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# Evaluation of chitooligosaccharide application on mineral accumulation and plant growth in *Phaseolus vulgaris*

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ABSTRACT

Chitooligosaccharides (COS) – water soluble derivatives from chitin, are an interesting group of molecules for several biological applications, for they can enter plant cells and bind negatively charged molecules. Several studies reported an enhanced plant growth and higher crop yield due to chitosan application in soil grown plants, but no studies have looked on the effect of COS application on plant mineral nutrient dynamics in hydroponically grown plants. In this study, *Phaseolus vulgaris* was grown in hydroponic culture and the effect of three different concentrations of COS on plant growth and mineral accumulation was assessed. There were significant changes in mineral allocations for Mo, B, Zn, P, Pb, Cd, Mn, Fe, Mg, Ca, Cu, Na, Al and K among treatments. Plant morphology was severely affected in high doses of COS, as well as lignin concentration in the stem and the leaves, but not in the roots. Chlorophyll A, B and carotenoid concentrations did not change significantly among treatments, suggesting that even at higher concentrations, COS application did not affect photosynthetic pigment accumulation. Plants grown at high COS levels had shorter shoots and roots, suggesting that COS can be phytotoxic to the plant. The present study is the first detailed report on the effect of COS application on mineral nutrition in plants, and opens the door for future studies that aim at utilizing COS in biofortification or phytoremediation programs.



## 1. Introduction

Chitosans are a wide group of molecules, derived from chitin, that have a specific reactive amine group able to bind negatively charged molecules such as proteins. Their production results either from treatment of shellfish and crustaceous industry's waste waters, purification of fungi cell wall or from enzymatic production [1]. Chitooligosaccharides (COS) are homo- or heterooligomers of N-acetylglucosamine and D-glucosamine, and can be produced from either chitin itself, or from chitosan. The effect of chitosans and COS on plant growth depends heavily on the specific properties of the polymers. The degree of deacetylation (DD), degree of polymerization (DP – number of units of the polysaccharide, also referred to as molecular weight (MW)) and the oligomer structure pattern (OSM) are the most important parameters for their application in biology [2–6]. Furthermore, the applied dose, application method [7], pH and temperature of the medium, viscosity degree and the presence or absence of an active unit will modify the activity of the molecule [8]. The general lack of nomenclature standardization in literature makes comparisons between various studies difficult. Nevertheless, it is thought that COS are the most interesting chitosans for biological applications, for they are soluble in water, more biocompatible and less cytotoxic [9], although a recent study revealed that application of high doses of COS can lead to red blood cell aggregation and/or adhesion [10].

Typically, chitosans are used either per se, since they have interesting biochemical properties such as antimicrobial activities, or as a support matrix for other compounds (i.e. drug-delivery systems). Many chitosan-based applications are currently being investigated in various areas such as biomedical and cosmetic industries, biotechnology, water treatment, biomaterials, food safety and agriculture. In the latter instance, chitosans have demonstrated important abilities to enhance plant growth and increase disease and pest control for several important crops [8,11,12]. Chitosan effects will therefore depend on the intrinsic properties of the applied compound and on the application method. Foliar application has shown promising results in lowering transpiration rate in peppers [13], reducing susceptibility of *Vitis vinifera* to *Botrytis cinerea* [14] and increasing production of artemisinin in *Artemisia annua* [15]. Medium amendments with chitosans increased tomatoes' resistance to *Fusarium* disease [16,17] and adding chitosans in biofertilizers has resulted in a lower susceptibility to pine wilt disease in *Pinus pinaster* [18]. It seems that the capacity of chitosans to

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induce plant defense is linked to its binding to pathogen-associated molecular pattern (PAMP) receptors [19,20]. This induces a cascade of defensive responses, such as production of phytoalexins, increased lignification rate, chitinase activity, biosynthesis of phenolic compounds and expression of defense-related genes [19–22].

Chitosan seed coating is already a common practice in agriculture, as it can modulate germination rate and time, disease control and plant growth [23–27]. For example, Ziani and colleagues [28] reported increased germination rates, enhanced resistance to fungi and increased plant growth in artichoke treated with chitosans. Due to their physico-chemical properties, chitosans can interact with many negatively-charged intracellular compounds, resulting in diverse molecular responses [8]. In a study conducted in cowpea, chitosans induced differential allocation of potassium (K) and nitrogen (N) [29]. Given that chitosans have the potential to modulate plant mineral uptake, their application in programs of biofortification and/or phytoremediation is ponderable [30]. However, a more detailed analysis on the effect of COS on mineral nutrition is warranted. In the present study, the effect of COS application on plant growth and mineral allocation was investigated to better understand the effect of this plant elicitor on plant mineral nutrition. To this end, common bean plants (*Phaseolus vulgaris*) were grown in aerated hydroponic conditions for two weeks supplemented with low, medium and high concentrations of COS. The present study demonstrates that COS application affects mineral allocation in the plant in a dose-responsive manner and that there are several macro-level consequences to this shift.

## 2. Materials and methods

### 2.1. Plant material

Common bean, *P. vulgaris*, cv. "Papo-de-Rola", was utilized in this study. Seeds were germinated in the dark for seven days at 25 °C and then transferred to aerated hydroponic culture for 14 days. Seedlings were randomly distributed among the four tested conditions: untreated control, 0.01 g/L COS solution (hereinafter COS.01), a 0.05 g/L COS solution (COS.05) and a 0.1 g/L COS solution (COS.1). Each treatment had five replicates and three independent replications of the experiment were performed to ensure data robustness, for a total of $N_{plants} = 60$. The nutritive solution was changed every three days and consisted of an optimized bean solution containing the following macro- and micronutrients: 1.2 mM $KNO_3$, 0.8 mM $Ca(NO_3)_2$, 0.2 mM $Mg(SO_4)_2$, 0.3 mM $NH_4H_2PO_4$, 25 μM $CaCl_2$, 25 μM $H_3BO_3$, 0.5 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $MoO_3$ and 0.1 μM $NiSO_4$ in 1 mM MES buffer stabilized at pH 5.5. Plants were grown at 20 μM Fe(III)-EDDHA [ethylenediamine-N,N'bis(o-hydroxyphenyl)acetic acid]. COS were diluted at the previously described concentrations in 1% acetic acid, stabilized at pH 5.5 and added to the nutritive solutions. Plants were kept in a growth chamber (Aralab Fitoclima 10000EHF, Portugal) with 16 h day/8 h night photoperiod. The temperature was kept at 25 °C during the light period, with 70% of relative humidity and at 18 °C during the dark period, with 80% of relative humidity. Plants were exposed to a constant photon flux during the day of about 370 μmol $m^{-2}$ $s^{-1}$.

### 2.2. Chitosan treatments

Chitooligosaccharides (COS) were purchased from Shangai NiceChem Co., Ltd. (Shangai, China); the DD ranged from 80% to 85%, the MW was less than 3 kDa and neither the DP nor the OSM were provided by the producer. The COS used in the present study were obtained from crab shells. COS were diluted in 1% acetic acid solution at pH 5.5 to ensure solubility.

### 2.3. Germination assay

A germination test was conducted on 140 seeds, randomly assigned to one of four treatments: untreated control, COS.01, COS.05 and COS.1. The seeds were soaked in a known volume of solution for 25 min and then dried at room temperature for one hour or until they were not sticky. The solution volume was recorded to determine the amount of COS absorbed by the seeds. Germination rate and seedling size were recorded every day since day 3 up to day 7.

### 2.4. Morphological trait assessment

Root length, plant height, number of leaves and stem diameter were measured every two days until the end of the experiment. Dry weight of root, shoot and stem were taken at 14 days, and shoot:root ratio was also calculated.

### 2.5. Mineral nutrient analysis

After 14 days, three plants per treatment were dissected in the different plant parts and dried at 60 °C for five days or until stable dry weight was reached. Two hundred mg of each individual sample were mixed with 5 ml of 65% $HNO_3$ in a Teflon reaction vessel and heated in a Speedwave™ MWS-3+ (Berghof, Germany) microwave system. Digestion procedure was conducted in five steps: 1–130 °C/10 min, 2–160 °C/15 min, 3–170 °C/12 min, 4–100 °C/7 min, 5–100 °C/3 min. The resulting clear solutions of the digestion procedure were then brought to 20 ml with ultrapure water for further analysis.

Mineral concentration determination for molybdenum (Mo), boron (B), zinc (Zn), phosphorus (P), lead (Pb), cadmium (Cd), cobalt (Co), nickel (Ni), manganese (Mn), iron (Fe), magnesium (Mg), calcium (Ca), copper (Cu), sodium (Na), aluminum (Al) and potassium (K) was performed using the inductively coupled plasma optical emission spectrometer Optima 7000 DV (PerkinElmer, USA) with radial configuration.

### 2.6. Pigments and lignin analysis

Immediately after collection, two plants per experiment were frozen in liquid nitrogen and stored at −80 °C for pigment and lignin analysis. Chlorophyll A, B and carotenoids were extracted and quantified as described in Sims and Gamon [31]. Briefly, 12.5 ml of calcium carbonate 0.4% (w/v) in methanol were added to 0.5 g of leaves. After 48 h, the supernatant was collected and absorbances were measured at 663 and 645 nm using a NanoPhotometer™ (Implen GmbH). Lignin purification and quantification was performed using the acetyl bromide method as based on Nunes da Silva et al. [32]. In brief, tissues were lyophilized for 72 h and ground to a fine powder. One hundred and fifty mg of powder were methanol-extracted for 24 h in the dark at 4 °C. The pellet was treated three times with water, acetone and hexane and dried overnight at 60 °C. Five hundred μl of acetic acid and 500 μl of acetyl bromide 25% (v/v) in acetic acid were added to the pellet and the mixture was incubated at 50 °C for 2 h with agitation. One hundred μl of the supernatant were mixed with 200 μl of acetic acid, 150 μl of NaOH 3 M, 50 μl of hydroxylamine hydrochloride 0.5 M and 500 μl of acetic acid. Each sample was analyzed in triplicate. The absorbance was measured at 280 nm using a NanoPhotometer™ (Implen GmbH) and lignin was quantified using a lignin (Aldrich) standard calibration curve.