochemical assays were performed. An increase of 1.2 folds of 37-kD immunoreactive PR2 (Fig. 5A) and 34-kD immunoreactive PR3 (Fig. 5B) was recorded in leaves from 24 day old seedlings treated with 1 mg mL^{-1} of CS-MP at 16 h after treatment (Fig. 1), compared with non-treated. In our assay PR2 and PR3 accumulation was unchangeable in seedlings treated with 0.01 mg mL^{-1} and 0.1 mg mL^{-1} CS-MP. In order to get insights into the temporal pattern, PR2 accumulation we measured at 40 h after CS-MP application. Apparently, a gradual accumulation of PR2 was detected according to concentrations and times here assayed (Fig. 5C).

4. Discussion

The conventional commercial propagation of tomato seedlings currently requires greenhouse conditions. The establishment of roots in the first period of seedling development in cell flats is critical and often demands the application of plant growth promoters and biostimulants (Kubota et al., 2001). An improvement in these early stages of development before the transplantation to the soil is associated to a positive impact on crop productivity (Servin et al., 2015). In this work, the action of CS-MP as an environmental friendly growth promoter including its efficacy as immunomodulator of defense response was investigated during early developmental stages of tomato seedlings cultivated under commercial greenhouse conditions. Tomato seed germination, vigor index and seedling biomass were favored by CS-MP treatment (Table 1; Fig. 2). In term of applied doses and biological action, CS-MP proved to have differential responses compared to CS. While CS-MP evidenced a promotion of seed germination, the control with bulk CS had no effect on any of the doses tested. Similar results on germination were obtained when CS nanoparticles were applied on rice and wheat seeds (Divya et al., 2019). Moreover, CS-MP demonstrated to

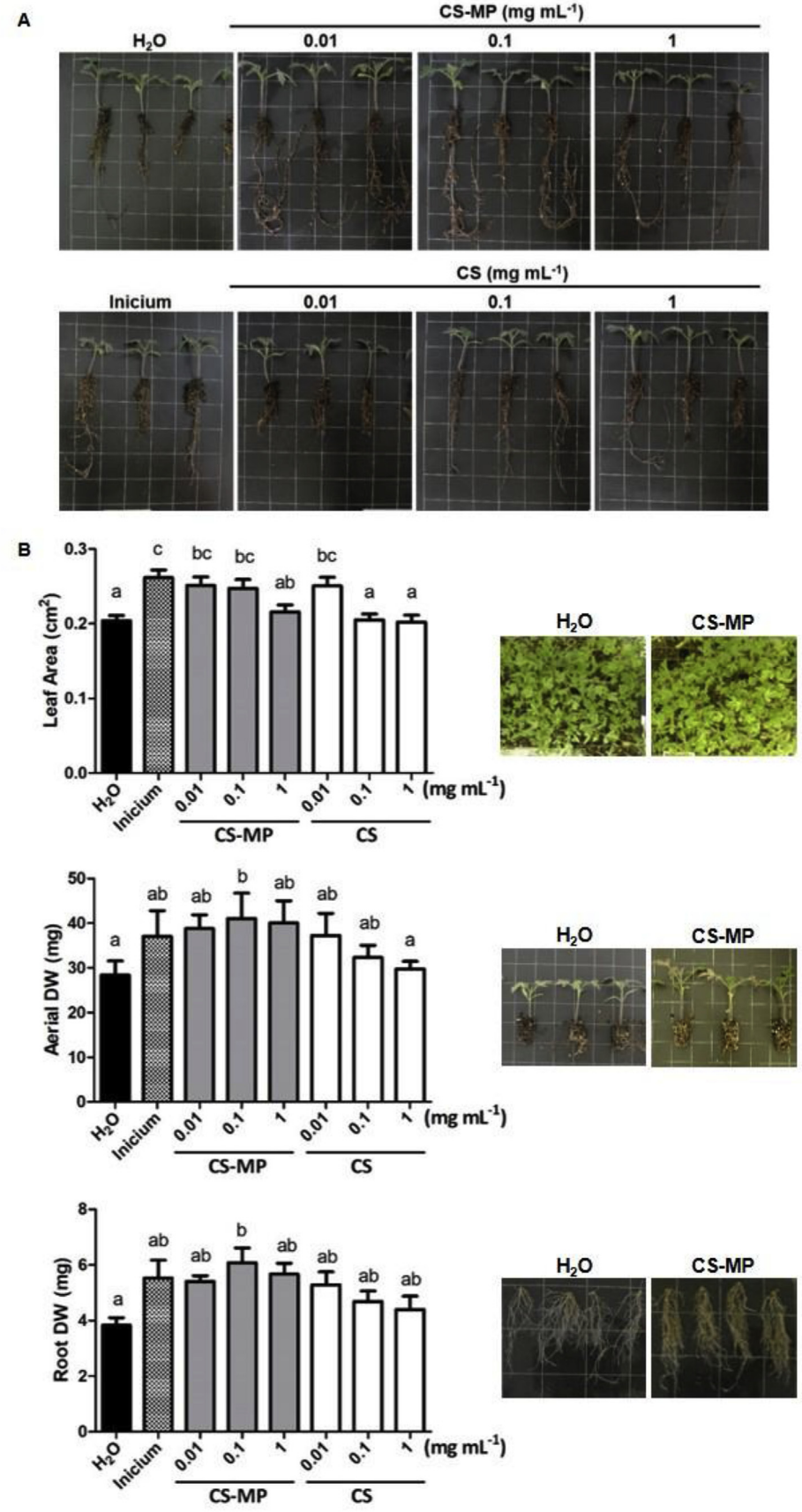

Fig. 2. Effect of CS-MP on tomato plants biomass. Tomato seedings were sprayed with 0.01, 0.1 and 1 mg mL^{-1} of MP-CS, bulk CS and H_2O or 0.1% Inicium as controls. A- Representative 26 day old seedlings are shown. B- Left panel show histograms indicating the quantification of growth parameters: leaf area (upper), aerial DW (middle) and root DW (lower) and right panel show a representative image of 0.1 mg mL^{-1} CS-MP treated plantlets at the final of the assay. Values are the mean (± SE) of three independent experiments. Different letters indicate significant difference between treatments ($p < 0.05$; Tukey-test, n = 100).

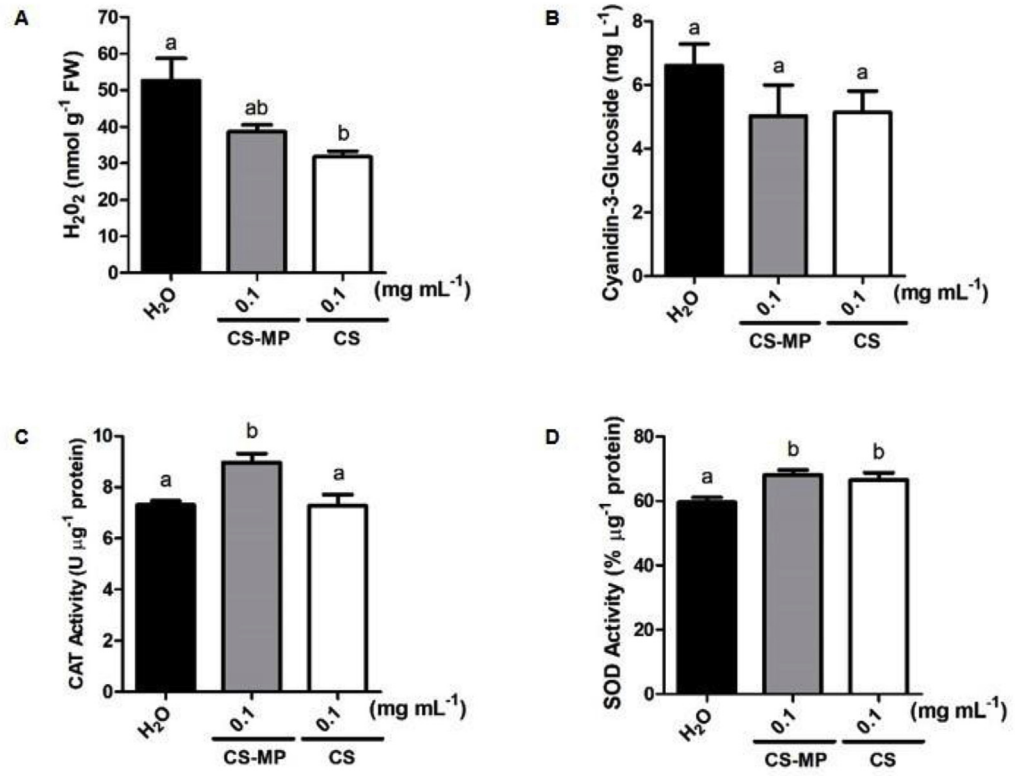

Fig. 3. Effect of CS-MP on redox state of tomato seedlings. Fifteen day old tomato seedlings were sprayed with 0.1 mg mL^{-1} of CS-MP, bulk CS and H_2O as control. Peroxides (A) and anthocyanins (B) content and enzymatic activities of CAT (C) and SOD (D) were measured 24 h after treatments. Anthocyanins content was expressed as cyanidin-3-glucoside according to Giusti and Wrolstad (2001). Values are the mean (± SE) of three independent experiments. Different letters indicate significant difference between treatments ($p < 0.05$; Tukey-test, n = 15).

exert better performance than CS on the promotion of foliar area, and root and shoot biomass. The biostimulant action of CS-MP was evidenced in a wide range of concentrations (0.01–1 mg mL^{-1}) with no cytotoxicity effects. Thus, we assume that our results convincingly establish CS-MP as a sustainable growth promoter of tomato plants grown under greenhouse and probably also, under field conditions. So far, some studies in Arabidopsis, wheat, rice, tomato, and pepper plants including the analysis of growth parameters regulated by comparable concentrations of bulk CS or CS-nanoparticles has described an inhibition of root growth or alternatively, a promotion of root growth at narrow doses but showing cytotoxicity at higher concentrations (Divya et al., 2019; Li et al., 2019; Lopez-Moya et al., 2017). In addition, contrary to our findings, the TPP-nanoparticles of CS obtained from low MW (110 kDa) developed by Asgari-Targhi et al. (2018) exerted cytotoxicity evaluated as root growth cessation of *C. annuum* plants at lower concentrations (20 µg mL^{-1}) compared with the respective control CS (100 µg mL^{-1}). An increased toxicity of chitosan/TPP nanoparticles was also detected when analyzing germination and first stages of growth and development in *Zea mays*, *Pisum sativum* and *Brassica rapa* compared with bulk CS (Nakasato et al., 2017). Regarding to the effects

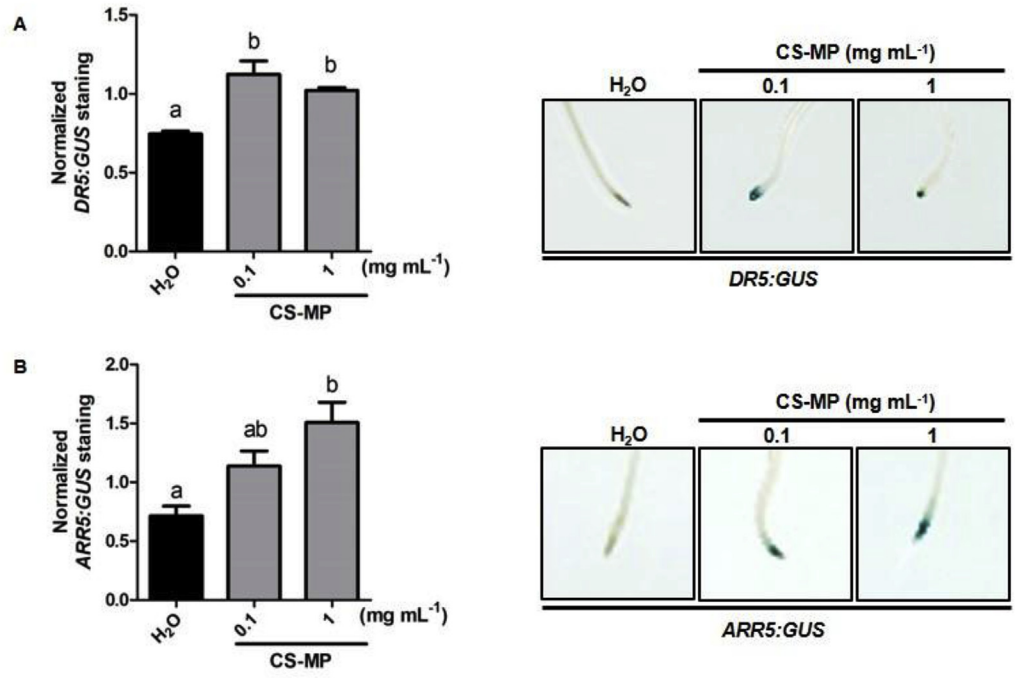

Fig. 4. Effect of CS-MP on *DR5:GUS* and *ARR5:GUS* reporter tomato plants. Five day old *DR5:GUS* (A) and *ARR5:GUS* (B) tomato seedlings were transferred to liquid ½ MS medium supplemented with 0.1 and 1 mg mL^{-1} CS-MP. GUS activity was revealed after incubation with X-Gluc at 37 °C. GUS activation in the root tip was analyzed 24 h post-treatment. Left panel show histograms indicating the quantification of GUS stained of three independent experiments. Values are the mean (± SE). In right panel shows representative GUS stained root tips after 24 h.

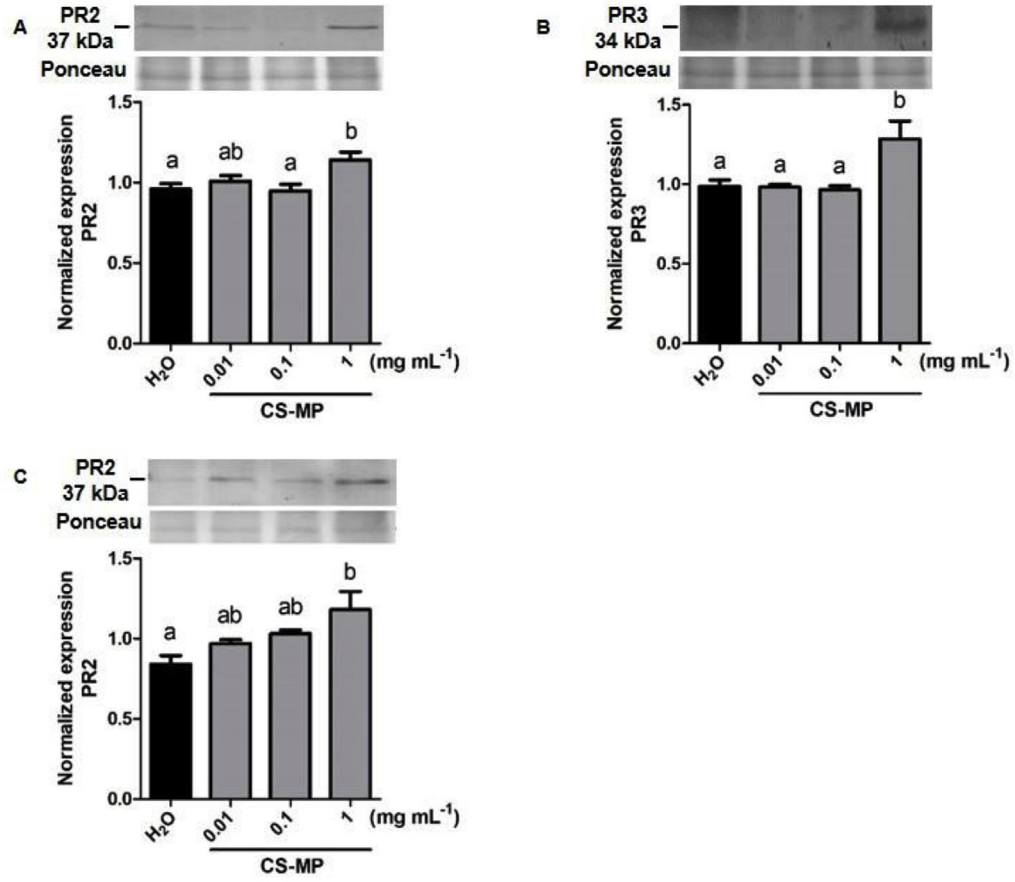

Fig. 5. Abundance of defense marker proteins (PR2 and PR3) detected in CS-MP treatment leaves. Soluble proteins were isolated at 16 h after the 3rd spray. Top: Immunoblotting showing relative levels of PR2 (A) and PR3 (B). Bottom: Histograms expressing PR2 and PR3 abundance related to the reference of an unknown-protein content detected by Ponceau staining indicated in respective upper panels (A and B). In C protein were isolated 40 h after the 3rd spray and PR2 was analyzed by Western Blot. Protein abundance was calculated by densitometry analysis using ImageJ software and expressed as a.u. Data are mean values of 3 independent experiments ± SE.

of CS based particulated systems most of them are mainly focused in the use of low molecular weight CS since is the most frequently used in the field of biomaterials and it is mostly associated to a suitable development of drug delivery systems with good properties in terms of size and polydispersity to reach specific biological and/or pharmacological targets (Baranwal et al., 2018). In addition, most of the CS-based materials described up to know are in the nano scale. Since, the CS used in this work exhibited a high Mw (1531 ± 372 kDa) we hypothesize that the differential biological properties of CS-MP are associated to the MW properties of the CS used for synthesis as well as the micro scale size of the resulting particles, and the new microstructure resulting from the electrostatic interaction between NH_3^+ groups of CS and the TPP^- cross-linker. The interaction between TPP^- and NH_3^+ which allows the formation of the microstructure organization during the ionic gelation method confers new properties to the material if compared to bulk CS macrostructure. The reduction in the number of exposed NH_3^+ in CS-MP which disrupt cell membrane potential in plants, and the augmentation of the surface contact (Kohane, 2007; Pacheco et al., 2013) could be associated to a better biological performance of CS-MP in a wide range of concentrations compared with bulk CS. It has been well documented that plants exposed to CS trigger early hormonal and biochemistry responses which next unchained physiological responses impacting on root and shoot architecture (Pichyangkura and Chadchawan, 2015; Malerba and Cerana, 2018). Our results demonstrate that auxin and cytokinin signalings were activated in tomato plants by the action of CS-MP suggesting that these two phythormones could be responsible at least in part, of root growth and development in tomato seedlings. A more precise discernment about the mode of regulation could allow us to understand the different levels of hormonal regulation mediated by CS-MP in tomato. Fendrych et al. (2018) described that seedlings react to very sensible changes in auxin concentrations by extremely rapid adaptation of root growth. CS induced a strong accumulation of auxin in the root tip which at a molecular level negatively affects the expression of the transcription factor *Wuschel-related homeobox 5*, a master gene regulator of root stem cell activity, leading to growth arrest in Arabidopsis seedlings (Lopez-Moya et al., 2017). In Arabidopsis, bulk CS also induced a rapid repression of auxin signaling in the root tip of the auxin reporter transgenic seedlings, *BA3:GUS* associated to root gowth inhibition (Iglesias et al., 2019). The repression of auxin genes involved in root development has been also detected in CS-treated sweet orange plants (Coqueiro et al., 2015). These evidences are in concordance to the intricate negative feedback regulations important to limit the auxin signal (Salehin et al., 2015). However, CS-MP seem to exert a differential regulation of auxin signaling compared to bulk CS since induced an activation of the auxin promoter *DR5* at multiple doses in Micro-Tom tomato (Fig. 4) and Arabidopsis (Iglesias et al., 2019). We therefore, hypothesize that differential physico-chemical characteristics of CS-MP over bulk CS could unchained a moderate and accurate activation of auxin signaling which lead to the promotion of root growth. In addition, by analyzing *ARR5:GUS* transgenic seedlings in Micro-Tom varietal we suggest that cytokinin signaling might also contribute to promote tomato root growth (Fig. 4). In addition to the role of CS on the accumulation of abscisic acid, jasmonic acid, salicylic acid and auxin (Asgari-Targhi et al., 2018; Lopez-Moya et al., 2017; Pichyangkura and Chadchawan, 2015) our work highlights on the participation of cytokinin signaling as part of the putative pathways that act downstream of CS in tomato. Therefore, we suggest that a delicate crosstalk between auxins and cytokinins could be essential for the proper development of root architecture and plant growth (Schaller et al., 2015).

In addition to the impact on hormonal pathways, CS action involves the downstream modulation of ROS and RNS. In Arabidopsis, CS perception depends on the chitin receptor AtCERK1 downstream of which is established an early oxidative burst associated to the posterior

elicitation of plant defense responses (Gubaeva et al., 2017).The balance of redox status is very sensitive since ROS and RNS constitute signaling molecules during multiple developmental responses along plant life cycle but in excess they can lead to nitrosative and oxidative stress with toxic effects on cell metabolism (Mittler et al., 2011; Molassiotis and Fotopoulos, 2011). The positive effects triggered by H_2O_2 priming lies on the fact that primed plants show a more pronounced and early expression of a pool of transcripts that involved genes encoding enzymatic antioxidants and polyamine synthesis (Antoniou et al., 2016; Savvides et al., 2016). Therefore, we evaluated if these early reported burst of ROS triggered by CS (Miya et al., 2007) act as a priming signal to induce the activation of antioxidant metabolism which could protect tomato plants to eventual stresses during greenhouse growth. Indeed, tomato plants sprayed with 0.1 mg mL^{-1} CS-MP had a better redox status in terms of the activation of CAT and SOD antioxidant activities which consequently resulted in a decrease H_2O_2 level (Fig. 3). Since bulk CS presented reduced levels of H_2O_2 but the only activation of SOD activity we hypothesize that other scavengers of ROS such as glutathione and ascorbic acid could be also modulated by CS and CS-MP (Zeng et al., 2010). In concordance with our results in tomato plants, CS-treated *Zea mays* plants also evidenced a reduction in H_2O_2 and superoxide anion levels associated to an induction of the activity of CAT, SOD and ascorbate peroxidase after 3 d of treatment (Turk, 2019). The modulation of redox parameters was associated to the alleviating effect of CS on growth retardation and oxidative stress induced by salt stress in maize plants. Reduced levels of ROS accumulation and similar levels of SOD and CAT activities were detected in *Camellia sinensis* plants treated with CS-nanoparticles for 24 h (Chandra et al., 2015). Nitric oxide accumulation has been also found to be increased by CS- micro and nanoparticles in *A. thaliana* and *C. sinensis*, respectively (Iglesias et al., 2019; Chandra et al., 2015, 2017). We also analyzed the action of CS-MP as an elicitor agent during early developmental stage of tomato seedlings. The abundance of the defense marker proteins PR2 and PR3 was improved in CS-MP-treated seedlings demonstrating that CS-MP can act both as biostimulants and in the protection of plants against pathogen. Supporting our findings, CS nanoparticles have been found to induce innate immune responses in tea and tomato plants since unchained an induction of *PRs* transcript levels (Chandra et al., 2015, 2017; Chun and Chandrasekaran, 2019). Downstream modulation of redox metabolism and defense response genes triggered by CS-based materials of different sizes and physical-chemical properties seems to be much more similar compared with the modulation of hormonal pathways and developmental programs.

Since tomato yield crop is severely reduced by many pathogens including fungi, bacteria, virus and insects (Singh et al., 2017) we propose that our described CS based particulated system constitutes a valuable biomaterials that can act both as biostimulants and in the protection of plants against pathogens. It could be used as an alternative to synthetic pesticides with the concomitant environmental and chemical advantages. However, we assume that several aspects on the molecular mechanism underlying CS-MP action remain to be elucidated. Undoubtedly, a better understanding on CS-MP perception and transduction into metabolic changes will bring strong tools for a proper application of these biomaterials in the field.

5. Conclusions

CS-MP were non-toxic for tomato plants during the first stages of development in a wide range of doses, a very important property for agricultural applications. Indeed, CS-MP treatments improved seed germination, vigor index, and root and shoot biomass in tomato seedlings. CS-MP trigger the modulation of auxin and cytokinin hormonal signaling pathways; induce the activity of antioxidant enzymes and the induction of plant defense marker proteins. Our findings validate a promising scenario for the application of CS-MP as a sustainable growth promoter for the establishment of tomato plantlets, augmenting crop productivity with reduced environment impact.

Author contributions

S.L.C., M.F.S., A.Y.M. and C.A.C. designed research; S.L.C., M.F.S., A.Y.M and M.J.I. performed research; D.F.F, S.M-S, A.A.C. contributed new reagents/analytic tools; S.L.C. and M.F.S. analyzed data; S.L.C, D.F.F., V.A.A. and C.A.C. wrote the paper with input from the other authors.

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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.2019.09.002.

References

Antoniou, C., Savvides, A., Christou, A., Fotopoulos, V., 2016. Unravelling chemical priming machinery in plants: the role of reactive oxygen–nitrogen–sulfur species in abiotic stress tolerance enhancement. Curr. Opin. Plant Biol. 33, 101–107.

Asgari-Targhi, G., Iranbakhsh, A., Ardebili, Z.O., 2018. Potential benefits and phytotoxicity of bulk and nano-chitosan on the growth, morphogenesis, physiology, and micropropagation of *Capsicum annuum*. Plant Physiol. Biochem. 127, 393–402.

Baranwal, A., Kumar, A., Priyadharshini, A., Oggu, G.S., Bhatnagar, I., Srivastava, A., Chandra, P., 2018. Chitosan: an undisputed bio-fabrication material for tissue engineering and bio-sensing applications. Int. J. Biol. Macromol. 110, 110–123.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.

Beers, R.F., Sizer, I.W.A., 1952. Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133–140.

Bellincampi, D., Dipierro, N., Salvi, G., Cervone, F., De Lorenzo, G., 2000. Extracellular H_2O_2 induced by oligogalacturonides is not involved in the inhibition of the auxin-regulated rolB gene expression in tobacco leaf explants. Plant Physiol. 122, 1379–1386.

Cerchiara, T., Abruzzo, A., Di Cagno, M., Bigucci, F., Bauer-Brandl, A., Parolin, C., Vitali, B., Gallucci, M.C., Luppi, B., 2015. Chitosan based micro-and nanoparticles for colon-targeted delivery of vancomycin prepared by alternative processing methods. Eur. J. Pharm. Biopharm. 92, 112–119.

Chandra, S., Chakraborty, N., Dasgupta, A., Sarkar, J., Panda, K., Acharya, K., 2015. Chitosan nanoparticles: a positive modulator of innate immune responses in plants. Sci. Rep. 5, 15195.

Chandra, S., Chakraborty, N., Panda, K., Acharya, K., 2017. Chitosan-induced immunity in *Camellia sinensis* (L.) O. Kuntze against blister blight disease is mediated by nitric-oxide. Plant Physiol. Biochem. 115, 298–307.

Chun, S.C., Chandrasekaran, M., 2019. Chitosan and chitosan nanoparticles induced expression of pathogenesis-related proteins genes enhances biotic stress tolerance in tomato. Int. J. Biol. Macromol. 125, 948–954.

Considine, M.J., Foyer, C.H., 2014. Redox regulation of plant development. Antioxidants Redox Signal. 21, 1305–1326.

Coqueiro, D.S.O., de Souza, A.A., Takita, M.A., Rodrigues, C.M., Kishi, L.T., Machado, M.A., 2015. Transcriptional profile of sweet orange in response to chitosan and salicylic acid. BMC Genomics 16 (1), 288.

Divya, K., Vijayan, S., Nair, S.J., Jisha, M., 2019. Optimization of chitosan nanoparticle synthesis and its potential application as germination elicitor of *Oryza sativa* L. Int. J. Biol. Macromol. 124, 1053–1059.

El Hadrami, A., Adam, L.R., El Hadrami, I., Daayf, F., 2010. Chitosan in plant protection. Mar. Drugs 8, 968–987.

Fendrych, M., Akhmanova, M., Merrin, J., Glanc, M., Hagihara, S., Takahashi, K., Uchida, N., Torii, K.U., Friml, J., 2018. Rapid and reversible root growth inhibition by TIR1 auxin signaling. Nature Plants 4 (7), 453–459.

Giusti, M.M., Wrolstad, R.E., 2001. Characterization and measurement of anthocyanins by UV-visible spectroscopy. Current Protocols in Food Analytical Chemistry F1. 2.1–F1. 2.13.

Gubaeva, E., Gubaev, A., Melcher, R., Cord-Landwehr, S., Singh, R., El Gueddari, N.E., Moerschbacher, B., 2017. Chitosan perception in Arabidopsis requires the chitin receptor AtCERK1 suggesting an improved model for receptor structure and function. Mol. Plant Microbe Interact. 170092.
Hernández-Hernández, H., González-Morales, S., Benavides-Mendoza, A., Ortega-Ortiz, H., Cadenas-Pliego, G., Juárez-Maldonado, A., 2018. Effects of chitosan–PVA and Cu nanoparticles on the growth and antioxidant capacity of tomato under saline stress. Molecules 23, 178.
Iglesias, M.J., Colman, S.L., Terrile, M.C., Paris, R., Martín-Saldaña, S., Chevalier, A.A., Alvarez, V., Casalongue, C., 2019. Enhanced properties of chitosan microparticles over bulk chitosan on the modulation of auxin signaling pathway with beneficial impacts on root architecture in plants. J. Agric. Food Chem. 67, 6911–6920.
Iglesias, M.J., Terrile, M.C., Bartoli, C.G., D'Ippólito, S., Casalongué, C.A., 2010. Auxin signaling participates in the adaptative response against oxidative stress and salinity by interacting with redox metabolism in Arabidopsis. Plant Mol. Biol. 74 (3), 215–222.
Iglesias, M.J., Terrile, M.C., Windels, D., Lombardo, M.C., Bartoli, C.G., Vazquez, F., Mark, E., Casalongué, C.A., 2014. MiR393 regulation of auxin signaling and redox-related components during acclimation to salinity in Arabidopsis. PLoS One 9 (9), e107678.
Iriti, M., Varoni, E.M., 2015. Chitosan-induced antiviral activity and innate immunity in plants. Environ. Sci. Pollut. Control Ser. 22, 2935–2944.
Kashyap, P.L., Xiang, X., Heiden, P., 2015. Chitosan nanoparticle based delivery systems for sustainable agriculture. Int. J. Biol. Macromol. 77, 36–51.
Kohane, D.S., 2007. Microparticles and nanoparticles for drug delivery. Biotechnol. Bioeng. 96, 203–209.
Kubota, C., Kakizaki, N., Kozai, T., Kasahara, K., Nemoto, J., 2001. Growth and net photosynthetic rate of tomato plantlets during photoautotrophic and photomixotrophic micropropagation. Horticulture Science 36, 49–52.
Kumaraswamy, R.V., Kumari, S., Choudhary, R.C., Pal, A., Raliya, R., Biswas, P., Saharan, V., 2018. Engineered chitosan based nanomaterials: bioactivities, mechanisms and perspectives in plant protection and growth. Int. J. Biol. Macromol. 113, 494–506.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Li, R., He, J., Xie, H., Wang, W., Bose, S.K., Sun, Y., Hu, J., Yin, H., 2019. Effects of chitosan nanoparticles on seed germination and seedling growth of wheat (*Triticum aestivum* L.). Int. J. Biol. Macromol. 126, 91–100.
Lopez-Moya, F., Escudero, N., Zavala-Gonzalez, E.A., Esteve-Bruna, D., Blázquez, M.A., Alabadí, D., Lopez-Llorca, L.V., 2017. Induction of auxin biosynthesis and WOX5 repression mediate changes in root development in Arabidopsis exposed to chitosan. Sci. Rep. 7, 16813.
Malerba, M., Cerana, R., 2018. Recent advances of chitosan applications in plants. Polymers 10, 118.
Mansilla, A.Y., Albertengo, L., Rodríguez, M.S., Debbaudt, A., Zúñiga, A., Casalongué, C., 2013. Evidence on antimicrobial properties and mode of action of a chitosan obtained from crustacean exoskeletons on *Pseudomonas syringae* pv. tomato DC3000. Appl. Microbiol. Biotechnol. 97, 6957–6966.
Martín-Saldaña, S., Chevalier, M.T., Iglesias, M.J., Colman, S.L., Casalongué, C.A., Álvarez, V.A., Chevalier, A.A., 2018. Salicylic acid loaded chitosan microparticles applied to lettuce seedlings: recycling shrimp fishing industry waste. Carbohydr. Polym. 200, 321–331.
Maynard, D.N., Hochmuth, G.J., 2006. Knott's Handbook for Vegetable Growers, fifth ed. Wiley, Hoboken, New Jersey, pp. 1–621.
Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V.B., Vandepoele, K., Gollery, M., Shulaev, V., Van Breusegem, F., 2011. ROS signaling: the new wave? Trends Plant Sci. 16, 300–309.
Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., Shibuya, N., 2007. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. Proc. Natl. Acad. Sci. 104, 19613–19618.
Molassiotis, A., Fotopoulos, V., 2011. Oxidative and nitrosative signaling in plants: two branches in the same tree? Plant Signal. Behav. 6, 210–214.
Nakasato, D.Y., Pereira, A.E., Oliveira, J.L., Oliveira, H.C., Fraceto, L.F., 2017. Evaluation of the effects of polymeric chitosan/tripolyphosphate and solid lipid nanoparticles on germination of *Zea mays, Brassica rapa* and *Pisum sativum*. Ecotoxicol. Environ. Saf. 142, 369–374.
Pacheco, P., White, D., Sulchek, T., 2013. Effects of microparticle size and Fc density on macrophage phagocytosis. PLoS One 8, e60989.
Pichyangkura, R., Chadchawan, S., 2015. Biostimulant activity of chitosan in horticulture. Sci. Hortic. 196, 49–65.
Pirbalouti, A.G., Malekpoor, F., Salimi, A., Golparvar, A., 2017. Exogenous application of chitosan on biochemical and physiological characteristics, phenolic content and antioxidant activity of two species of basil (*Ocimum ciliatum* and *Ocimum basilicum*) under reduced irrigation. Sci. Hortic. 217, 114–122.
Salehin, M., Bagchi, R., Estelle, M., 2015. SCFTIR1/AFB-based auxinperception: mechanism and role in plant growth and development. The Plant Cell 27, 9–19.
Savvides, A., Ali, S., Tester, M., Fotopoulos, V., 2016. Chemical priming of plants against multiple abiotic stresses: mission possible? Trends Plant Sci. 21, 329–340.
Schaller, G.E., Bishopp, A., Kieber, J.J., 2015. The yin-yang of hormones: cytokinin and auxin interactions in plant development. The Plant Cell 27 (1), 44–63.
Servin, A., Elmer, W., Mukherjee, A., De la Torre-Roche, R., Hamdi, H., White, J.C., Bindraban, P., Dimkpa, C., 2015. A review of the use of engineered nanomaterials to suppress plant disease and enhance crop yield. J. Nanoparticle Res. 17, 92.
Shah, Z.H., Rehman, H.M., Akhtar, T., Alsamadany, H., Hamooh, B.T., Mujtaba, T., Daur, I., Al Zahrani, Y., Alzahrani, H.A., Ali, S., Yang, S.H., Chung, G., 2018. Humic substances: determining potential molecular regulatory processes in plants. Front. Plant Sci. 9, 263.
Siddaiah, C.N., Prasanth, K.V.H., Satyanarayana, N.R., Mudili, V., Gupta, V.K., Kalagatur, N.K., Satyavati, T., Dai, X.F., Chen, J.Y., Mocan, A., Singh, B.P., Srivastava, R.K., 2018. Chitosan nanoparticles having higher degree of acetylation induce resistance against pearl millet downy mildew through nitric oxide generation. Sci. Rep. 8, 2485.
Silva, W.B., Vicente, M.H., Robledo, J.M., Reartes, D.S., Ferrari, R.C., Bianchetti, R., et al., 2018. SELF-PRUNING acts synergistically with DIAGEOTROPICA to guide auxin responses and proper growth form. Plant Physiol. 176, 2904–2916.
Singh, V.K., Singh, A.K., Kumar, A., 2017. Disease management of tomato through PGPB: current trends and future perspective. 3 Biotech 7, 255.
Smith, P.K., Krohn, R.I., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.
Sreekumar, S., Goycoolea, F.M., Moerschbacher, B.M., Rivera-Rodriguez, G.R., 2018. Parameters influencing the size of chitosan-TPP nano-and microparticles. Sci. Rep. 8, 4695.
Sudisha, J., Sharathchandra, R., Amruthesh, K., Kumar, A., Shetty, H.S., 2012. Pathogenesis Related Proteins in Plant Defense Response in Book: Plant Defence. Springer, Dordrecht, pp. 379–403.
Tilman, D., Balzer, C., Hill, J., Befort, B.L., 2011. Global food demand and the sustainable intensification of agriculture. Proc. Natl. Acad. Sci. 108, 20260–20264.
Tittonell, P., 2014. Ecological intensification of agriculture—sustainable by nature. Current Opinion in Environmental Sustainability 8, 53–61.
Turkan, I., 2018. ROS and RNS: key signalling molecules in plants. J. Exp. Bot. 69, 3313–3315.
Turk, H., 2019. Chitosan-induced enhanced expression and activation of alternative oxidase confer tolerance to salt stress in maize seedlings. Plant Physiol. Biochem. 141, 415–422.
van Loon, L.C., Rep, M., Pieterse, C.M., 2006. Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol. 44, 135–162.
Xia, X.J., Zhou, Y.H., Shi, K., Zhou, J., Foyer, C.H., Yu, J.Q., 2015. Interplay between reactive oxygen species and hormones in the control of plant development and stress tolerance. J. Exp. Bot. 66, 2839–2856.
Yin, H., Zhao, X., Oligochitosan, Du Y., 2010. A plant diseases vaccine—a review. Carbohydr. Polym. 82, 1–8.
Zeng, K., Deng, Y., Ming, J., Deng, L., 2010. Induction of disease resistance and ROS metabolism in navel oranges by chitosan. Sci. Hortic. 126, 223–228.