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# Effect of chitooligosaccharides with different degrees of acetylation on wheat seedlings under salt stress

Ping Zou[a,b], Kecheng Li[a], Song Liu[a,*], Ronge Xing[a], Yukun Qin[a], Huahua Yu[a], Miaomiao Zhou[a,b], Pengcheng Li[a,*]

[a] Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[b] Graduate University of the Chinese Academy of Sciences, Beijing 100049, China

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ABSTRACT

In this study, chitooligosaccharides (COSs) with varying degrees of acetylation (DAs) were applied to wheat seedlings in order to investigate their effect on the plants' defence response under salt stress. The results showed that treatment with exogenous COSs that had different DAs could promote the growth of plants, decrease the concentration of malondialdehyde (MDA), improve the photosynthetic efficiency and enhance the activities of antioxidant enzymes. The mRNA expression level examination of several salt stress response genes suggested that COS could protect plants from the damage of salt stress by adjusting intracellular ion concentration and enhancing the activities of antioxidant enzymes. Furthermore, COS with DA 50% was the most effective in alleviating salt stress to wheat seedlings, which indicated that the activity of COS was closely related with its DAs.



## 1. Introduction

Soil salinity is an important factor in crop growth, and variations within the salinity can cause abiotic stress and lead to significant inhibition of germination, growth and productivity of crops. High salt concentration can cause an imbalance of cellular ions and disrupt the equilibrium between production and scavenging of reactive oxygen species (ROS) (Apel & Hirt, 2004). Salinity disturbs the $Na^+/K^+$ ratio leading to severe toxic effects on genes and enzymes – causing impairments on metabolism (Rivero et al., 2014).

Increased level of $Na^+$ in cells disrupts physiological processes, especially photosynthesis, by reducing the photosynthetic efficiency (Munns, 2002). Additionally, salt stress can lead to the generation of ROS such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals, which in turn can generate other destructive species such as lipid peroxides (Mittler, Vanderauwera, Gollery, & Breusegem, 2004). Plants have evolved complex mechanisms to combat against the salt stress-induced oxidative stress. These mechanisms include a host of antioxidants, including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), which play significant roles in scavenging ROS (Bose, Rodrigo-Moreno, & Shabala, 2013).

With increases in population growth and the development of industry, the amount of farmland worldwide is decreasing, and the saline land is gradually increasing (Diby & Harshad, 2014). Therefore, it is crucial to develop techniques to increase crop yield and quality in saline land. One current technique to improve salt tolerance of plants is transgenic technology which has limited effectiveness and is enveloped in controversy. Another technique is the application of exogenous biostimulators, which is able to decrease the negative effect of abiotic stress and increase yield and quality of crops. Chitosan has been reported to possess diverse biological activities such as antifungal activity (Ma, Yang, Yan, Kennedy, & Meng, 2013, Souza, Hallan, Mirelli, & Oliveira, 2013), antibacterial activity (Li, Liu, et al., 2013) and antiviral activity (Kulikov, Chirkov, Ilina, Lopatin, & Varlamov, 2006). Although chitosan appears to improve the tolerance of plants, such as safflower and sunflower (Jabeen & Ahmad, 2013), to salt stress (salt concentration 0–0.8%), the exact physiological mechanisms are not currently understood. How the DAs exactly works in altering activities of chitosan is unclear, but the DA is the most important parameter influencing the chitosans' various properties (Kasaai, 2010). The effectiveness of chitosan in various applications appears to be dependent on the DA (Je & Kim, 2006; Maksimov, Valeev, Cherepanova, & Burkhanova, 2013). When compared with chitosan,

* Corresponding authors. Tel.: +86 532 82898707; fax: +86 532 82968951.
*E-mail addresses:* sliu@qdio.ac.cn (S. Liu), pcli@qdio.ac.cn (P. Li).

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COS has lower molecular weight and higher water solubility and, in some studies, is reported to exhibit better antifungal (Meng, Yang, Kennedy, & Tian, 2010; Rabea et al., 2005) and antiviral (Kulikov et al., 2006) activity and better ability to promote plant growth (Nguyen, Vo, & Tran, 2011). However, most of these reports fail to mention the function of COS with different DAs in improving the capacity of plants against salt stress.

Accordingly, in this study, we exposed wheat seedlings to salt stress and investigated the effect of exogenous COSs with different DAs on wheat seedlings. Furthermore, we evaluated the expression of a series of salt-associated genes in wheat by quantitative RT-PCR to explore the physiological mechanisms of exogenous COS with different DAs on wheat tolerance to salt stress.

## 2. Materials and methods

### 2.1. Preparation of COS with different DAs

Highly deacetylated chitosan (DA < 10%) was obtained according to the method reported by Song, Li, Li, and Ding (2005). Highly deacetylated COS was prepared by the degradation of highly deacetylated chitosan as reported by Li et al. (2012). The chitosan powder (3 g) was introduced in 100 mL 2% acetic acid. Then 1 mL 30% $H_2O_2$ was added into the chitosan solution. The degradation assisted with microwave radiation was carried out with the power of 600 W at 70 °C for 60 min. When cooled to room temperature, the reaction mixture was adjusted to pH 7.0 and then dialyzed to remove salts and the rest of $H_2O_2$. The dialysis fluid was lyophilized to yield powdered products.

The N-acetylation was performed according to the method reported by Li, Liu, Xing, Qin, and Li (2013). The highly deacetylated COS powder (20 mg) was dissolved in 5 mL methanol/water (50:50, v/v) solution. Different amount of acetic anhydride was added stoichiometrically into the COS solution under magnetic stirring at room temperature for 1 h. Subsequently, the resulting solution was concentrated and lyophilized to yield powdered COS with different DAs.

The weight average molecular weight (Mw) were measured by an Agilent 1260 gel permeation chromatography (Agilent Technologies, USA) equipped with a refractive index detector. Chromatography was performed on TSK G3000-$PW_{XL}$ columns, using 0.2 M $CH_3COOH$/0.1 M $CH_3COONa$ aqueous solution as mobile phases at a flow rate of 0.8 mL/min with column temperature at 30 °C. The sample concentration was 0.4% (w/v). The standards used to calibrate the column were dextrans Mw 80,000, 50,000, 25,000, 12,000, 5000 and 1000 Da (Sigma, USA). Fourier transform infrared (FT-IR) spectra of samples were measured in the range of 4000–400 $cm^{-1}$ regions using a Thermo Scientific Nicolet iS10 FT-IR spectrometer in KBr discs. The DA value was measured by ultraviolet spectrophotometry (Muzzarelli & Rocchetti, 1985). The highly deacetylated COS powder (10–20 mg) was introduced in 0.001 mol $L^{-1}$ HCl. After complete dissolution, the absorbance of the solution was measured at 199 nm. The DA value was quantified by comparison with a standard curve using N-acetyl glucosamine.

### 2.2. Plant material and treatments

The following experiments were conducted with wheat (*Triticum aestivum* L. Jimai 22) seeds, which were surface sterilized with a 1% sodium hypochlorite solution for 10 min and thoroughly washed with distilled water. Seeds were soaked in distilled water for 5 h and then transferred to a Petri dish with moist gauze for germination at 25 °C for 24 h in the dark. Germinated seeds were sowed in Petri dishes with nylon mesh and were grown in Hoagland solution in a growth incubator at a day/night cycle of 14 h/10 h, at 25 °C/20 °C, respectively, with a relative humidity of 65% and a light intensity of 800 μmol $m^{-2}$ $s^{-1}$. When the second leaf of wheat seedlings was fully expanded, 100 mM NaCl was added into the solution. Preliminary studies using various Mw of COS (1300, 3300, 5300, 9300 Da) showed that 0.01% 1300 Da COS solution was close to optimal for enhancing salt tolerance of wheat seedlings. Thus, the experiments were divided into seven groups, including a control check (CK, neither COS nor NaCl), NaCl stressed as a negative control, salicylic acid (SA) treated as a positive control, and 4 COS-NaCl stressed (treated with 0.01% COS of 1300 Da with various DAs of 2, 29, 50 and 68%) groups. The nutrient solution was renewed every other day.

### 2.3. Growth parameters

After 10 days of NaCl treatment, wheat seedlings of each group were harvested for determination of shoot length and wet weight; after which samples were dried at 105 °C for 2 h to obtain dry weight.

### 2.4. Lipid peroxidation degrees

The level of lipid peroxidation in plants was determined based on malondialdehyde (MDA) content. MDA, a product of lipid peroxidation, was determined using a thiobarbituric acid (TBA) reaction (Seckin, Sekmen, & Türkan, 2008). After 10 days of NaCl treatment, leaf samples (0.5 g) were homogenized in 10% trichloroacetic acid (TCA). The homogenate was centrifuged at 4000 × *g* for 10 min. The supernatant was used for estimating the MDA content. Then, 2 mL of 0.6% TBA was added to 2 mL supernatant, and the mixture was heated at 100 °C for 15 min and cooled in an ice bath immediately afterwards. Next, the mixture was centrifuged at 10,000 × *g* for 15 min. The absorbance of the supernatant was recorded at 450, 532 and 600 nm, separately. The MDA content was expressed as μmol MDA $g^{-1}$ fresh weight (FW).

### 2.5. Chlorophyll content, fluorescence and photosynthetic characters

After 10 days of NaCl treatment, chlorophyll was extracted with 95% ethanol. Chlorophyll a (Chl-a), chlorophyll b (Chl-b) and total chlorophyll (a + b) content were determined spectrophotometrically (Pongprayoon, Roytrakul, Pichayangkura, & Chadchawan, 2013). Photosynthetic rate (Pn), transpiration rate (*E*), stomatal conductance (gs) and intercellular $CO_2$ concentration (Ci) were measured with a portable photosynthesis system (L.MAN-LCPro-SD, BioScientific Ltd., UK). Atmospheric conditions consisted of 25 ± 2 °C, gas flow rate of 200 μmol $s^{-1}$, photosynthetic photon flux density of 800 μmol $m^{-2}$ $s^{-1}$ and $CO_2$ concentration of 400 ± 5 μmol $m^{-2}$ $s^{-1}$. Chlorophyll fluorescence was measured using a portable fluorometer (PAM-2100, Walz, Germany). The maximum quantum yield of PSII (Fv/Fm) was determined after dark adaptation for 30 min.

### 2.6. Determination of soluble sugar

After 10 days of NaCl treatment, soluble sugar was measured by the following procedure: 0.5 g of leaf samples were cut up and heated at 100 °C for 30 min in 5 mL distilled water. The extract was diluted 5-fold for determination. 500 μL diluents, 1 mL 5% phenol and 5 mL sulfuric acid were mixed and after standing for 3 min, the absorbance was read at 485 nm. Soluble sugar concentration was quantified by comparison with a standard curve using the criterion of glucose.