Moolphuerk N and Pattanagul W (2020)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca 48(4):2072-2084
DOI:10.15835/48412018
Research Article

Pretreatment with different molecular weight chitosans encourages drought tolerance in rice (*Oryza sativa* L.) seedling

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Abstract

Drought is a critical environmental constraint limiting plant growth and productivity. Chitosan has been utilized as a potential biostimulant and proven to be effective against drought stress in many plant species. The objective of this study was to determine the effects of pretreatment with different molecular weight (MW) chitosans on some physiological characteristics of rice seedlings under drought stress. Rice seedlings were treated with low (50-190 kDa), medium (190-310 kDa) and high (310-375 kDa) MW chitosans by seed priming and foliar spray. The seedlings were subjected to drought by withholding water for four days. The relative water content (RWC) was reduced from 93% in the control plants to 74% in the droughted plants. The results revealed that treating with chitosan, especially with low MW chitosan, promoted root growth under drought stress. All chitosan treatments led to higher relative water content and photosynthetic pigment under drought condition. Pretreatment with chitosan also induced sugar accumulation, and treating with low MW chitosan significantly increased starch accumulation under drought stress. In addition, chitosan treatments alleviated the effects caused by drought stress as represented by the decreases in electrolyte leakage, malondialdehyde (MDA) as well as hydrogen peroxide (H_2O_2), corresponding with the increases in activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) activity.

Keywords: antioxidant enzymes; chitosan; drought stress; foliar application; rice; seed priming

Introduction

The ongoing increase of the severity and frequency of drought in a warming climate negatively affects agricultural production and threaten food security worldwide. Drought stress is one of the critical limiting factors affecting both the quality and quantity of many agricultural products, especially rice. Thailand is one of the world's largest producer and exporter of rice, but current rice production is still limited by rainfall variability and drought due to the most of farmers' popular and famous rice varieties are susceptible to drought stress. Among these varieties, 'Khao Dawk Mali 105' ('KDML105') is famous in either the country or worldwide. Demand for this variety is increasing in both domestic and international markets; however, the cultivation is limited by infertile and drought soil (Yoshihashi *et al.*, 2004). The 'KDML105' rice is mainly cropped in Northeastern Thailand, the highest quality rice production area. Approximately 90% of rice cultivation in

Received: 30 Jul 2020. Received in revised form: 02 Dec 2020. Accepted: 07 Dec 2020. Published online: 22 Dec 2020.

northeast Thailand is rain-fed, therefore rainfed rice yields in this region are generally below potential due to water shortages (Prabnakorn *et al*., 2018).

Drought stress limits plant growth by reducing cellular turgor pressure as well as limiting photosynthesis due to stomatal closure. Drought stress also induces free radicals and reactive oxygen species (ROS) causing damage to various cellular components. Accumulation of ROS results in the loss of membrane stability and integrity along with the reduction of photosynthetic pigments such as chlorophylls. Naturally, plants display a range of mechanisms to withstand drought stress; however, these mechanisms vary between plant species (Toldi *et al*., 2009). In the case of the drought-susceptible plants, these mechanisms are not sufficient to overcome water deficit or drought conditions (Fang and Xiong, 2015). Various management strategies have been proposed to cope with drought stress. Among these, plant biostimulants received considerable attention during the past few years due to their efficiency to enhance abiotic stress tolerance and improve plant productivity (Du Jardin, 2015). Plant biostimulants include diverse formulations of compounds, substances, and other products that are applied to plants or soils to regulate and enhance the crop's physiological processes, thus making them more efficient (Sharma *et al*., 2014). There are several classes of plant biostimulants, but chitosan has been proven to be effective in term of improving abiotic stresses.

Chitosan is a natural polymer and one of the chitin derivatives when the degree of deacetylation of chitin reaches about 50% (Rinaudo, 2006). Chitosan is obtained after deacetylation of chitin in which its chemical structure composed of a linear polymer consisting of two subunits, D-glucosamine and *N*-acetyl-D-glucosamine linked together by glycosidic bond (Hidangmayum *et al*., 2019*).* The functional properties of chitosan such as solubility, biodegradability, and diverse bioactive attributes are related to molecular weight and the degree of deacetylation (Rajoka *et al*., 2019*).* Many studies have differently determined classes of chitosan based on its molecular weight; however, the specific categories are still unclear. Commercially, chitosan is classified into three main different classes: low (50-190 kDa), medium (190-310 kDa), and high (310-375 kDa) molecular weight (MW) (Prashanth and Tharanathan, 2007*).* The cationic nature of chitosan is somewhat unique because most of the polysaccharides are usually either neutral or negatively charged in an acidic condition. The unique property allows it to generate electrostatic complexes with other negatively charged synthetic or natural polymers (Rinaudo, 2006). Chitosan has been, therefore, investigated and developed as a plant biostimulant (Katiyar *et al*., 2015; Hidangmayum *et al*., 2019). In plants, chitosan elicits numerous defense responses related to biotic and abiotic stresses. It has been utilized effectively in many plant-related applications to increase plant productivity as well as protect plants against the attack of pathogens (Malerba and Cerana, 2018). Previous studies revealed that chitosan has a potential to enhance plant growth as well as increase yield in many crops including apple, wheat, maize and rice (Yang *et al*., 2009; Lizárraga-Paulín *et al*., 2011; Zeng and Luo, 2012; Seang-Ngam *et al*., 2014). In drought stress, chitosan treatment alleviates the adverse effect of drought stress by increasing antioxidant enzyme activities, promoting root water absorption capability by increasing root growth and enhancing photosynthetic activities. It has been suggested that chitosan binds specific receptors located in plant cell membranes, which triggers signal transduction cascade via various secondary messengers, including ROS, hydrogen peroxide (H_2O_2), nitric oxide, and phytohormones. H_2O_2 leads to abscisic acid production and induces ROS scavenging mechanism, while nitric oxide initiates ABA synthesis leading to stomatal closure as well as triggering stress-responsive genes (Hidangmayum *et al*., 2019). Nevertheless, it was reported that different molecular weight chitosan exhibit different level of protein and compound, suggesting chitosan have varied functionality depending upon its class (Lin *et al*., 2005).

Due to the ongoing and severity of drought stress in Thailand, it is essential to find a solution to mitigate the problem and improve the quality of rice production. Plant biostimulants, chitosan in particular, have received much attention in the research related to abiotic stress. Although chitosan has been proven to be effective in promoting stress tolerance in plants, relationships between MW and biological properties as well as its mechanism remain unclear. Our research aims to investigate the effects of pretreatment with different MW chitosans on drought tolerant in the 'KDML105' rice seedlings. Three classes of chitosan, including low,

medium, and high MW chitosan, were used in our experiment. Growth parameters, including root and shoot lengths, root and shoot fresh and dry weights, were determined. In addition, some physiological characteristics related to drought tolerant response, such as relative water content, chlorophyll content, electrolyte leakage, malondialdehyde (MDA) concentration, total soluble sugar and starch content as well as antioxidant enzyme activities were examined.

Materials and Methods

Chitosan solution preparation

Three types of commercial chitosan including low MW (50-190 kDa with 20-300 cP viscosity), medium MW (190-310 kDa with 200-800 cP viscosity) and high MW (310-375 kDa with 800-2000 cP viscosity) with 75-85% deacetylation was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Chitosan solution for seed priming, five mg of chitosan powder was entirely dissolved in 2 ml of 1% acetic acid, 98 ml of distilled water was then added to obtain 100 ml of 50 mg l^{-1} chitosan solution, the solution was adjusted to pH 6.0 using 1 M NaOH. Acidified distilled water used in no-chitosan treatment was prepared by the combination of 2 ml of 1% acetic acid and 98 ml of distilled water then the pH of the solution was adjusted to 6.0 by using 1 M NaOH. Chitosan solution, 200 ml of 50 mg l^{-1}, and acidified water for foliar spray were prepared using the same method as mentioned above and Tween 20 (Sigma-Aldrich, Saint Louis, MO, USA) was added as leaf surfactant in foliar application.

Plant material and experimental design

Rice seeds (*Oryza sativa* L.) cv. ''KDML105 obtained from Khon Kaen Rice Research Center, Khon Kaen, Thailand were surface sterilized by soaking in 80% ethanol for 30 seconds followed by soaking in 3% sodium hypochlorite solution for 10 min and washing with sterile distilled water for 10 min three times. The experiment which was divided into five treatments with four replicates was consisted of control (no drought and no chitosan treatment), drought (withholding water and no chitosan treatment), low, medium, and high MW chitosan (withholding water and chitosan-treated treatment). For control and drought treatments, sterilized seeds were primed in acidified water, meanwhile chitosan treatments seeds were primed in chitosan solutions for 48 h. Seven seeds were implanted in a plastic pot (20.32 cm in diameters and 17.78 cm in height) filled with 2 kg of paddy soil obtained from the rice-growing field (Khon Kaen, Thailand) (loamy sand with pH 6.04, EC= 0.040 dS m^{-1}, organic matter= 0.235%, total N= 0.023 mg kg^{-1}, total P= 36.65 mg kg^{-1}, and total K= 234.50 mg kg^{-1}) and allowed to grow in greenhouse at Department of Biology, Faculty of Science, Khon Kaen University, Thailand for 21 days under natural light (11/13 of day/night), temperature (37 °C of average) and humidity (63% of average) conditions during February 2018. For control and drought groups, twenty-one-day old seedlings were foliar-sprayed with 200 ml per pot of acidified water. For chitosan treatements, plants were foliar-sprayed to runoff by a manual sprayer with 200 ml per pot of 50 mg l^{-1} chitosan solution two times (9 a.m. and 10 a.m.) at day 14 and 21 after planting. Control group was watered daily to field capacity (500 ml per pot), meanwhile drought group and three groups of chitosan treatments was imposed to drought stress by withholding water for four days which rice leaves showed drought stressed symptom as the O shape of leaf rolling (International Rice Research Institute, 2002).

Growth parameter measurement

Rice seedlings were randomly selected from each pot and gently uprooted. The seedlings were separately measured for shoot and root lengths as well as shoot and root fresh weights. Dry shoot and root weights were obtained after oven-dry at 70 °C for 72 h.

Relative water content determination

Middle leaf blade was cut to piece in the size of 1.5 × 1.5 cm^2, immediately weighed the fresh weight (FW). The leaf piece was then soaked in deionized water for 4 h under fluorescent light and weighed as turgid weight (TW). It was subsequently dried at 70 °C for 48 h and weighed as dry weight (DW). RWC=[(FW-DW)/(TW-DW)] ×100 (Turner,1981).

Chlorophyll content determination

Approximately 20-30 mg of leaf tissue was immersed in 80% acetone for 24 h. Chlorophyll content was spectrophotomically determined at wavelength of 645 and 663 nm and calculated according to Arnon (1949).

Total soluble sugar and starch extraction and analyses

Approximately 50 mg of leaf tissue was used for total soluble sugar and starch analyses. Soluble sugars were extracted from the tissues in hot 80% (v/v) ethanol three times in a 65 °C water bath for 60 min each. Total sugar in leaf tissue was determined colorimetrically using phenol sulfuric acid method (Dubois et al., 1956). The leaf residue remaining after ethanolic extraction was hydrolyzed in 1 ml of 0.2 M KOH and boiled for 30 min. Samples were cooled and adjusted to about pH 5.5 by adding 2 ml of 1 M acetic acid. Starch in the leaf residue was digested with amyloglucosidase overnight, and released glucose was quantified enzymatically using hexokinase/glucose-6-P dehydrogenase.

Electrolyte leakage measurement

Leaf pieces about 50-60 mg were immersed in 10 ml deionized water for 24 h. Electrical conductivity (EC) was measured by conductivity meter and recorded as EC1. The leaf tissue was then boiled at 100 °C for 20 min and cooled down at room temperature. EC was measured and recorded as EC2. The electrolyte leakage was calculated according to Baninasab and Ghobadi (2011).

Malondialdehyde determination

The production of MDA was estimated base on thiobarbituric acid (TBA) reactivity. Leaf samples were ground thoroughly with 2 ml of 0.1% (w/v) trichloroacetic acid, followed by centrifugation at 1000 rpm for 5 min. A mixture of 0.5 ml of supernatant and 1.5 ml of 0.5% TBA was boiled in a 95 °C water bath for 30 min. The reaction was stopped by freezing on ice for 10 min. The absorbance was determined at wavelength of 532 nm. The concentration of TBA reacting substance (TBARS) was calculated using a molar extinction coefficient of 155 mM^{-1} cm^{-1} (Sunohara and Matsumoto, 2004).

H_2O_2 determination

Leaf samples were ground thoroughly with 1 ml of 0.1% (w/v) trichloroacetic acid, followed by 1500 rpm centrifugation at 4 °C for 15 min. The reaction mixture was prepared by mixing 0.5 ml of supernatant, 0.5 ml of 10 mM potassium phosphate buffer, and 1 M of KI and spectrophotomically measured at a wavelength of 390 nm (Terzi *et al*., 2014).

Antioxidant enzyme activity assay

Approximately 0.05 g of leaf sample was thoroughly ground on ice using mortar and pestle in 4 ml of extraction buffer [50 mM potassium phosphate buffer (pH 7.8), 0.4 mM EDTA, 1 mM ascorbic acid and 2% polyvinyl polypyrrolidone]. The extract was filtered through 4 layers of cheesecloth, then centrifuged at 10,000 rpm for 1 min. The filtrate was then used as an enzyme extract for superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (GPX, EC 1.11.1.7) assays (Lu *et al*., 2009). The amount of protein in the enzyme extract was determined by the Bradford method (Bradford, 1976).

SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of NBT. The reaction mixture (2 ml) contained 50 mM potassium phosphate buffer (pH 7.8), 10 mM methionine, 50 μM NBT, 0.025% Triton X-100 and 10 μM riboflavin. The photo-reduction of NBT was measured at 560 nM. One unit of SOD was defined as the amount of enzyme that produced 50% inhibition of NBT. CAT activity was assayed in a 2 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 25 mM H_2O_2, and 0.1 ml of enzyme extract. The subsequence decomposition of H_2O_2 was observed at 240 nm (E = 0.0394 mM^{-1} cm^{-1}). APX activity was assayed in a 2 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM ascorbic acid, 0.4 mM EDTA, 12.5 mM H_2O_2 and 0.1 ml of enzyme extract. The subsequent decrease in ascorbic acid was observed at 290 nm (E = 2.8 mM^{-1} cm^{-1}) (Sunohara and Matsumoto, 2004). GPX activity was assayed in a 2 ml reaction mixture containing 50 mM potassium phosphate buffer, 3% guaiacol, 20 mM H_2O_2, and 0.1 ml of enzyme extract. GPX activity was calculated from the formation of tetraguaiacol per minute at 470 nm (E= 26.6 mM^{-1} cm^{-1}) (Uarrota *et al*., 2016).

Statistical analysis

The experiment was designed as a completely randomized design with four replicates. Data were analyzed by one-way analysis of variance (One-way ANOVA) using SPSS version 24 (IBM Corp., Armonk, N.Y., USA). The mean differences were compared by Duncan's multiple range comparison test. *P*-values<0.05 were considered significantly different. Data were expressed as means ± standard errors.

Results

Chitosan pretreatment enhanced rice seedling growth under drought stress

Drought stress resulted in the overall reduction of shoot and root growths of rice seedling as indicated by the decreases in shoot length, root length, shoot and root fresh weights as well as shoot and root dry weights (Figure 1A-F). Although exogenous chitosan application had little effect on shoot growth (Figure 1A, 1C, 1E), its application promoted root growth in rice seedling as seen by the increases of root fresh and dry weight (Figure 1D, 1F). Low MW chitosan was the most effective in promoting root growth, followed by medium MW and high MW chitosan, respectively (Figure 1B, 1D, 1F).

Effects of chitosan on some physiological characteristics of rice plant under drought stress

The relative water content of leaf was significantly reduced when the plant was subjected to drought stress. All chitosan treatments caused a significant increase in relative water content compared to the untreated plants (Figure 2A). Additionally, drought stress resulted in increases of electrolyte leakage as well as MDA in the leaf. All chitosan treatments showed a reduction in electrolyte leakage and MDA (Figure 2B, 2C). The electrolyte leakage and MDA of all chitosan treatments were similar to those of the control plants, and there was no difference between each chitosan treatment. H_2O_2 significantly increased when the plant was subjected to drought. Treating with three different MW of chitosans resulted in a lower concentration of H_2O_2 in which the high MW chitosan had a significant lowest H_2O_2 among the treated groups (Figure 2D).

Drought stress also caused reductions in both total soluble sugar and starch content in leaf. Treating with chitosans, however, significantly increases the accumulation of total soluble sugar and starch in the leaf (Figure 2E, 2F). Also, low MW chitosan caused the highest increase in starch level in the leaf, followed by medium and high MW chitosan, respectively (Figure 2F).

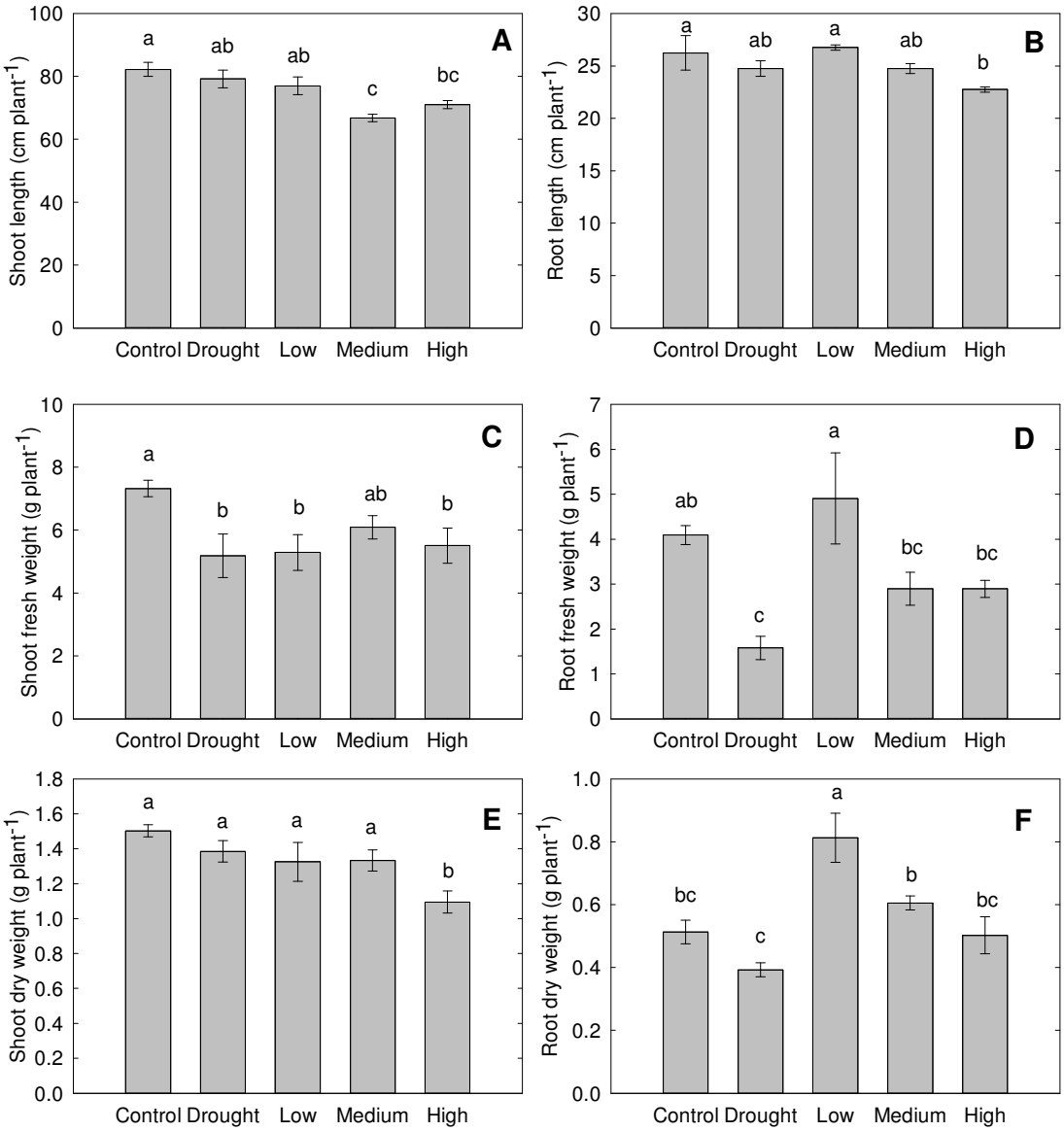

Figure 1. Effects of chitosan pretreatment shoot length (A), root length (B), shoot fresh weight (C), root fresh weight (D), shoot dry weight (E) and root dry weight (F) of rice seedling under drought stress
Values are expressed as mean ± S.E. (n= 4). Different letter indicates significant difference ($P < 0.05$) by Duncan's multiple range comparison test

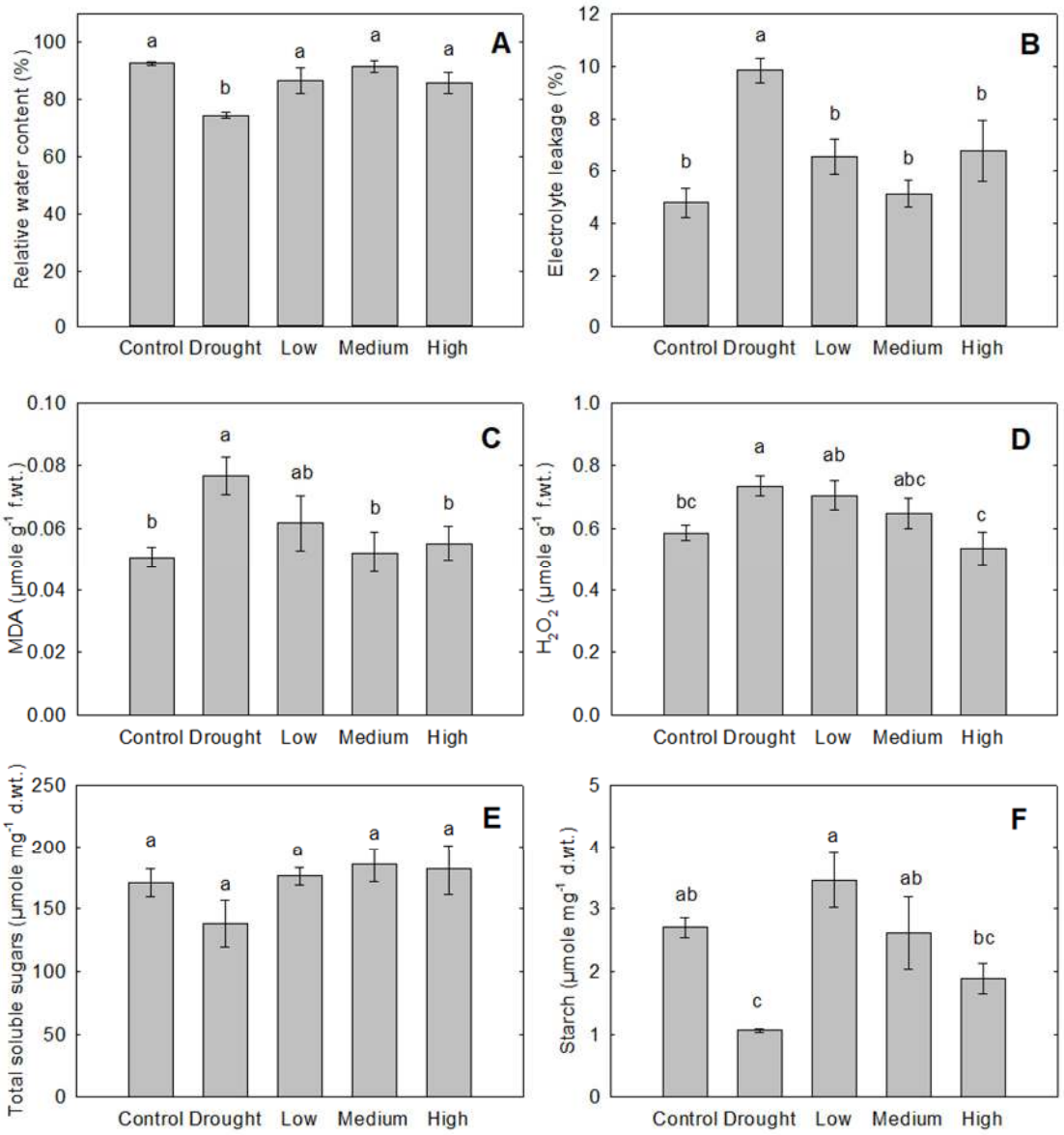

Figure 2. Effects of chitosan pretreatment on relative water content (A), electrolyte leakage (B), MDA (C), H_2O_2 (D), total soluble sugar (E), and starch (F) in rice seedling under drought stress

Values are expressed as mean ± S.E. (n= 4). Different letter indicates significant difference ($P < 0.05$) by Duncan's multiple range comparison test

Chitosan pretreatment improves chlorophyll content in rice seedling under drought condition

The results showed that drought stress negatively affected chlorophyll *a*, chlorophyll *b*, and total chlorophyll content in the leaf of rice seedling. Treating with low, medium, and high MW chitosans resulted in a significantly higher concentration of chlorophyll *a* compared to the droughted plant (Figure 3A). Chitosan treatments also increase chlorophyll *b* concentration compared to the untreated plant in which high MW chitosan caused the highest increase in chlorophyll *b* concentration, followed by medium MW and low MW chitosan, respectively (Figure 3B). In addition, leaf total chlorophyll content was highest in the high MW chitosan group compared to other chitosan treatments and the droughted plant; however, the values are not significantly different within the chitosan treatments (Figure 3C).

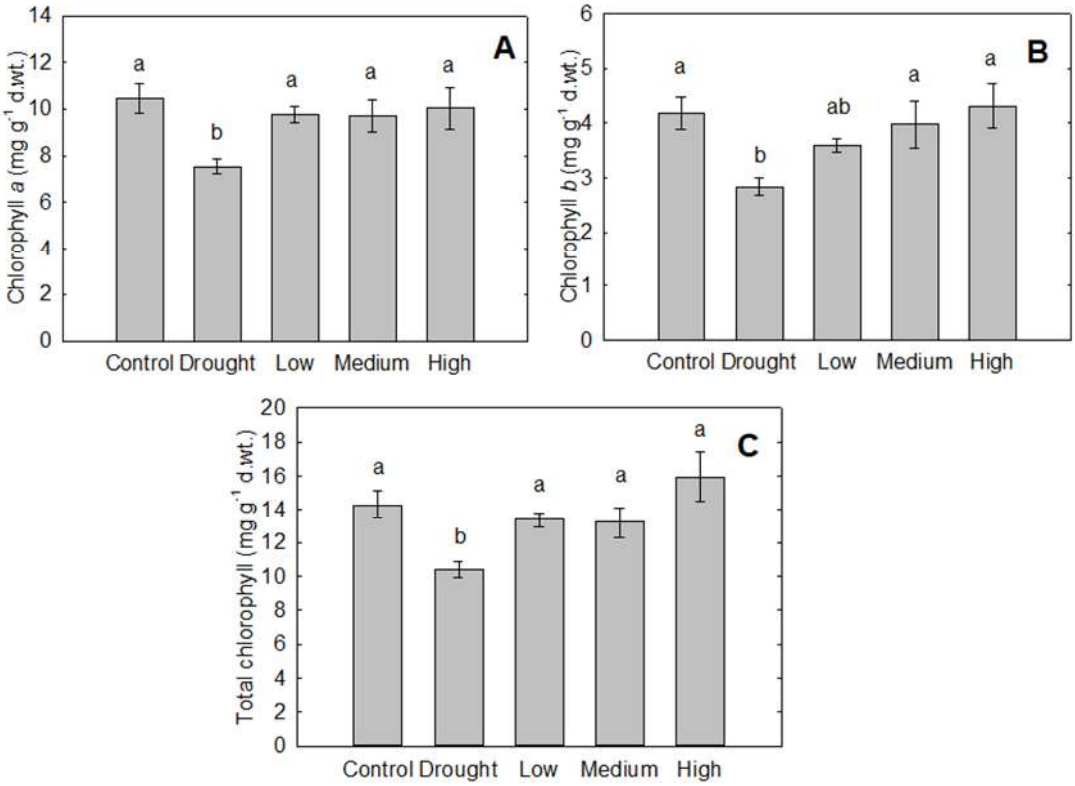

Figure 3. Effects of chitosan pretreatment on chlorophyll *a* (A), chlorophyll *b* (B), and total chlorophyll (C) contents in rice seedling under drought stress

Values are expressed as mean ± S.E. (n= 4). Different letter indicates significant difference ($P < 0.05$) by Duncan's multiple range comparison test

Chitosan application elicited ROS scavenging system

The activities of antioxidant enzymes, including SOD, CAT, APX, and GPX increased slightly when the plant was subjected to drought stress. Treating with medium MW chitosan resulted in the highest activity of SOD, followed by high MW and low MW chitosan, respectively (Figure 4A). Their activities, however, were not significantly different compared to the untreated group. Similarly, treating with all type of chitosans also increase the activity of CAT in which low MW chitosan resulted in the highest CAT activity, followed by high MW and medium MW, respectively (Figure 4B). The activity of APX also increased when the plant was treated with all type of chitosans, and their activities were not significantly different (Figure 4C). Besides, low MW and high MW chitosans caused a significant increase in GPX activity compared to the untreated plant; however, its activity in the medium MW group was not different from the untreated plant (Figure 4D).

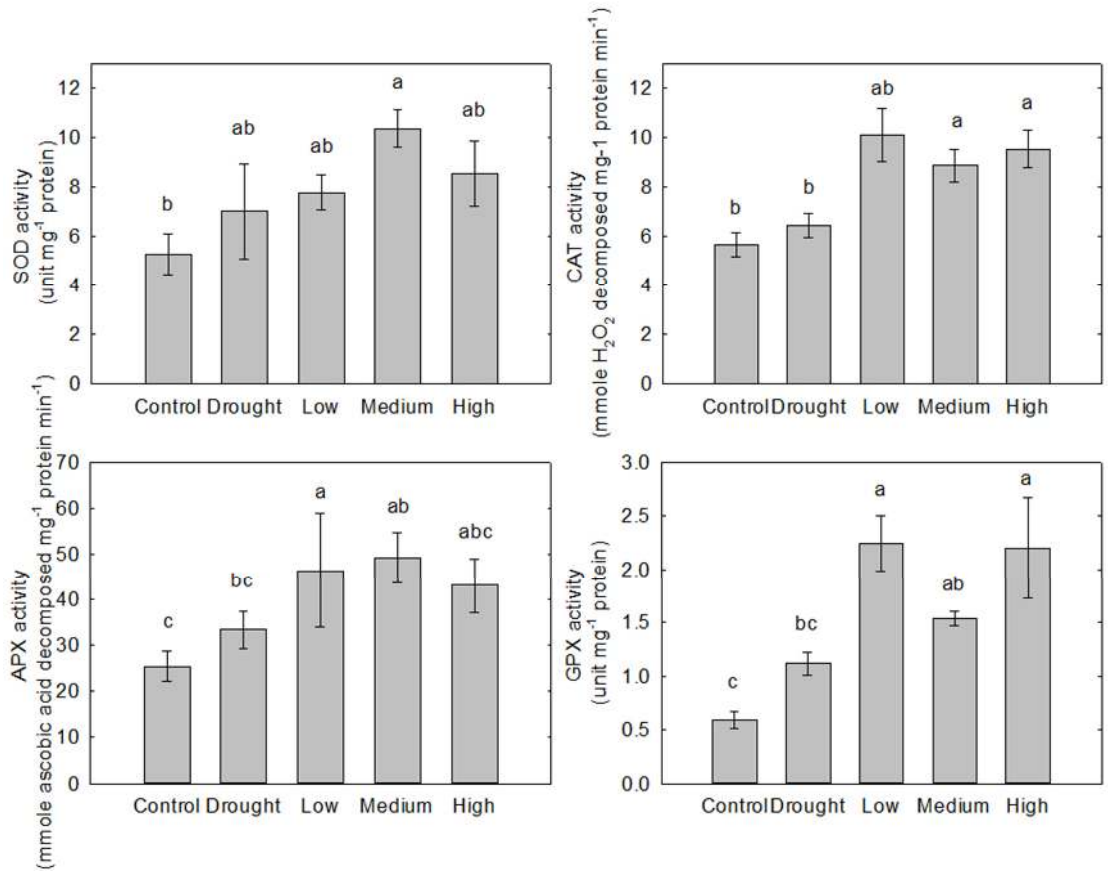

Figure 4. Effects of chitosan pretreatment on SOD (A), CAT (B), APX (C), and GPX (D) activities in rice seedling under drought stress. Values are expressed as mean ± S.E. (n= 4)
Different letter indicates significant difference ($P < 0.05$) by Duncan's multiple range comparison test

Discussion

The effectiveness of chitosan on increase plant growth has been reported in various plants. Foliar spray with different type and deacetylation degree of chitosan promoted the growth of 'Leung Pra Tew 123' rice seedlings under normal condition via induction of many growth-related genes, and the best enhancement was observed in rice seedlings treated with oligomeric chitosan (Chamnanmanoontham, 2015). The present study revealed that chitosan enhanced rice seedling growth by increase root fresh and dry weights, especially treating with low MW chitosan. Ma *et al.* (2014) also reported that chitosan promotes wheat growth in terms of germination capacity, root length, and increase in root activity. Increased root growth strengthens the capacity of water absorption, therefore, enhance drought resistance of the seedlings (Katiyar *et al.*, 2015). Drought stress also has a negative effect on relative water content as well as membrane stability and integrity as indicated by increases of electrolyte leakage and MDA. These effects, however, are alleviated when the plant was pretreated with chitosan. The previous research also indicated that using chitosan nanoparticle through the soil and foliar application significantly increased relative water content in barley (*Hordeum vulgare* L.) under late-season drought stress (Behboudi *et al.*, 2018). It is also shown that chitosan application increased cell membrane permeability and decreased MDA content in *Thymus daenensis* as well as wheat under drought stress (Rebolledo *et al.*, 2012; Zeng and Luo, 2012).

Photosynthetic pigments are also considerably reduced by drought stress. This effect also leads to the reduction of total soluble sugar and starch accumulation in the leaf. Treating with chitosan helps the plant to maintain photosynthetic function as indicated by higher concentration of chlorophyll *a*, chlorophyll *b*, and total chlorophyll under drought stress. Sugar and starch accumulations are also observed in the chitosan-treated plant exposed to drought stress. In basil and white cover plants, chitosan application significantly increased soluble sugar content under severe drought (Pirbalouti *et al*., 2017). Besides providing energy, sugars play a crucial role in downstream events of gene expression and signal transduction of a wide range of abiotic stresses. Sugar accumulation has been known to play an essential role in an osmotic adjustment under drought condition (Wu *et al*., 2016). Sugar metabolism is also involved in membrane stabilization, conferring plant resistance against drought stress (Gupta and Thind, 2019). Treating with low MW chitosan also leads to the highest starch accumulation, compared to the other chitosan treatments as well as the untreated plant. Starch accumulation during the drought period is believed to be a key component of drought tolerance mechanism (Dong and Beckles, 2019).

Generally, drought stress generates the excessive ROS which damages plants by oxidizing photosynthetic pigments, membrane lipids, proteins, and nucleic acids (Yordanov *et al*., 2000). In this study, H_2O_2 significantly increased in response to drought stress. Plants normally cope with oxidative damage by increasing the activity of antioxidant enzymes in which indicates drought resistance in plants (Weng *et al*., 2015). The present study demonstrated that chitosan pretreatment induces the activities of SOD, CAT, APX as well as GPX. It was also reported that chitosan alleviates the adverse effect of water stress by enhanced production of antioxidant enzymes (Hidangmayum *et al*., 2019). The result also corresponded to the previous studies, which also revealed that chitosan application increases APX in rice cv. 'LPT123-TC171', as well as SOD, POX, and CAT activities in wheat under drought stress (Zeng and Luo, 2012; Pongprayoon *et al*., 2013). Also, application of chitosan at low concentrations increased antioxidant enzyme, including CAT and POX, activities in wheat and maize seedlings under salt stress condition; however, the activities are reduced at higher concentrations of chitosan (Peykani and Sepehr, 2018).

Conclusions

The results obviously suggested that chitosan pretreatment through the method of seed priming combined with foliar spray could alleviate the effect of drought stress in the rice seedlings. Chitosan, especially low MW, enhances root growth which may increase water absorption. Chitosan also helps the plant to maintain water content under drought condition. Photosynthetic capacity is improved, possibly by preventing pigment disintegration. Rice plant treated with chitosan displays a better osmotic adjustment via accumulation of sugar as well as starch, especially those treated with low MW chitosan. In addition, chitosan reduced the severity of drought by improving membrane stability and integrity corresponded with the increase in antioxidant enzyme activity.

Authors' Contributions

Both authors read and approved the final manuscript.

Acknowledgements

This work was supported by Science Achievement Scholarship of Thailand (SAST) and Salt Tolerant Rice Research Group (STRG), Department of Biology, Faculty of Science, Khon Kaen University, Thailand.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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